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École doctorale **R**essources **P**rocédés **P**roduits **E**nvironnement **(RP2E)**

Laboratoire d'**I**ngénierie des Biomolécules **(LIBio)**

THÈSE

Présentée à l'Université de Lorraine par

Mohamed Hussein Hamdy ROBY

En vue d'obtenir le grade de

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Synthèse et caractérisation de biomolécules antioxydantes

Synthesis and characterization of antioxidant biomolecules

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Abstract

An efficient solvent-free bioprocess was developed for the synthesis of DHA phenolic ester, using the lipase B from *Candida antarctica*. The protocol developed here led to high-level production (440 g/L) of DHA vanillyl ester (DHA-VE) that exhibits interesting application potential as food ingredient. DHA-VE was characterized by a high stability and a high radical scavenging activity towards DPPH, ABTS and hydroxyl radicals. Neuroprotective properties of DHA-VE were also demonstrated in rat primary neurons exposed to amyloid-β oligomers. Enzymatic esterification of DHA with vanillyl alcohol (VA) led to increased DHA levels in erythrocytes and brain tissues of mice fed DHA-VE-supplemented diet comparing with DHA. No visible toxicity of the ester was found. Enrichment of emulsions with DHA-VE improved significantly their oxidative stability whatever the conditions of storage, showing the potential of DHA-VE to enrich various food matrices with DHA while protecting them against oxidation. The enzymatic process was applied to salmon oil as a source of omega-3 polyunsaturated fatty acids (PUFA). The total conversion of VA (50 g/L) was achieved after 24 h of reaction, leading to the production of a wide variety of esters that mirror the initial composition of the oil. The crude reaction medium recovered from salmon oil alcoholysis exhibited a high stability together with high antioxidant properties in comparison with native salmon oil. In conclusion, the approach that consists in bringing phenolic compounds and PUFA-rich lipids together within a single structure is expected to provide stable bioactive ingredients that should broaden the scope of application of omega-3 PUFAs whose health benefits are increasingly sought.

Key words:

Omega-3, DHA, phenolic compounds, vanillyl alcohol, enzymatic esterification, oxidative stability, neuroprotection, and fish oil

Résumé

Un procédé enzymatique sans solvant a été développé permettant la synthèse d'un ester phénolique de DHA. L'optimisation des paramètres réactionnels a permis d'atteindre des rendements élevés (440 g/L) d'ester de DHA et d'alcool vanillique (DHA-VE), dont les activités biologiques et le potentiel applicatif ont été évalués. L'activité inhibitrice du DHA-VE vis-à-vis des radicaux ABTS, DPPH et hydroxyle a été démontrée. Un effet neuroprotecteur de l'ester a également été mis en évidence sur des neurones primaires de rat, exposés aux oligomères du peptide β-amyloïde. Une étude *in vivo* a permis de montrer que le greffage d'alcool vanillique conduit à une augmentation du taux de DHA au niveau des globules rouges et des neurones, indiquant une biodisponibilité accrue du DHA lorsque celui-ci est couplé au composé phénolique. Aucune toxicité visible de l'ester n'a été constatée. Par ailleurs, l'incorporation de DHA-VE dans divers systèmes émulsionnés a permis d'accroître leur stabilité à l'oxydation, quelles que soient les conditions de stockage. Ceci montre le potentiel de cet ester pour enrichir diverses matrices alimentaires en DHA, tout en améliorant leur stabilité à l'oxydation. Le procédé enzymatique développé a été appliqué à de l'huile de saumon, utilisée comme source d'acides gras polyinsaturés de la série oméga-3. L'incorporation totale de l'alcool vanillique (50 g/L) a été obtenue après 24 h de réaction, conduisant à la production d'une grande variété d'esters, représentatifs de la composition initiale de l'huile en acides gras. Le milieu réactionnel brut issu de l'alcoolyse de l'huile présente une grande stabilité et des propriétés antioxydantes importantes par rapport à l'huile de saumon native. En conclusion, l'approche consistant à assembler des composés phénoliques et des lipides polyinsaturés au sein d'une même structure semble prometteuse pour renforcer le potentiel applicatif de ces deux familles de biomolécules et produire de nouveaux ingrédients bioactifs stables.

Mots clés :

Omega-3, DHA, composés phénoliques, alcool vanillique, estérification enzymatique, stabilité à l'oxydation, neuroprotection et l'huile de poisson.

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List of abbreviations

ω-3	Omega-3
ω-6	Omega-6
AA	Arachidonic acid
$ABTS^{+\bullet}$	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
ALA	Alpha - Linoleic acid
AOCS	American Oil Chemists' Society
ANOVA	Analysis of variance
aw	Water activity
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoulene
DHA	Docosahexaenoic acid
DHA-EE	Docosahexaenoic ethyl ester
DHA-VE	Docosahexaenoic vanillyl ester
EPA-VE	Eicosapentaenoic vanillyl ester
DPA-VE	Docospentaenoic vanillyl ester
DAG	Diacylglycerol
$DPPH^{\bullet}$	2, 2-diphenyl picryl hydrazyl
EFA	Essential fatty acid
EPA	Eicosapentaenoic acid
FAs	Fatty acids
FFAs	Free fatty acids
GLC	Gas-liquid chromatography
HPLC	High-performance liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
MAG	Monoacylglycerol
PL	Phenolic lipids
PUFAs	Polyunsaturated fatty acids
PV	Peroxide value
SFA	Saturated Fatty acids
SFM	Solvent-free medium
SO	Salmon oil
TAG	Triacylglycerol

TBARS	Thiobarbituric acid reactive substances
TBHQ	*tert*-Butylhydroquinone
TLC	Thin-layer chromatography
UV	Ultraviolet
VA	Vanillyl alcohol

Introduction

Introduction

In the recent years, interests for natural substances with beneficial activity to human have sharply risen. In fact, there is a significant increase in nutraceuticals and pharmaceutical products, based on natural compounds. The main interest has been observed for natural substances with strong antioxidant activity, because oxidative stress induced by multiple factors is the main cause of many pathological conditions such as inflammation, cancer, coronary heart disease and even skin aging. Also there has been a significant consumer interest in health enhancing role of specific foods or physiologically-active food components.

Unsaturated lipids have been widely recognized for their role in the maintenance of human health. These lipids, especially those from the omega-3 (ω-3) series have been linked to inhibitory effects on atherosclerosis cardiovascular and Alzheimer diseases (Gogus andSmith, 2010 ; Prostek *et al.*, 2014 ; Ruxton *et al.*, 2004). However the use of such lipids remains strongly limited due to their high susceptibility to auto-oxidation. To overcome this difficulty, a lot of research has been carried out focusing on the development and the use of antioxidants that could delay or even prevent omega-3 lipids oxidative degradation. In this context, natural plant phenols were perceived by many researchers as potential substitutes for controversial synthetic antioxidants; however, the major drawback of these compounds is their low solubility in matrices that strongly restrains their use in food applications (Acosta-Estrada *et al.*, 2014 ; Pinchuk *et al.*, 2012).

The hydrophilic nature of phenolic compound reduces their effectiveness in oil based formulae and emulsions (Laguerre *et al.*, 2013). The synthesis of more

lipophilic derivatives, especially esters, could help to increase their lipophilicity, and then their interactions with lipidic phases that need to be stabilized. To achieve this goal, acylation with fatty acids appears as a promising way (lipophilization) that could extend the scope of application of phenolic antioxidants in lipid-rich food matrices. When applied to polyunsaturated lipids, this approach is expected to provide stable ingredients with high nutritional value and high antioxidant potential. Additional effects could be an increased bioavailability of phenols as well as cumulative and even synergistic biological activities (Shahidi F. and Zhong, 2010 ; Stasiuk andKozubek, 2010).

Many studies reported the enzymatic synthesis of phenolic lipids basing on the ability of lipases to catalyze the acylation of phenolic compounds with either fatty acids or triacylglycerols (TAG) (Bouallagui *et al.*, 2011 ; Liu L. Y. *et al.*, 2014b ; Mbatia B. *et al.*, 2011 ; Stergiou *et al.*, 2013 ; Wang J. K. andShahidi, 2014). Main advantages of enzyme-catalyzed processes include the use of mild reaction conditions that limit substrate degradation and high selectivity that avoids the production of undesirable compounds and facilitates further purification protocols (Christopher *et al.*, 2014).

According to all these knowledge we can ask these questions:

The grafting of phenolic compound with omega-3 fatty acid will improve the oxidative stability of these PUFA omega-3 fatty acids?

What is the effect of the process on the bioavailability of Phenols and PUFA?

The antioxidant activity of phenolic compound going to be maintained?

What about new activities molecules produced?

What about the health functions of omega-3 fatty acids?

What about the possibility of application of these new molecules in food industries?

So that, the general context of this PhD work was to develop new biological active food ingredients, from their structural design to their formulation in food matrices, together with the evaluation of their nutritional properties and biological activities. Two classes of biomolecules were studied: natural phenolic compounds and omega-3 polyunsaturated fatty acids from marine sources. Numerous studies reported the beneficial effects of these biomolecules in the prevention of certain diseases related to the cardiovascular system and the brain (Murray, 2014; Velderrain-Rodriguez *et al.*, 2014; Wu F. J. *et al.*, 2014; Zhang H. Y. *et al.*, 2014).

More specifically, this PhD project aimed to overcome the problems related to the poor solubility of some natural phenolic compounds in lipidic phases and the high instability of omega-3 polyunsaturated fatty acids. Vanillyl alcohol was chosen as phenolic partner. Vanillyl alcohol (VA) is a powerful aroma constituent of many food products. This phenol has been proved to be as intense as vanillin, the most abundant component in vanilla (*Vanilla planifolia*) (Perez-Silva *et al.*, 2006). In addition to this interesting olfactory property, vanillyl alcohol exhibits a high radical scavenging capacity that was suggested to be responsible for its anticonvulsive and antioxidant properties (Shyamala *et al.*, 2007). Polyunsaturated fatty acids and especially DHA were chosen as fatty acid partners, because of their health beneficial activities. Vanillyl alcohol and polyunsaturated lipids were associated within a single structure through a solvent-free lipase-catalyzed bioprocess. Products were purified, characterized, and then their properties and biological activities were studied. Their

incorporation into food matrix models was investigated aiming to clarify their application potential.

The main objectives of this research project were to:

1. Optimize the enzymatic process for the synthesis of docosahexaenoic acid vanillyl ester (DHA-VE) through an alcoholysis reaction between vanillyl alcohol (VA) and docosahexaenoic ethyl ester (DHA-EE) as model substrates, with immobilized lipase B from *Candida antarctica* (Novozym 435®).

2. Identify, purify the DHA-VE phenolic ester, and then characterize its chemical structure.

3. Study oxidative stability of DHA-VE phenolic ester under different storage conditions.

4. Study the biological activities of DHA-VE ester (antioxidant activity, neuroprotective activity).

5. Study incorporation of DHA-VE phenolic ester in food models, (margarine and oil in water emulsion system) and oxidative stability of the lipid phase.

6. Apply optimized alcoholysis process to the production of phenolic lipids using vanillyl alcohol and salmon oil as a source of polyunsaturated fatty acids.

7. Study oxidative stability of the crude reaction medium recovered from salmon oil alcoholysis under different storage conditions.

This PhD report was organized around four chapters: the first chapter untitled "**Literature Review**" summarized the previous studies about beneficial effects of omega-3 and antioxidant activity of phenolic compounds. In addition, the various chemical and/or enzymatic processes allowing the synthesis of phenolic lipid esters were mentioned as well as the methods used to study their properties and their fields

of application. The second chapter, "**Material and Methods**" presented the various experimental techniques used to achieve all the objectives as well as the materials and the associated equipment. Chapter three untitled «**Results and Discussion**» consists of four parts. The first part aimed to develop an efficient and environment-friendly solvent-free bioprocess for the synthesis of DHA vanillyl ester (DHA-VE) in sufficient quantities to allow further *in vitro* and *in vivo* experiments as well as potential applications. The main objective of part two is study the effect of alcoholysis process on the oxidative stability of DHA stored at different storage conditions. Meanwhile the third part refers to the potential application of synthesized DHA-VE in retard lipid oxidation emulsions system. Finally the fourth part discusses the possibility of applying the optimized synthesis method with characterized salmon oil to produce vanillyl fatty acid esters. Oxidative stability and antioxidant capacity of crude synthesis reaction medium was compared with salmon oil. The obtained results were shown, interpreted, and then discussed. The last chapter "**Conclusions and Perspectives**" summarized most important contributions and conclusions, and then proposed new prospects for project further development.

Introduction

Les produits riches en lipides tels que les huiles de poisson, sont préconisés pour leur teneur élevée en oméga-3, une famille d'acides gras polyinsaturés (AGPI) ayant des effets bénéfiques sur l'homme (Uauy andValenzuela, 2000). Ces effets ont notamment été observés au niveau de la peau mais également dans le cadre de la prévention et du traitement de certains cancers, de maladies neurodégénératives et de troubles inflammatoires (Ait-Yahia *et al.*, 2003 ; Braga *et al.*, 1998 ; Gogus andSmith, 2010 ; SanGiovanni *et al.*, 2000 ; Sies, 2004 ; Uauy *et al.*, 1992). Toutefois, l'homme synthétise ces AGPI d'une façon limitée. C'est pourquoi un complément de ces lipides par l'alimentation peut parfois s'avérer nécessaire.

L'utilisation des acides gras oméga-3 pour la préparation d'aliments enrichis reste néanmoins limitée par leur sensibilité particulière à l'auto-oxydation. Ce phénomène est responsable du rancissement des aliments, associé à une perte de leur valeur nutritionnelle et sensorielle (Uauy andValenzuela, 2000).

Diverses solutions peuvent être mises en œuvre pour limiter cette voie de dégradation. La plus couramment employée par les industriels, consiste en l'ajout d'agents antioxydants (Wanasundara andShahidi, 1996). Ces composés peuvent minimiser le phénomène de rancissement au sein des aliments et retarder la formation des produits toxiques d'oxydation. Ceci conduit à un maintien de la qualité nutritionnelle et organoleptique des matrices alimentaires ainsi qu'à une augmentation de la durée de vie des produits (Jadhav *et al.*, 1996). Parmi les substances antioxydantes disponibles, les composés phénoliques d'origine naturelle suscitent de plus en plus d'intérêt auprès des chercheurs et des industriels pour protéger les produits à base de lipides (Figueroa-Espinoza andVilleneuve, 2005).

Toutefois, l'efficacité de ces molécules est souvent limitée par leur faible solubilité dans de nombreux milieux, conduisant à des phénomènes de migration et de séparation de phases dans les matrices alimentaires, et par suite à un éloignement des espèces oxydables et des agents antioxydants.

Pour contourner cette difficulté, une solution envisageable consiste à combiner les AGPI et les antioxydants au sein d'une même structure moléculaire. Les effets attendus d'une telle association sont une protection efficace des AGPI contre l'auto-oxydation et une augmentation de la solubilité du composé phénolique dans la phase hydrophobe des aliments. Par ailleurs, de tels composés bimodulaires sont susceptibles de cumuler les effets bénéfiques des AGPI et des phénols lorsque ces derniers sont pourvus de propriétés biologiques particulières (Mbatia B. *et al.*, 2011). On peut ainsi espérer obtenir des molécules actives plus stables, facilement incorporables et pourvues de propriétés biologiques accrues ou caractérisées par de nouvelles activités biologiques (Shahidi F. and Zhong, 2010). L'intérêt de ces composés est certain et justifie la mise en œuvre de travaux spécifiques pour préciser leur potentiel applicatif et répondre à **un certain nombre de questions scientifiques :**

- Est-ce que le fait de greffer un composé phénolique antioxydant sur les AGPI améliore vraiment leur stabilité ?
- Ce greffage va-t-il jouer sur la biodisponibilité du composé phénolique et des AGPI?
- L'activité antioxydante du composé phénolique va-t-elle être maintenue ?
- Quelles sont les nouvelles activités des molécules produites ?
- Est-il possible de produire ces nouvelles molécules à l'échelle industrielle et de les incorporer dans des produits alimentaires ?

Dans ce contexte, et au vu de ces questionnements, l'objectif de ce travail de doctorat est le développement d'un procédé enzymatique sans solvant, permettant de produire de nouveaux ingrédients alimentaires, combinant des AGPI (plus spécifiquement le DHA) et un composé phénolique antioxydant au sein d'une même structure moléculaire. A l'issu de la synthèse, il s'agira de caractériser les nouvelles molécules produites et de déterminer leurs propriétés et activités biologiques. Enfin, leur incorporation dans des matrices alimentaires sera étudiée dans l'objectif de préciser leur potentiel applicatif. Les travaux porteront dans un premier temps sur une molécule modèle associant le DHA et l'alcool vanillique ; dans un second temps, l'étude sera étendue à des milieux plus complexes, issus de l'incorporation d'alcool vanillique dans de l'huile de saumon par un procédé enzymatique d'alcoolyse.

Les principaux objectifs de ce projet de recherche étaient les suivants:

1. Développer et optimiser un procédé enzymatique pour la synthèse de l'ester de DHA et de l'alcool vanillique (DHA-VE) en présence de la lipase B de *Candida antarctica* immobilisée (Novozym 435®).

2. Purifier et caractériser la structure de l'ester DHA-VE.

3. Etudier la stabilité à l'oxydation de l'ester DHA-VE sous différentes conditions de stockage.

4. Etudier les activités biologiques de l'ester DHA-VE (activité antioxydante, activité neuroprotectrice)

5. Etudier l'effet de l'incorporation de l'ester DHA-VE dans des denrées alimentaires modèles (margarine et émulsion huile dans eau) sur la stabilité à l'oxydation de la phase lipidique.

6. Appliquer le procédé enzymatique d'alcoolyse pour la production d'esters d'alcool vanillique et d'acides gras polyinsaturés issus de l'huile de saumon.

7. Etudier la stabilité à l'oxydation et le potentiel antioxydant du milieu réactionnel brut issu de l'alcoolyse de l'huile de saumon.

Ce rapport sera organisé en quatre chapitres :

Le premier chapitre, "***Recherche Bibliographique***", résumera des exemples de travaux montrant les effets bénéfiques des oméga-3 et l'activité antioxydante des composés phénoliques. Seront abordées également, les différentes méthodes de synthèse chimique et enzymatique des composés associant composés phénoliques et AGPI, leurs propriétés, ainsi que leurs domaines d'application.

Le second chapitre, **"Matériels et Méthodes"**, présentera les différentes techniques expérimentales utilisées pour la réalisation de ce travail ainsi que les matériels et équipements associés.

Le chapitre «**Résultats et discussion**» se compose de quatre parties. La première partie vise à développer un procédé enzymatique sans solvant pour la synthèse de l'ester de DHA et d'alcool vanillique (DHA-VE) dans des quantités permettant la mise en place d'études *in vitro* et *in vivo* et des essais de formulation dans des matrices alimentaires modèles. L'objectif principal de la deuxième partie est d'étudier l'effet du greffage de l'alcool vanillique sur la stabilité à l'oxydation du DHA stocké dans différentes conditions. La troisième partie porte sur la possibilité d'appliquer le procédé enzymatique développé à la synthèse d'extraits riches en esters phénoliques d'acides gras polyinsaturés, à partir d'huile de saumon. Il s'agira également de vérifier la stabilité à l'oxydation de ces extraits et de préciser leur potentiel antioxydant. Enfin, ce chapitre se termine par une étude relative à l'incorporation de l'ester DHA-VE dans deux matrices modèles.

Le dernier chapitre, **Conclusions et Perspectives**", soulignera les principales contributions de cette étude et proposera de nouvelles suggestions pour la poursuite de ce projet de recherche.

Literature review

Chapter 1: Literature review

Dietary fat is an essential component for digestion, absorption, and transport of fat soluble vitamins and phytochemicals, such as carotenoids and lycopenes. Dietary fat contributes approximately 34% of the energy in the human diet. Because fat is a main source of energy (9 kcal/g), humans are able to obtain adequate energy with a reasonable daily composition of fat-containing food items products.

1.1. Fatty acids

Fatty acids are classified as saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), and polyunsaturated fatty acid (PUFA). The term essential fatty acids (EFA) refer to those polyunsaturated fatty acids (PUFA) that must be provided by food because these cannot be synthesized in the body yet, and they are necessary for health. There are two families of EFA, omega-3 (ω-3) and omega-6 (ω-6). ω-3 and ω-6 structures are based on the position of the double bond from the methyl (omega) terminal of the aliphatic carbon chain (Gogus andSmith, 2010 ; Murray, 2014). The parent fatty acid of the ω-6 series is linoleic acid (18:2n-6), and the parent fatty acid of the ω-3 series is linolenic acid (18:3n-3.). ω-3 includes a- linolenic acid (ALA), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA) (Fig. 1).

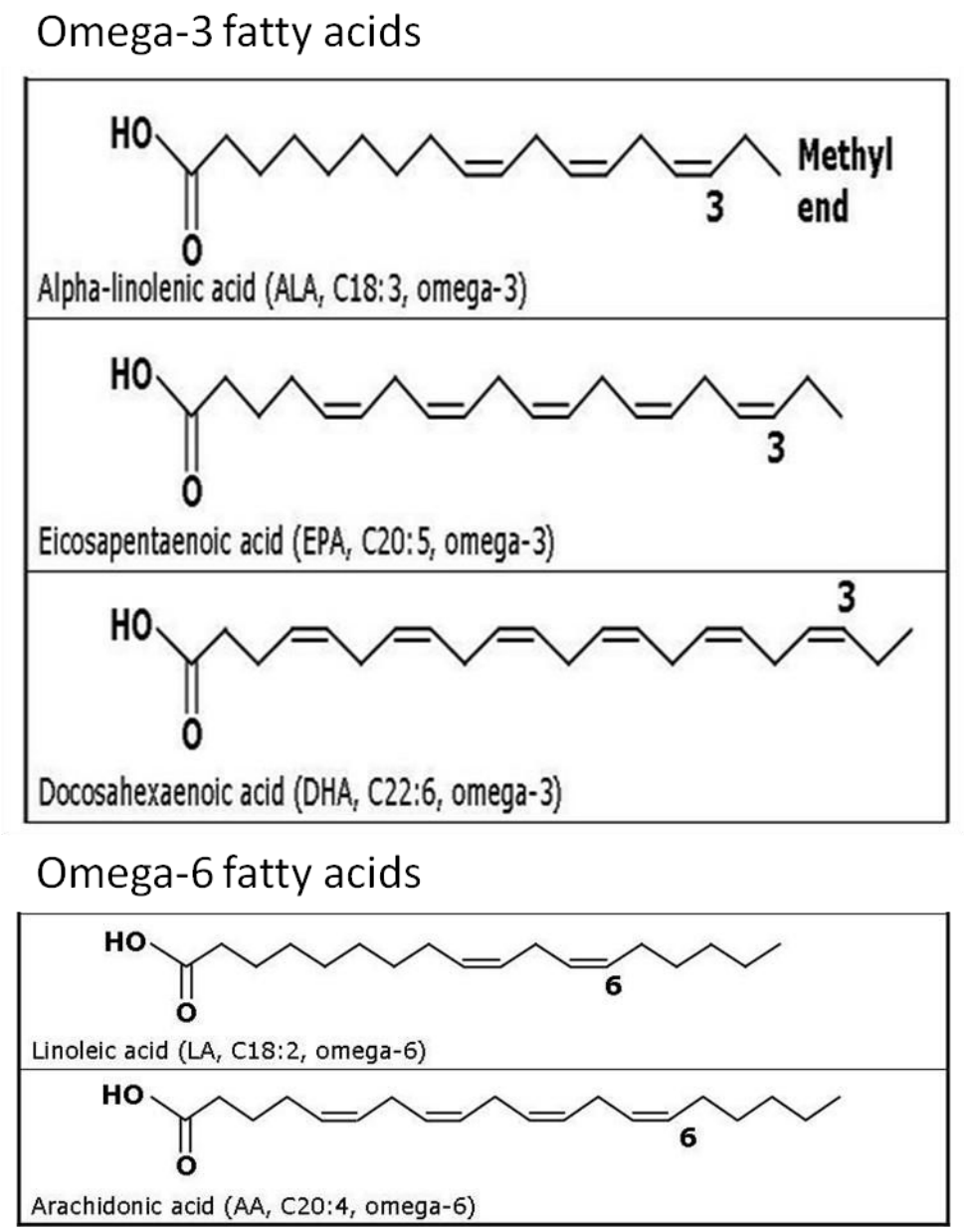

Figure 1 . Omega-3 and omega-6 fatty acids

Humans body can synthesized long-chain (20 carbons or more) omega-6 fatty acids, such as dihomo-gamma-linolenic acid (DGLA; 20:3n-6) and arachidonic acid (AA; 20:4n-6) from linoleic acid and long-chain omega-3 fatty acids, such as eicosapentaenoic acid (EPA; 20:5n- 3) and docosahexaenoic acid (DHA; 22:6n-3) from linolenic acid through a series of desaturation (addition of a double bond) and elongation (addition of two carbon atoms) reactions (Domenichiello *et al.*, 2014). Unlike the linolenic and linoleic acid, oleic acid (18:1n-9), is consumed in substantial

amounts in the typical Western diet and is not an essential fatty acid. There is little eicosatrienoic acid (ETA; 20:3n-9) in cell membranes, however, probably because of the overwhelming competition from dietary linoleic acid for the relevant desaturase and elongase enzymes. The pathways for desaturation and elongation of ω-3 and ω-6 fatty acids are given in Fig. 2.

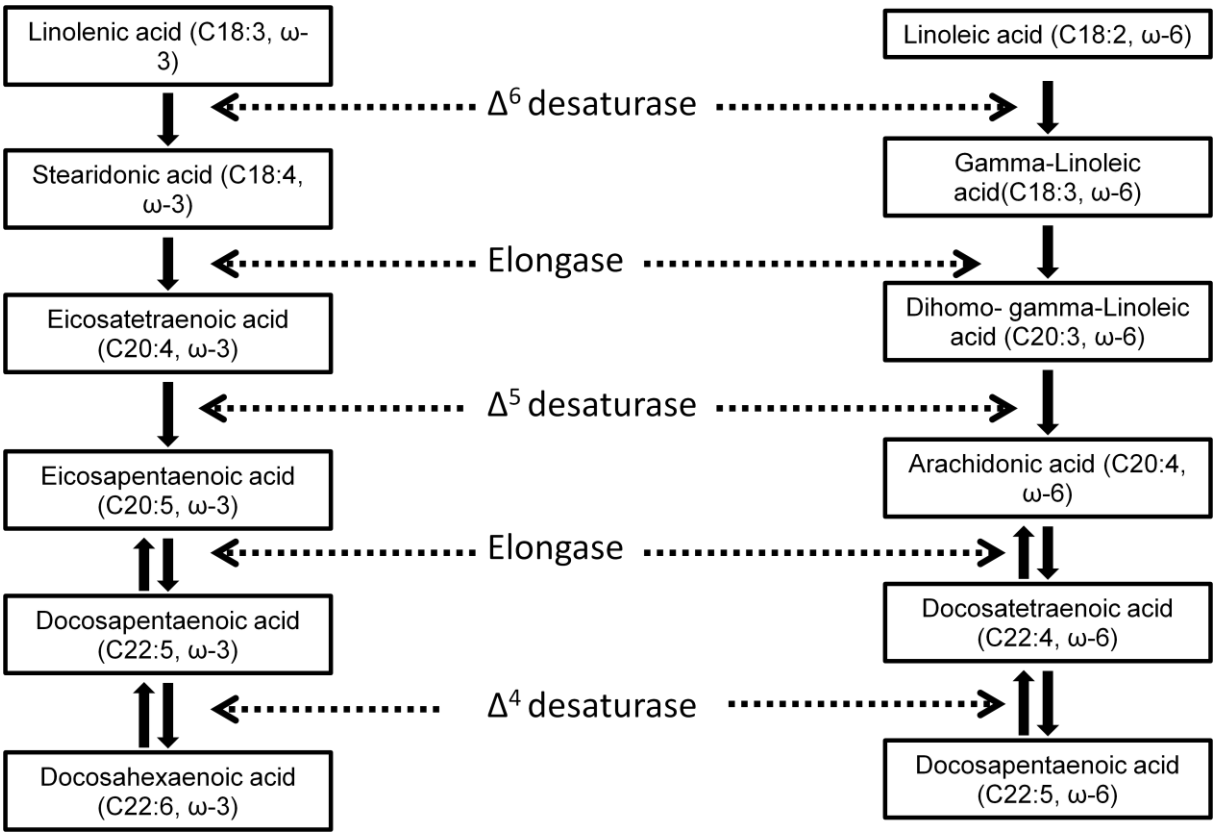

Figure 2. Desaturation and elongation pathway of ω-3 and ω-6 fatty acids.

1.2. Health Benefits of omega-3 fatty acids

The ω-3 fatty acids provide a wide range of benefits from general improvements in health to protect against inflammation and disease. Several studies have indicated that the consumption of ω-3 fatty acids provide benefits in reducing the risk of cardiovascular diseases (Gogus andSmith, 2010 ; Ruxton *et al.*, 2004). DHA and EPA have been used in a number of small clinical trials to understand their efficacy and shown to possess immunomodulatory properties depending on their

localization in different cell types. DHA is selectively incorporated into retinal cell membranes and postsynaptic neuronal cell membranes, suggesting that it plays important roles in vision and nervous system function (Igarashi *et al.*, 2013 ; Kuratko *et al.*, 2013 ; Swanson *et al.*, 2012a). Brain DHA content may be particularly important, since animal studies have shown that depletion of DHA in the brain can be resulted in learning deficits. It is not clear how DHA affects brain function, but changes in DHA content of neuronal cell membranes could alter the function of ion channels or membrane-associated receptors, as well as the availability of neurotransmitters (Arab-Tehrany *et al.*, 2012 ; Cunnane *et al.*, 2013). Increasing ω-3 fatty acid intake enhances the DHA content of cell membranes, resulting in higher proportions of DHA in the body.

The ω-3 fatty acids are reported to associate with the brain development; also it is important for the vision and the functions of the reproductive system. This may be due to the fact that DHA is a component of brain nerve synapses, in the eye's retina, in the testes and sperms and plays a vital role in the development and functions of these organs and systems (Cunnane *et al.*, 2013). The nervous system contains approximately 35% PUFAs as its lipid content; most of which are LC PUFAs. In addition, higher prenatal intake of DHA has been shown to be associated with improved visual, cognitive, and motor development in off-spring. Children given ω-3 PUFAs supplemented formula demonstrated enhanced visual and mental capabilities (Swanson *et al.*, 2012a). While in human adults, clinical studies have suggested a low intake or inadequate. The ω-3 fatty acids possess anti-thrombotic properties, which in combination with their anti-inflammatory effect is likely to positively aid cardiovascular disease treatment. DHA and EPA also appear to possess anti-cancer and anti-apoptotic effects. Additionally, these PUFAs suppress

gene expression of lipogenic genes in the liver and trigger adipose fatty acid oxidation, suggesting a potential role against obesity (Murray, 2014 ; Wu F. J. *et al.*, 2014).

The benefits of ω-3 fatty acids to human health are widely acknowledged. Dietary consumption of ω-3 fatty acids by incorporation in the foods is ultimately the most effective mechanism of providing them to the average consumer. However, the fishy odor and flavor that pervades products naturally rich in ω-3 fatty acids, like fish oil, inhibits direct consumption. Developed new techniques to eliminate these odors would allow the fortification of more foods with ω-3 fatty acids, which would then increase the probability that Western diets would have the overall and consistent increase in ω-3 fatty acids, needed to bring about long term health improvements.

1.3. Phenolic compounds

Phenolic compound is chemically defined as a substance that contains an aromatic ring containing one or more hydroxyl substitute including functional derivatives (Rice-Evans *et al.*, 1996). Phenolic compounds are a large group of the secondary metabolites widespread in the plant kingdom. They are categorized into classes depending on their structure and subcategorized within each class according to the number and position of hydroxyl group and the presence of other substituent. In general, phenolic compounds are present in a wide variety of food plants as esters or glycosides conjugated with other compounds, such as flavonoids, alcohols, hydroxyl fatty acids, sterols and glucosides. Phenolic compounds found in foods may be categorized accordingly to three groups, simple phenols and phenolic acids, hydroxycinnamic acid derivatives and flavonoids. The simple phenols include the monophenols, such as p-cresol found in berry fruits (e.g. raspberry, blackberry) and

diphenols, such as hydroquinone found commonly in vanilla (Acosta-Estrada *et al.*, 2014 ; Velderrain-Rodriguez *et al.*, 2014).

Phenolic compounds play a major role in the protection against oxidation processes. The antioxidant properties of phenolic compounds can act as free radical scavengers, hydrogen donators, metal chelators and singlet oxygen quenchers (Carocho andFerreira, 2013 ; Gulcin andBeydemir, 2013). The antioxidant properties of phenolics are mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers. Commonly, the *in vitro* methods often do not correlate with the ability of phenolic compounds to inhibit oxidative deterioration of foods. To accurately evaluate the potential of antioxidants in foods, models must be developed that have the chemical, physical and environmental conditions expected in food products. Because these factors are not consistent throughout all food systems, individual models must be developed.

The use of natural phenolic as antioxidants has been increasing because the most widely used and commercially available antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and tert-butylhydroquinone (TBHQ) are not considered as safe due to their suspected role as carcinogenesis promoters (Karre *et al.*, 2013). However, the effectiveness of these antioxidants can sometimes be limited due to their poor solubility in hydrophobic media, migration phenomena and phase separation in food matrices, thus leading to a shift away from oxidizable species and antioxidants. One of the possible solutions is combining PUFA with phenolic antioxidant compounds within the same molecular structure named phenolic lipids (PL) by forming an ester bond via an enzymatic esterification (Liu L. Y. *et al.*, 2014b ; Zhang H. Y. *et al.*, 2014 ; Zhu *et al.*, 2014). Lipase-catalyzed synthesis of different types of PL, either in solvent-free media or in presence of an

organic solvent, in operating batch wise or in continuous mode, has been widely described in the literature.

1.3.1. Nutritional and antioxidant properties

Phenolic compound are natural antioxidants that present in food or in the body, to delay or stop the oxidation of that substance. The main advantage of these natural antioxidant are : (1) they are readily acceptable by the consumers; (2) they are considered to be safe; (3) no safety tests are required by legislation; and (4) these natural antioxidant is identical to the food which people have taken over a hundred years or have been mixing with food. Phenolic compounds are associated with nutritional and organoleptic qualities of foods from plant origin (Gulcin andBeydemir, 2013 ; Velderrain-Rodriguez et al., 2014). Phenolic compounds at low concentration protect foods from autoxidation, but at high concentration, they can cause undesirable discoloration as a result of their interaction with the carbohydrate or protein components. The autoxidation reaction of PUFA is a three step reaction including initiation, propagation and termination (Fig. 1.3). The initiation step could be induced through the exposure of the lipid to light, heat, ionizing radiation, metal ions, and metalloprotein catalysts or by enzyme lipoxygenase.

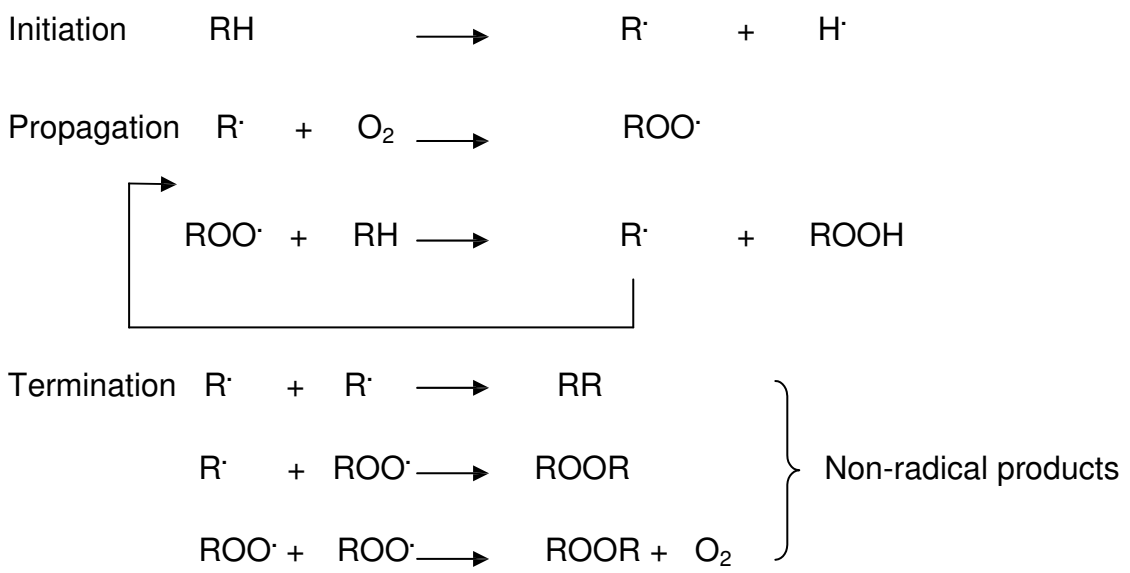

Figure 3. Autoxidation reaction, species identification is as follows: lipid (RH), Oxygen (O2), free radicals, (R. and H.), peroxy radical (ROO.) and non-radical products (RR, ROOR), including hydroperoxides (ROOH).

Phenolic antioxidants are able to delay the oxidation process of the lipid by acting as free radical quenchers (Fig. 1.4).

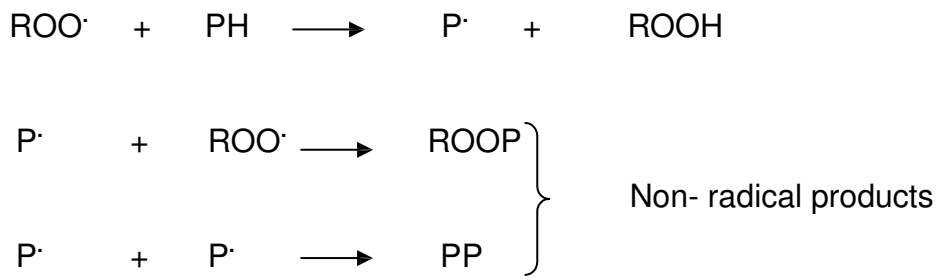

Figure 4. Radical scavenging action of phenolic antioxidants, where PH is the phenolic antioxidant, P· is phenolic radical, ROO· is the peroxide radical and ROOH is the hydroperoxide.

The phenoxy radical intermediates formed are relatively stable due to resonance delocalization of unpaired electrons around the aromatic ring and the lack of appropriate sites for attack by molecular oxygen (Fig. 1.5).

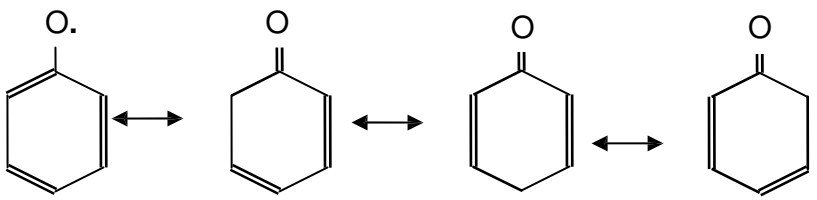

Figure 5. Phenoxy radical resonance stabilization

Among naturally found phenolic compounds, phenolic acids are of high interest due to their potential biological properties (Torres de Pinedo A. T. et al., 2007a ; Torres de Pinedo A. T. et al., 2007b). Many phenolic acids are known to be potent antioxidants through their radical scavenging activity and due to their chemical structure, the reactivity of phenolic acids increases as the number of hydroxyl and methoxyl groups increases (Silva et al., 2000). The consumption of fruits, vegetables and soft drinks such as tea and coffee, which contain phenolic compounds, has been linked to lower risk of some diseases; such as cancer and CVD (Kuriyama, 2008 ; Yang Chung S. andLandau, 2000). However, the use of phenolic acids as natural antioxidants in foods and nutraceutical supplements has the limitation of low solubility in oily-based media . Nevertheless, lipase-catalyzed reactions of lipids with phenolic acids could produce structured lipids with phenolic moieties, which would have health benefits and improved solubility characteristics (Sabally K. et al., 2006b ; Sabally Kebba et al., 2007 ; Sorour Noha et al., 2012a ; Stamatis et al., 2001).

4. Synthesis of phenolic lipids (PL) compounds

Phenolic lipids (PL) are types of fats and oils modified to improved nutritional or physical properties by incorporate phenol compound on the glycerol backbone. Phenolic lipids play an important role as antioxidant and biological active compounds, but their contents in the nature are minor and the procedures for separation and purification are not easy, very expensive and take a long time, which makes their applications in the food or cosmetic industry very inconvenient. Consequently, the synthesis of PL has attracted more attention in recent years due to it is a good way to improve the hydrophobic nature of phenolic compounds, which could be achieved by chemical or enzymatic synthesis.

1.4.1. Chemical synthesis of phenolic lipids

Chemical synthesis is a traditional method that used for PL preparation. Synthesis of PL through chemical synthesis could be done by using Friedel–Crafts acylation reaction or Fisher acid catalysis esterification. These processes are generally carried out at relatively high temperatures and pressures under anhydrous conditions, using rather unspecific alkali metal or alkali catalysts. Some related works have been provided in this topic, one of them is the work of (Qianchun *et al.*, 2011) about the chemical synthesis of phytosterols esters of polyunsaturated fatty acids (PUFA), that could be used in different formulations of functional foods. Direct esterification of phytosterols with PUFA was catalyzed by sodium bisulfate to produce sterol esters of PUFA without organic solvent. The modeling of sodium bisulfate with superfluous fatty acids as solvents to synthesize phytosterols esters of PUFA was successfully performed with degree of esterification up to 96% and less oxidative products in the reaction process (Qianchun *et al.*, 2011).

The chemical esterification of flavonoids with some fatty acids was provided by (Mainini *et al.*, 2013) and its product exhibited lipophilic, antiradical and antioxidant properties. Works reported by (Zhong andShahidi, 2011;2012) on epigallocatechin gallate (EGCG) the predominant catechin in tea was structurally modified by esterification with fatty acids, including stearic acid (SA), docosapentaenoic acid (DPA), eicosapentaenoic (EPA) and docosahexaenoic (DHA). The esterification of EGCG with these fatty acid using acylating agents, namely, the acyl chlorides, resulted in yields of 65.9, 42.7 and 30.7 for SA, EPA and DHA respectively (Zhong andShahidi, 2011). This esterification leads to produce various compounds that have anti-inflammatory effect and also showing higher inhibition effect against hydroxyl and peroxyl radical-induced DNA scission (Zhong andShahidi, 2012). Chemical synthesis of PL meets some partial needs to a certain extent, this pattern possesses

a low degree of regioselectivity and is generally accompanied by drastic reaction conditions, many intermediary stages and purification steps to remove byproducts and catalyst residues and generating extra wastes to dispose of. The main drawbacks to chemical transesterification are: (1) non-selectivity leading to random distribution of FAs, (2) isomerization of sensitive PUFAs by the alkali catalyst, (3) production of fatty acid soaps and unwanted by products and (4) requiring substantial post treatment and downstream processes, especially when food applications are concerned.

1.4.2. Enzymatic synthesis of phenolic lipids (PL)

Enzymes are used more and more in various applications of different fields such as pharmaceutical, cosmetic or food industry. In the past years, a better understanding of enzymes functionalities and catalytic behaviors, together with the progress of molecular engineering has led to new applications for various types of enzymes. Enzymatic synthesis of PL from fats and oils is receiving a lot of attention as a method for their modification because of the enzymes offers the advantages of milder reaction conditions, minimization of side reactions and by product formation, a selective specificity, a wider variety of pure synthetic substrates, fewer intermediaries and purification steps, and a more environmentally friendly process (Torres de Pinedo A. T. *et al.*, 2005b). Even if most reactions of enzymes may be more expensive than chemical reagents, the enzyme catalyzed acylation is a well-mastered technique for synthesis of selectively modification of PL at present. A high degree of conversion to the desired products could be achieved under the optimal reaction conditions. The enzymatic processes can be used in the production of fats and oils containing beneficial fatty acids and phenolic compounds. Some reviews have given a comprehensive understanding and shown a whole outline on the enzymatic synthesis of PL (Aziz andKermasha, 2014b ; Karboune *et al.*, 2008 ;

Sabally Kebba *et al.*, 2006a ; Sorour N. *et al.*, 2012b ; Tan *et al.*, 2012 ; Zheng Y. *et al.*, 2010).

In particular, enzymes appear to be very effective for the synthesis of molecules involving the grafting of a lipophilic moiety or a hydrophilic one. This review will be described and discussed some of the recent works in the field of enzymes assisted acylation of fatty acids with phenolic compounds in order to modify the hydrophilic/lipophilic properties of the initial molecules to obtain new products with multi-functional properties combining for example, antimicrobial, antioxidant and emulsifying properties. The enzymatic synthesis of phenolic lipids have been reported previously (Hong *et al.*, 2012 ; Mbatia B. *et al.*, 2011 ; Speranza andMacedo, 2012 ; Tan *et al.*, 2012 ; Tan andShahidi, 2013). A lot of enzymes can be used in the synthesis of PL and selectivity is the most important characteristics of enzymes used in phenolic lipid synthesis. Lipase is the most enzymes used in this type of process because of high selectivity, lower overall reaction time and fewer side reactions when compared with chemical methods (Li C. *et al.*, 2014). An example of a synthesis reaction catalyzed by the lipase is shown in Figure 1.6. This overwhelming interest is based largely on consumers' desire to maintain overall well-being with minimal effort and an industries' ability to respond to this need. Furthermore, with the consumption of manufactured foods continually on the rise, there is a distinct advantage to providing more healthful choices for consumers. The concept of a natural phenolic lipid composed of a long-chain aliphatic and phenolic moiety readily fits this mould, particularly since the inclusion of unsaturated lipids into these compounds could result in additional nutritional benefits. Lipases constitute the most important group of biocatalysts for biotechnological applications.

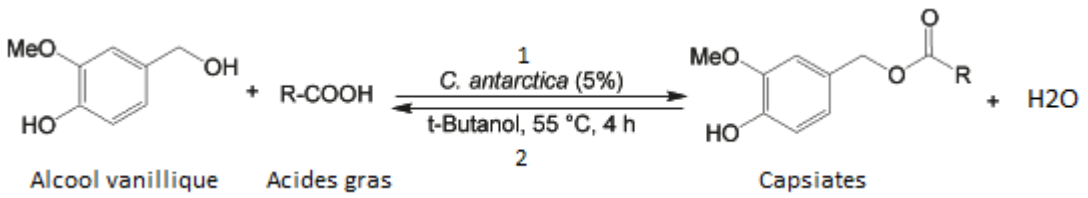

Figure 6. Enzymatic synthesis reaction capsiates (Fatty alcohol-vanillic acid ester) catalyzed by lipase Novozym 435® (Reddy *et al.*, 2011).

1.5. Lipases

1.5.1. Definition, sources and applications

Lipases enzymes are defined as glycerol ester hydrolyses that can hydrolyze tri-, di- and monoacylglycerols (Houde *et al.*, 2004 ; Sandoval *et al.*, 2012b). Lipases are soluble in water as a result of their protein nature but it could be act on lipids, which are water insoluble, at the interface between oil and water (Jaeger andEggert, 2002 ; Sandoval *et al.*, 2012b) and catalyze esterification, transesterification in addition to the hydrolytic activity on TAG (Gog *et al.*, 2012 ; Leung *et al.*, 2010 ; Rodrigues andAyub, 2011). Lipases are originated from a wide variety of sources including animals, plants and microorganisms. Animal lipases include pregastric esterase, pancreatic lipase and lingual lipases (Sandoval *et al.*, 2012b). Plants such as wheat germ and castor beans also contain lipases (Barros *et al.*, 2010 ; Villeneuve, 2003). Finally microbial sources including yeast (*Candida* and *Geotrichium*), moulds (*Rhizopus, Aspergillus*) and bacteria (*Bacillus,Pseudomonas*) (Sandoval *et al.*, 2012d). Lipases are widely used because of their ready availability, low cost of production, utility in food, biotechnology and pharmacology (Sandoval *et al.*, 2012c). Novel biotechnological applications have been successfully established using lipases for the synthesis phenolic lipids, the production of pharmaceuticals, agro-chemicals, and flavor compounds (Dhake *et al.*, 2013 ; Houde *et al.*, 2004 ; Sandoval *et al.*, 2012a). Moreover, the use of lipases in the food industry is

increasing due to the need for the production of esters, biodegradable polyesters and specific FAs (Jala *et al.*, 2012).

1.5.2. Mechanism of action

Lipase catalyzed reactions have been gained a lot of interest over the last years; the major reason for this is lipase can promote either ester formation or ester hydrolysis. Moreover, lipase can control the acylation and deacylation to produce specific fatty acids and triacylglycerols (i.e. phenolic lipids). Lipase-catalyzed reactions can be classified into three groups which are hydrolysis, esterification and transesterification (Kamal *et al.*, 2013).

1.5.2.1. Hydrolysis

Hydrolysis of lipids by lipases refers to the splitting of fat into its constituent acids and alcohols in the presence of water. Lipase catalyzed hydrolysis can be used for the preparation of fatty acids from oils, especially for the selective hydrolysis and concentrations of PUFAs from edible oils (Mbatia B. *et al.*, 2011). Furthermore, lipase catalyzed hydrolysis reactions only in the presence of amount of water. This is due to the fact that water molecules participate in the breaking of covalent bond in the substrate as well as subsequent incorporation of their elements into these bonds to form reaction products (Hampson and Foglia, 1999).

Different products are determined during the extent of hydrolysis reaction as shown in Fig. 1.7 Mixtures of monoacylglycerols, diacylglycerols and free fatty acids are produced; the more complete the hydrolysis, the higher the concentration of free fatty acids in the final reaction medium. Lipase-catalyzed hydrolysis reactions are ideal for removal of fatty acids from unstable oils, including conjugated or highly unsaturated fatty acids, which effectively reduce unwanted oxidation reactions (Bispo *et al.*, 2014). Lipase- catalyzed hydrolysis reactions to produce glycerol esters enriched in *w*-3 fatty acids from fish oil (Cerdàn *et al.*, 1998 ; Miranda *et al.*, 2013).

Because natural fish oils do not contain more than about one-third of their fatty acids from the ω-3 family, hydrolysis reactions are particularly helpful for the purpose of concentration.

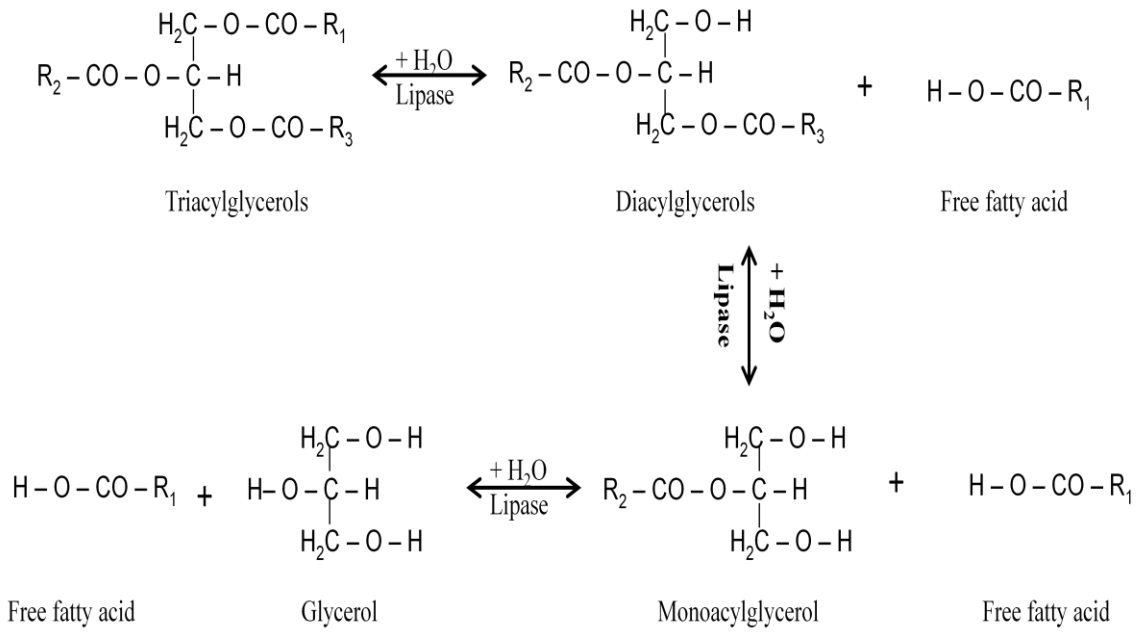

Figure 7. Enzymatic hydrolysis of triacylglycerol molecule. Reverse reaction corresponds to synthesis by esterification. R1, R2 and R3 are different acyl groups.

1.5.2.2. Esterification

Esterification is the reverse reaction of hydrolysis and is used to synthesize selected products under appropriate reaction conditions (Zniszczol andWalczak, 2014). The products of an esterification reaction are usually an ester and water. The water content of esterification reaction system strongly effects on lipase activity. Low water content shifts the equilibrium of the reaction to favour the synthesis of lipids. So that additional techniques were used to drive synthesis reaction including removal of water that formed during the process by evaporation under reduced pressure (Herbst *et al.*, 2012) or by adding molecular sieves to adsorbed the water. Direct enzymatic esterification of some primary alcohols and selected carboxylic acids were catalyzed

by the *Candida antarctica* and *Rhizomucor miehei* lipases. The reactions were performed in solvent-free medium with the removal of water (Sanchez *et al.*, 2014).

5. 2. 3. Transesterification

Transesterification is a process of acyl exchange between two molecules. This process normally takes place between an ester and alcohol (alcoholysis), an ester and an acid (acidolysis) or an ester with another ester (interesterification), and no water is involved in the reaction. Acidolysis is one of the most frequently used reactions to incorporate novel fatty acids into TAG in several researches (Kocak *et al.*, 2013 ; Ray *et al.*, 2013 ; Wang J. K. andShahidi, 2014). While interesterification involving hydrolysis and esterification, firstly hydrolysis of the TAG molecule, then followed by re-synthesis of the liberated fatty acids onto the glycerol molecule. Interesterification is another main strategy to incorporate PUFAs into TAGs. The literature reported extensive research work on the interesterification reaction (Ilyasoglu, 2013 ; Tarnowska *et al.*, 2013). Lipase catalyzed alcoholysis, acidolysis and interesterification reactions are described clearly in Fig. 1.8.

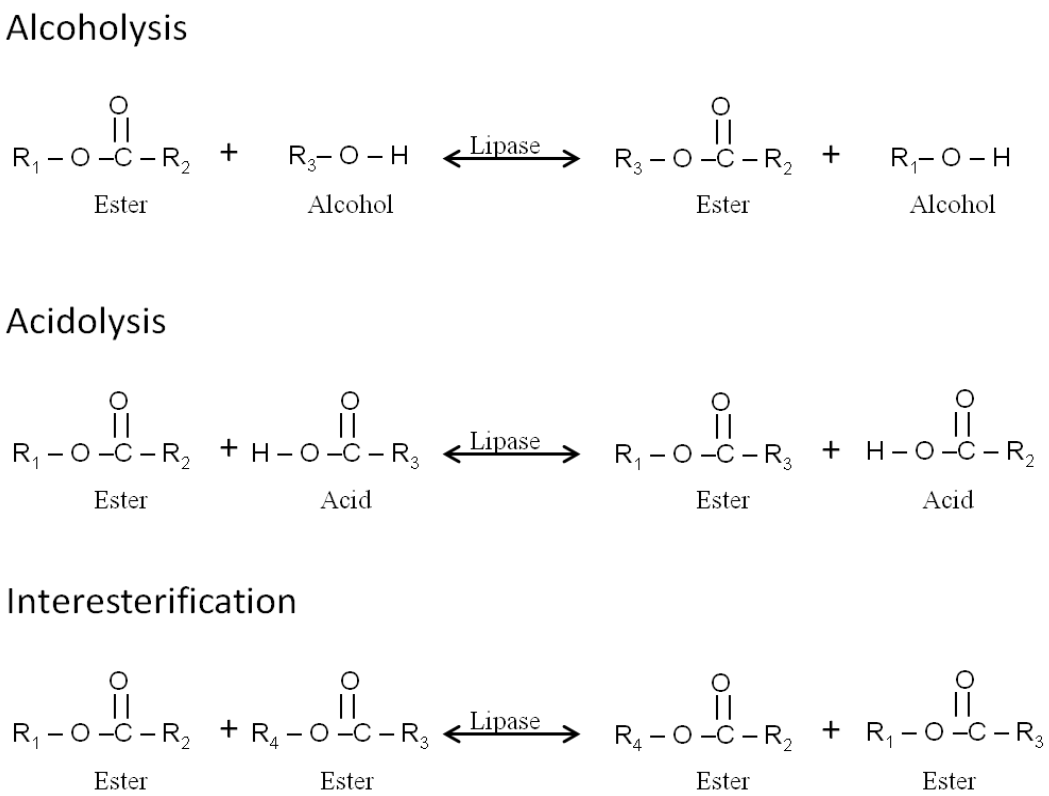

Figure 8. Lipase-catalyzed transesterification reactions. R1, R2 and R3 are different acyl groups.

1.5.3. Selectivity and Specificity of lipase

It is beneficial to have knowledge about lipase selectivity/specificity to guide research to the best choice of lipase for particular fatty acid or for synthesis of PL containing ester of a specific fatty acid. Specificity generally refers to the ability of enzyme to differentiate between several substrates. Lipases can be divided according to their specificity into three groups; (i) non-specific lipases, (ii) acyl group specific and (iii) positional specific lipases. Non-specific lipase can catalyze the release of FA from any position on the glycerol molecule. Acyl-group specific lipases catalyze the release of a particular type of FA from the TAG molecules, while positional lipases attack sn-1,3 positions on the TAG molecule. The use of positional specific lipases has lead to the production of useful TAG mixtures whose composition could not be produced by simple chemical transesterification. In recent years,

positional specific lipases have been intensively used in research purposes and food industry sectors (Hermansyah *et al.*, 2010 ; Mbatia B. *et al.*, 2010b ; Suo *et al.*, 2013)

1.6. Enzyme reactions in organic solvent media (OSM)

Enzymes in organic solvents have manifested good selectivity and stability; however, catalytic activities in this environment are generally lower than in aqueous solutions. This could be partly explained by the fact that in low water environments, enzymes are less flexible. On the other hand, the activities of enzymes also depend on the type of organic solvent, since some are known to inactivate or denature biocatalysts. Meanwhile the advantages of using organic solvent media are increased solubility of hydrophobic compounds that permits for greater interactions between substrates and enzymes as well as advantageous, partitioning of substrates and products; specifically, this is because partitioning of products away from the enzyme can decrease the possibility of inhibition due to excess product around the biocatalyst (Reddy *et al.*, 2011 ; Sanchez *et al.*, 2014).

When enzymes are placed in OSM they exhibit novel characteristics such as altered chemo- and stereo-selectivity, enhanced stability, increased rigidity, insolubility and high thermal stability (Dossat *et al.*, 2002). It has also been reported that the thermal stability of lipases can be improved in organic solvent systems since the lack of water prevents the unfolding of the lipase at high temperatures (Rahman *et al.*, 2005). The activity of lipase in OSM depends on the nature and concentration of the substrate and source of the enzyme. Moreover, the organic solvent used can dramatically affect the activity of the lipase. Lipases are more active in n-hexane, n-heptane and isooctane as compared to other solvents, such as toluene, ethyl acetate and acetonitrile (Lima *et al.*, 2004 ; Namal Senanayake andShahidi, 2002). It has been reported that the hydrophobicity of the solvent can affect the degree of acyl

migration during transesterification using a 1,3-specific lipase (Kim *et al.*, 2002). Since the choice of organic solvents based on minimization of acyl migration may conflict with maximization of transesterification, acyl migration is usually minimized by reducing reaction times (Jennings andAkoh, 1999). With increasing concern for the environment, synthesis of PL in solvent-free systems (Chaibakhsh *et al.*, 2009 ; Sun *et al.*, 2007 ; Zheng Yan *et al.*, 2009b) and ionic liquids systems (Guo andXu, 2006) has been extensively studied.

(Mbatia B. *et al.*, 2011) study the enzymatic synthesis of vanillyl esters from fish oil and vanillyl alcohol in acetone solvent medium. Lipase catalyzed esterification of vanillyl alcohol with different fatty acids was carried out by (Reddy *et al.*, 2011) to synthesis of capsiate analogues. Equimolar concentration of vanillyl alcohol and fatty acid were solubilized in *tert*-butanol and esterified using *Candida antartica* lipase (Novozyme 435) at 55°C for 4h.

1.7. Enzyme reactions in solvent free medium (SFM)

Enzymatic catalysis in solvent-free medium (SFM) has attracted considerable interest in the recent years (Feltes *et al.*, 2012). It used as an efficient approach to synthesis of natural products, pharmaceuticals, and food ingredients. Under non-aqueous conditions, the industrial utility of enzymes can be improved; recovery of product and enzyme are ease, and the ability to catalyze reactions that are not favorable in aqueous solutions (Jin *et al.*, 2003). However, it would be technically beneficial if the enzymatic reactions were performed in mixtures of substrates in the absence of solvents. Lipase catalyzed PL have been extensively studied in systems using organic solvents; however, if such a process is intended to be used in the food industry, it is preferred to develop solvent-free systems. The downside of organic solvents is that they are expensive, toxic, and flammable and their use involves

higher investment costs to meet safety requirements (Dossat *et al.*, 2002). On the other hand, solvent-free systems, which are a simple mixture of reactants and the biocatalyst, present the advantages of using nearly non-aqueous organic solvents, while offering greater safety, reduction in solvent extraction costs, increased reactant concentrations and consequently higher volumetric productivity defined as kg product per unit of reactor volume (Dossat *et al.*, 2002 ; Sandoval *et al.*, 2012b).

Lipase-catalyzed transesterification in SFM is important in industrial applications, and several studies reported that the immobilized *Candida antarctica* lipase (Novozym 435) could effectively catalyze the transesterification of oils in SFM (Aziz *et al.*, 2012 ; Feltes *et al.*, 2012). A study of (Dossat *et al.*, 2002) on transesterification of sunflower oil with butanol-1 by Lipozyme® was carried out in a SFM, and the reactor was maintained without any loss in activity for 3 months. This result was very different to that obtained using hexane, which leads to a total loss of the enzyme activity within a few hours. The mixture has interesting lubricant and surfactant properties.

Phenolic lipids have been received increasing attention in the food area, since they are a good way for providing nutraceutical FA to consumers. (Hong *et al.*, 2012) studied the esterification of vanillyl alcohol with conjugated linoleic acid under vacuum in solvent free system. Further studies on the enzymatic synthesis of structured phenolic lipids in SFM have also been conducted by (Sorour Noha *et al.*, 2012a ; Sorour N. *et al.*, 2012b ; Sun *et al.*, 2012). In these studies, Phenolic acids were esterified with fatty acids resulted in the formation of more lipophilic constituents that can be used as a nutraceutical product. In addition, feruloylated mono- and diacylglycerols were synthesized in SFM using *Candida antarctica* lipase, and the yield was 96% (Sun *et al.*, 2012).

Lipase-catalyzed synthesis in SFM has a number of advantages as compared to that in OSM, including the use of a smaller reaction volume, maximization of substrate concentration and with no additional solvent-recovery. In addition, downstream processing is easier as fewer purification steps are required providing significant cost savings, as well as toxic organic solvents are completely avoided (clean conversions), and an increase in the volumetric productivity can be achieved (Dossat *et al.*, 2002). However, there are some problems with the use of SFM, mainly, the high viscosity of the medium as well as the production of high amounts of glycerol, free FAs as by-products. These by-products affect the reaction equilibrium and limit the mass transfer rate (Zheng Yan *et al.*, 2009a). Thus the development of a bioprocess for the lipase-catalyzed synthesis in SFM is of major interest, but with great challenge.

1.8. Parameters affecting the enzyme activity and conversion yield of phenolic lipids

Grafting of phenolic compound substrates with lipids are the major difficulty to overcome in such lipase catalyzed reactions. Several key parameters are to be considered in order to achieve the reaction in satisfactory kinetics and yields and to overcome the fact that the two substrates greatly differ in term of polarity and solvent affinity.

1.8.1. Influence of solvent

The most interesting strategy is to carry out the synthesis reaction in solvent-free conditions. However, when it is not possible, the choice of an adequate solvent is important. The type of organic solvent employed can dramatically affect the reaction kinetics and catalytic efficiency of lipases (Fu Boyi andVasudevan, 2010). Two factors must be considered when solvent is selected; solvent affects the enzyme

activity and solvent effect on the equilibrium position of the desired reaction. Polarity of the solvents is an important characteristic which determine the effect of solvents on enzymatic catalysis reactions. Log *P* value, the partition coefficient between water and octanol, is used as the indicator of solvent polarity. (Laane *et al.*, 2009) reported that solvents with log P < 2 are not suitable for enzyme catalyzed systems, since they strip off the essential water from the enzyme and therefore inactivate them. However, solvents with log *P* values in the range of 2 - 4 were weak water distorters, in which enzymes display medium activity in and solvents with log P > 4 are ideal media for enzyme catalyzed systems since they do not distort the essential water from the enzyme. Therefore intermediate polarity media are often chosen. Other factors that must be taken into account in determining the most appropriate solvent for given reaction include solubility of reactants, solvent inertness, density, viscosity, surface tension, toxicity, flammability, waste disposal and cost (Akoh, 2002). A good contacte between the substrates must be obtained and the selected solvent must be solubilize them at least partially.

Various authors have tried to find original strategies to improve enzyme activity in organic solvent (Fu Boyi andVasudevan, 2010 ; Herbst *et al.*, 2012 ; Zheng Yan *et al.*, 2009a). The effect of solvent concentration on the conversion yield of phenolic lipids synthesized from flaxseed oil and phenolic acids was demonstrated by (Sabally Kebba *et al.*, 2006a). Solvent concentration of 7 % was the best concentration with 61.1 % of conversion yield. (Yang Zhiyong *et al.*, 2012) investigated and optimized the synthesis of lipophilized esters between selected phenolic acids and fatty alcohols in a binary solvent system, composed of hexane and butanone. The effect of proportions of hexane and butanone was first studied by varying the volume ratio of hexane/butanone from 85:15 to 45:55. It was found that

the conversion of phenolic acids strongly depended on the proportions of hexane and butanone. The optimal mixture ratio of hexane to butanone was found to be 65:35 with conversion yield of 96.7%.

1.8. 2. Lipase conditioning

Another important parameter in the synthesis reactions of phenolic lipids (PL) is concerning with the enzyme itself and especially its conditioning. Various techniques for lipases conditioning have greatly improved during the last ten years in the field of enzymes immobilization, chemical modification or molecular engineering (Mateo *et al.*, 2007 ; Plou *et al.*, 2002). In a very large majority, lipases are used after immobilization on a support. Different carrier materials are employed and the resulting immobilized enzyme usually exhibits an improved thermo-stability compared to its free form. Moreover, the use of immobilized enzymes allows an easy removal and recovery of the biocatalyst once the reaction is over (Horchani *et al.*, 2010). Lipase from *C. rugosa* was immobilized onto montmorillonite via two techniques i.e., adsorption and covalent binding montmorillonite (Reshmi andSugunan, 2013). The hydrolytic activity of free and immobilized lipases in water (emulsified substrate) and in heptane (insoluble enzyme powder) was assayed using p-nitrophenyl palmitate as substrate. A very promising result of this work was the observation that the activity of the immobilized enzyme becomes less sensitive to reaction conditions than that of its free counterpart. The immobilized enzymes displayed enhanced catalytic efficiency and exhibited a better storage stability. The K_m value of the covalently immobilized lipase was higher than that by adsorption. The activity of the free lipase in heptane (0.213 U/mg) was 0.51%of that in the aqueous medium (41.6 U/mg). The properties of the organo modified nanoclays and stability of immobilized lipases exhibited interesting characteristics that would be suitable for industrial biotransformation.

1.8. 3. Influence of water activity

Water content refers to the total amount of water present in the reaction system. Controlling of water activity is very important in lipid modification processes. Water content in the reaction system is a determining factor in whether the reaction equilibrium will progress toward hydrolysis or ester synthesis (Farnet *et al.*, 2013). While ester synthesis depends on low water content, too low water activity prevent all reaction from occurring. The monolayer of water on the surface of enzyme is required to maintain the three-dimensional structure of the enzyme, which is essential to enzymatic activity (Lue *et al.*, 2005). This layer acts as a buffer between the enzyme surface and the bulk reaction medium. However, too much water can cause hydrolysis of the TAG (Christopher *et al.*, 2014). The activity of lipases at different water activities is dependent on the source of the enzyme and the type of solvent and immobilization support used (Petersson *et al.*, 2007). Lipases from molds have shown to be more tolerant to low water activity than bacterial lipases. The optimal water content for most interesterification reactions by different lipases has been reported to be in the range of 0.04 to 11% (w/v) (Xiong *et al.*, 2014).

However, the amount of water in the system should be minimized in order to decrease the by-products. Lipases tend to retain the greatest degree of original activity, when immobilized on hydrophobic supports. Therefore, when the immobilized lipase contacts with oil in water emulsion, the oil phase tends to associate with and permeate the support, which can be assumed that an ordered hydrophobic network of lipid molecules will surround the support. Any water that reaches the enzyme for participation in the reaction must diffuse from the bulk emulsion. Thus, to avoid diffusional limitations, the oil phase must be well saturated with water (Chandel *et al.*, 2011).

Zhao *et al.*, (2007) investigated the effect of different reaction parameters on the enzymatic acidolysis of lard with capric acid catalyzed by Lipozyme TL-IM. They achieved the highest incorporation of capric acid (35.56 mol%) without added water. The amount of incorporation was almost constant up to 10% added water, but decreased significantly above this amount. Current research work shows that Lipozyme TL-IM catalyzed interesterification can easily be moved to the industrial sector for commercial exploitation. Both stirred tank reactors (Zhang Hong *et al.*, 2001) and PBR (Ronne *et al.*, 2005 ; Xu X. andAkoh, 2002) can be used for the production of plastic fats, and the control of water activity in the system presents no particular difficulty, as is often the case in other lipase-application systems, in which the lipase activity was not affected by the reduction of water content in the system (Alim *et al.*, 2008 ; Zhang Hong *et al.*, 2001).

1.8.4. Molecular sieve

In order to promote the synthesis of phenolic lipids by shifting the reaction towards synthesis rather than hydrolysis, a reduction of water content in the reaction mixture can be accomplished through the addition of molecular sieve pellets as dehydrating agents. (Li Zhen *et al.*, 2007) reported that the addition of molecular sieves increased the rate and conversion yield; this is due to the effect of the molecular sieves to sequester the water layer from the enzyme molecule which is essential for the water-enzyme interaction. (Mellou *et al.*, 2006) found that the conversion yield of rutin during esterification reaction with oleic acid catalyzed by immobilized *Candida antarctica* lipase B in different solvent was varied from 37 to 71 % under the use of molecular sieves (100mg/ml). However, (Karboune *et al.*, 2005) observed a 28 and 35% decrease in the maximum conversion yield upon the addition of 10 mg/mL of molecular sieves to the lipase catalyzed biosynthesis of cinnamoylated lipids. This could be explained by the fact that molecular sieves

promote the lipase-catalyzed-synthesis reactions by dehydrating; however, excess of molecular sieves will capture the necessary water of enzyme, which may inhibit the enzyme activity.

1.8.5. Substrate composition and concentration (molar ratio)

Chemical structures of the phenolic compounds have an effect on the conversion yield of the end products. Different studies presented the effect of chemical structure of phenolic compounds which are hydroxylated or methoxylated derivatives of cinnamic, phenyl acetic and benzoic acids on the conversion yield (Karboune *et al.*, 2008 ; Sorour Noha *et al.*, 2012a ; Sorour N. *et al.*, 2012b ; Stamatis *et al.*, 2001). The presence of a hydroxyl group in the sn-2 position has a negative inductive effect. Thus TAG is hydrolyzed at a faster rate as compared to DAGs, which are hydrolyzed faster than MAGs. Substrate conformation can also affect the reaction rate, since the hydrophobic tunnel in the lipase accepts aliphatic chains and aromatic rings easier than branched structures. Moreover, oxidation of substrates, such as PUFAs, could cause inhibition and decrease in lipase activity due to the production of hydroperoxides and their consequent breakdown to free radicals

Substrate concentration has an effect on the rate of enzyme hydrolysis and transesterification. So, it is very important to select a suitable substrate molar ratio in terms of reaction efficiency (incorporation level of acyl donors per unit time) and productivity (product quantity per unit time) in a reaction system. The choice of the proper substrate molar ratio is also related to the downstream processing expenses and associated difficulties of separating FFAs or acyl donors by evaporation and/or distillation. Previous studies have shown that high substrate molar ratio would require a shorter reaction time, move the reaction equilibrium to the product formation and improve the acyl incorporation ((Lee *et al.*, 2010 ; Yang Tiankui *et al.*, 2003). (Yang

Tiankui *et al.*, 2003) reported the positive effect of substrate molar ratio on the interesterification reaction between EPA and DHA ethyl esters and tripalmitin. They indicated that the optimization results suggested a molar ratio of 6 along with an enzyme load of 20% (Lipozyme TL-IM) and a 17.9 h reaction time would provide the highest incorporation. However due to the downstream purification expenses, they decided to select the optimal conditions to be a molar ratio of 5 along with a 20% enzyme load and 20 h reaction time. Lee et al. (Lee *et al.*, 2010)investigated the synthesis of 1,3-Dioleoyl-2-palmitoyl glycerol rich HMFS from tripalmitin-rich fraction and ethyl oleate by lipase-catalyzed interesterification. Similarly these authors reported an increase of OPO content (25.7%) with an increase of substrate molar ratio up to a ratio of 1:6 of tripalmitin-rich fraction to ethyl oleate.

(Sabally K. *et al.*, 2006b) investigated the enzymatic transesterification of selected PAs with TAGs, including trilinolein and trilinolenin in organic solvent media (OSM), and reported that the affinity of Novozym 435 was found to be greater for DHCA than that for ferulic acid; these authors suggested that the presence of both the methoxyl substituent and the double bond on the side of the aromatic ring of the ferulic acid could explain its lower affinity for the transesterification reactions with TAG.

(Karboune *et al.*, 2008) studied the effect of PA structure on the bioconversion yield (BY) of phenolic lipids (PLs) obtained by acidolysis of FSO with selected PAs, including hydroxylated and/or methoxylated derivatives of cinnamic, phenyl acetic and benzoic acids in OSM, using Novozym 435 as biocatalyst. The overall findings showed that the BY of PL was dependent on the structural characteristics of PAs. The highest BY was obtained with cinnamic acid (74%). In addition, Karboune et al. (2008) concluded that the presence of p-OH groups on the benzene cycle of

cinnamic acid derivatives may have an inhibitory effect on the lipase activity, since the BY decreased to 45 and 11%, respectively when *p*-coumaric and caffeic acids were used as substrates. The inhibitory effect of p-OH substituent was most likely due to their electronic donating effect rather than to their steric hindrance in the enzyme active site as the inhibition was much less significant (56%) in the presence of a double bond on the side chain conjugated with the aromatic ring of DHPA.

(Hong *et al.*, 2012) study the effects of molar ratio between vanillyl alcohol and conjugated linoleic acid (CLA) on the synthesis of capsiate analog by lipase-catalyzed esterification of vanillyl alcohol and CLA as a function of reaction time. At a molar ratio of 1:1, a rapid increase was observed in the reaction rate during the initial 3 h of reaction. However, no significant increase was observed when the reaction time was further increased, and the yield of capsiate analog at 12 h of reaction was only 40 mol%. Meanwhile, there was a remarkable difference between the yield of capsiate analog obtained at molar ratios of 1:1 and 1:2. However, no significant increase in the yield of capsiate analog occurred throughout entire reaction period when the molar ratio was increased from 1:2 to 1:5, and a maximum of 85 mol% was obtained. Thus, a molar ratio of 1:2 (vanillyl alcohol to CLA) was used in subsequent experiments.

1.8.6. Reaction Temperature

Temperature changes effect on different parameters including enzyme stability, affinity and preponderance of the competing reactions (Herbst *et al.*, 2012). Temperature normally affects lipase activity and high temperatures usually increase the initial transesterification rate. However, high reaction temperatures deactivate the enzyme due to its protein nature (Stamatis *et al.*, 2001). The optimal temperature used in transesterification reactions, is mainly based on considering properties of feedstock, such as melting behavior at different temperatures as well as the reaction

system that is with or without solvent. In a solvent-free system, the temperature is maintained high enough to keep the substrates in liquid state (Torres de Pinedo A. T. *et al.*, 2005b).

The optimal temperature for most immobilized lipases range of 30-60°C, while it tends to be lower for free lipases. Heat stability of lipase also depends on whether a substrate is present. This is because substrates remove excess water from the immediate vicinity of the enzyme, hence limiting its overall conformational mobility. (Ishihara *et al.*, 2010) studied the effect of temperature on vanillyl alcohol acylation with nonanoic acid to give vanillyl nonanoate in *n*-hexane solvent medium. The authors found that the optimum temperature for enzymatic acylation was 70°C. Higher temperature than 70°C leads to decrease the conversion yield due to the deactivation of enzyme at high temperature. The effect of temperature on the synthesis of capsiate analog by lipase catalyzed esterification of vanillyl alcohol and conjugated linoleic acid (CLA) was presented by (Hong *et al.*, 2012). The range of temperature tested was from 30°C to 60°C. The results demonstrated that the yield increased when the temperature increased from 30°C to 50°C. However, when temperature increased to 60°C there is no increase effect on the yield.

1.8.7. Enzyme concentration

Normally, as the enzyme concentration increases, the reaction equilibrium will be shifted quickly towards the synthesis (Carrin andCrapiste, 2008). However, for economic reasons, it is important to reduce the lipase loading and the reaction time. In addition, the presence of high enzyme concentration in the reaction medium may increase the probability of its collision with the substrate subsequently enhancing the reaction rate (Kumari *et al.*, 2009); however, after reaching certain enzyme concentration, the conversion yield was constant (Carrin andCrapiste, 2008). (Carrin andCrapiste, 2008) reported that during the Lipozyme IM-catalyzed acidolysis of

sunflower oil with palmitic acid and stearic acid mixture, the extent of palmitic and stearic acids incorporation was enhanced by increasing the amount of enzyme in the reaction; however, when the enzyme concentration was greater than 8% by weight of substrates, there was no significant increase in the esterification yield. The effects of lipase concentration on the synthesis of capsiate analog were depicted in the work of (Hong *et al.*, 2012) . The lipase loading range tested was between 1% and 20%. The yield was 82% during a 12 h reaction, but the reaction time required to approach yield was shortened as the lipase loading was increased up to 20%. A lipase loading of 20% would be unsuitable for this reaction because recovery of the reaction mixture might be reduced by the support of lipase. (Kobata *et al.*, 2002b) reported that excessive loading of Novozym 435 was not effective because too much swelling of immobilized lipase would take up too much space in the reaction solution. The yield obtained by a lipase loading of 10% approached equilibrium yield at a reaction time of 3 h. Consequently, the best lipase loading to produce a capsiate analog containing CLA was 10%, based on the weight of total substrates.

1.8.8. Agitation Speed

In a heterogeneous enzymatic system, it is important to ensure that the rate of substrate diffusion will not limit the rate of the synthesis reaction. The increase in agitation speed may decrease the boundary liquid layer surrounding the porous support, leading to lower diffusion limitations (Lue *et al.*, 2005) reported an increase of the enzymatic activity from 108.6 to 156.5 nmol/g/min, when the agitation speed of the system was increased from 0 to 200 rpm. The increase in the enzymatic activity indicated that external diffusion limitations of substrates did occur within the range of agitation applied. (Kumari *et al.*, 2009) reported that carrying the reaction at the optimum agitation speed can limit the external mass transfer limitations, in the case of immobilized enzymes, where the reactants need to diffuse from the bulk oil to the

external surface of the enzyme particles and from there, subsequently to the interior pores of the catalyst. In addition (Sorour N. *et al.*, 2012b) investigated the effect of agitation speed on the conversion yield of phenolic lipids synthesized from flaxseed oil and DHCA; the results have shown that the conversion yield increased significantly from 39 to 62.5% when the agitation speed was increased from 50 to 150 rpm, before it was decreased to 44.8% at agitation speed of 250 rpm. The low conversion yield could be attributed to insufficient agitation rate, a condition in which a hydrophilic layer of glycerol may be formed around the enzyme, limiting hence the mass transfer rate of the oil to the surface of the lipase.

1.8.9. Carbon chain length

The effect of carbon chain length of fatty alcohols on the reaction rate was examined by (Yang Zhiyong *et al.*, 2012), the esterification of C4–C18 straight-chain fatty alcohol with dihydrocaffeic acid (DHCA), as a model phenolic acid, were systematically evaluated. The results indicated that the conversion of DHCA was significantly affected by the number of carbon chain of fatty alcohols. Conversion yield of 95% was achieved within 3 days when hexanol was used as an acyl acceptor; while only 56% and 44% conversions were achieved when 1-butanol and octadecanol were employed, respectively. The conversions of ferulic and caffeic acids under the same conditions were much lower than was that of DHCA. In another by (Vosmann *et al.*, 2006) various alkyl cinnamates were formed in high to moderate yield by lipase-catalyzed esterification of cinnamic acid and its analogues with fatty alcohols in vacuo at moderate temperatures in the absence of drying agents and solvents.

Several carboxylic acids of different chain lengths from acetic, propionic, butyric, caproic and caprylic acids were tested via an enzymatic esterification reaction to produce hexyl ester in *n*-hexane and supercritical carbon dioxide

($SCCO_2$). The reactions were carried out at 40°C and the amount of enzyme used was 13.8 g/mol alcohol. Substrates were added at equimolar concentrations, with sufficient stirring to avoid external diffusion control. The results in both solvent shows that the reaction rate increases with the chain length of the acid, but the final yields were similar.

1.9. Analysis and characterization of phenolic lipids

The structural analyses of phenolic lipids have been carried out using a wide range of various techniques. These mainly include thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), gas liquid chromatography (GLC), and liquid chromatography mass spectrometry (LCMS). Thin-layer chromatography has been used for initial qualitative analyses of substrates by employing a wide range of organic solvent mixtures. Products from the esterification reactions characterized and analyzed by TLC using silica gel G-25 plates (Hong *et al.*, 2012 ; Mbatia B. *et al.*, 2011). The elution solvents used depend on the nature of synthesized compounds. In the study of Mbatia (Mbatia B. *et al.*, 2011) the elution solvent used was chloroform/methanol mixture (80:20, v/v) and pure chloroform; the plates were visualized under UV light (254nm) meanwhile in the work of (Hong *et al.*, 2012) the elution solvent was n-hexane/diethyl ether/formic acid (160:40:5.5, v/v/v) and the plate was visualized with 0.2 % (w/v) 2,7 dichlorofluorescein in methanol solution under UV light.

High-performance liquid chromatography (HPLC) has often been used over other instrumentation and has shown scientifically to be the overall preferred method of choice for quantification and separation of phenolic lipids following synthesis reactions (Moreno et al., 2003). Phenolic lipids were separated on C18 reverse phase column using a gradient elution system with UV detection at 280nm (Mbatia B.

et al., 2011). Gas-liquid chromatography (GLC) analysis has been conducted for determining the fatty acid composition of the synthesized phenolic lipids. REF has reported on the GC analysis of phenolic lipid esters through the use of a CP Sil CB-MS column linked to an FID detector.

Recent research on phenolic lipids has also made using liquid chromatography-mass spectrometry (LC-MS) that considered being one of the most powerful techniques used for the characterization of biomolecules due to its high sensitivity and specificity. Generally, its application is oriented towards the specific detection and potential identification of chemicals in the presence of other chemicals (in a complex mixture). LC-MS has been used for the structural characterization of lipids and phenolic lipids (Ishihara *et al.*, 2010 ; Mbatia B. *et al.*, 2011).

Many lipid systems have been studied by Fourier transform infrared spectroscopy (FTIR) in order to determine several aspects including the degree and the form of unsaturation of the acyl groups as well as their length (Mitra andBhowmik, 2000). The infrared region of the electromagnetic spectrum extends from 14,000 to 50 cm^{-1} and is divided into three areas; the far-infrared from 400 to 50 cm^{-1}; the mid-infrared region from 4,000 to 400 cm^{-1} and the near-infrared from 14,000 to 4,000 cm^{-1} (Guillén andCabo, 1997).

1.10. Application of phenolic lipids

Phenolic lipids, compounds which have been known for a century, are more recently being extensively studied not only from the biological but also from the chemical point of view. Phenolic lipids used as novel antioxidants that synthesized enzymatically. These natural antioxidants increased the antioxidant capacity and the oxidative stability of the edible oils. These products can be used as nutraceuticals for their nutritional value and antioxidant capacity as well as natural ingredients for their

physicochemical characteristics (Zhong andShahidi, 2011). Enzymatic esterification of omega-3 PUFAs with vanillyl alcohol lead to protect these compounds from oxidation and the PUFA – phenolic derivatives prepared confer the combined health beneficial properties of PUFA and the phenolic molecules (Mbatia B. *et al.*, 2011). Studies of (Zhong andShahidi, 2011;2012) indicated that antioxidant activity of esters produced from the esterification of EGCG with PUFA (EPA and DHA) was superior to that of parent compound in retarded the oxidation of bulk oil and emulsion. The results suggest that these lipophilic derivatives of EGCG could be considered for use in food preservation and health promotion (Zhong andShahidi, 2012).

Until now, most of the observed activities of phenolic lipids were rather nonspecific and resulted from their amphiphilic and phenolic nature. Further investigation on various aspects of biology may open new opportunities to exploit their properties, as, for example, chemo-preventive and antitumor agents, and to developed pharmaceuticals based on phenolic lipid compounds.

Materials and Methods

Chapter 2: Materials and Methods

2.1. Materials

2.1.1. Chemicals and enzyme

Candida antarctica lipase B (CALB) immobilized on a macro-porous acrylic resin (Novozym 435®, Novo Industry) was used to catalyze acylation reactions. Docosahexaenoic acid ethyl ester (DHA-EE) of 95% purity was supplied by KD-Pharma (Bexbach, Germany). Vanillyl alcohol (4-hydroxy-3-methoxybenzyl alcohol, VA) was purchased from Sigma-Aldrich Chemicals. α-tocopherol (purity of 98%), Tween 80, thiobarbituric acid (TBA) and 1,1,3,3-tetraethoxypropane (TEP) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Methanol and trichloroacetic acid (TCA) was obtained from Carlos Erba (Paris, France). Butylated hydroxytoluene (BHT) was purchased from Merck (Hohenbrunn, Germany). Margarine samples prima 4 were purchased from local markets in Nancy, France. According to the manufacturer, it contained approximately 60% oil. The oil phase was consisted of rapeseed oil, palm oil and copra oil. Other solvents of either analytic or HPLC grade were purchased from Merck. All other chemicals were purchased from Sigma-Aldrich Chemicals.

DHA vanillyl ester (DHA-VE) was obtained from an enzymatic esterification between DHA-EE and vanillyl alcohol. Vanillyl alcohol is a powerful aroma constituent of many food products. This phenol has been proved to be as intense as vanillin, the most abundant component in vanilla (Vanilla planifolia) (Perez-Silva *et al.*, 2006). In addition to this interesting olfactory property, vanillyl alcohol exhibits a high radical scavenging capacity that was suggested to be responsible for its anticonvulsive and antioxidant properties (Shyamala *et al.*, 2007).

Salmon heads (*Salmo salar*) supplied by a local company (Les Salaisons d'Orly, Orly, France).

2.2. Methods

2.2.1. Enzymatic synthesis of DHA vanillyl ester (DHA-VE)

2.2.1.1. Enzymatic synthesis of DHA-VE in organic medium

Enzymatic acylation reactions were achieved in organic solvent, under atmospheric pressure. Reaction media were prepared by solubilizing VA (100 mM, 15.4 g/L) and DHA-EE (200 mM) in 2 mL of acetonitrile. The solvent was pre-dried on 4-Å molecular sieves before use, aiming to low water activity below 0.1. Reactions were performed in 10-mL amber tubes submitted to orbital shaking (300 rpm) and initiated by adding 20 g/L of Novozym 435®. This protocol is further referred as solvent system.

2.2.1.2. Synthesis of DHA-VE in molten media

Reactions were performed under either atmospheric or reduced pressure. For syntheses achieved under atmospheric pressure, reaction media were prepared by solubilizing VA (200 mM, 30.8 g/L that corresponds to the maximal solubility of the substrate at 50°C) in a large excess of DHA-EE as acyl donor (2 mL), at 50°C. Reactions were performed in 10-mL amber tubes and initiated by adding 20 g/L of Novozym 435®. After 72 h of reaction, shaking was stopped allowing the decantation of enzyme particles. The supernatant was removed thus ending the reaction. This protocol led to reaction system A.

Syntheses achieved under reduced pressure were performed in the sample flask of a rotary evaporator Figure 9. Temperature and pressure conditions were set to 37°C and 500 mbar, so that the by-product of the reaction, *i.e.* ethanol, could be eliminated during the syntheses while avoiding VA evaporation. A rotation speed of 250 rpm was applied. Reaction media were prepared by solubilizing VA (162 mM, 25

g/L that corresponds to the solubility of the substrate at 37 °C) in 10 mL of DHA-EE. Reactions were started by adding 20 g/L of Novozym 435®. After 72 h, the enzyme was eliminated by filtering the reaction media. This protocol led to reaction system B.

Figure 9. Rotary evaporator

2.2.1.3. Process intensification

The production of DHA-VE was intensified by increasing VA intakes. A large quantity of VA (150 g/L) was introduced in several times (fed batch process) in 10 mL of DHA-EE. As described above, syntheses were performed in the sample flask of a rotary evaporator, at 37°C, under a 500-mbar pressure and a 250-rpm rotation speed. After the first 4 h of synthesis, the reaction medium was filtered and transferred to another flask. A new supply of VA was then carried out under a nitrogen stream. Once the phenolic compound was totally solubilized, the reaction was restarted by adding fresh enzymatic preparation. This protocol was repeated twice. At the end of the reaction, the enzyme was removed by filtration. This protocol led to reaction system C.

2.2.2. Kinetic following of the syntheses

Kinetic following of the reactions was performed by high performance liquid chromatography (HPLC) Figure 10, using a Shimadzu LC 10 system equipped with a computer-controlled system (LC Solution software). Separations were carried out on a reversed-phase Altima C18 column (150×2.1 mm, 5 µm, Grace-Alltech). VA and DHA-VE were detected at 280 nm on a multichannel photodiode-array detector (SPD-M10A VP). DHA-EE and free DHA were followed by evaporative light scattering detection (ELSD), using nebulizer and evaporator temperatures of 35°C and 45°C, respectively. Analyses were carried out with compressed air as ELSD gas at a pressure of 1.5 bars. Elution was performed using a gradient of solvent A (methanol/water 70/30 (v/v)) and B (methanol (100%)), at a flow rate of 0.2 mL/min. Elution protocol was as follows: 0-5 min: 100-0% A, 5-25 min: 100% B, 25-35 min: 100-0% B, 35-45 min: 100% A. Calibrations were made using analytical standard compounds. An aliquot from each reactor was withdrawn at predetermined times during synthesis, and then diluted 100 times in solvent A. All samples were filtered through a 0.2-µm membrane before injection.

Figure 10. High performance liquid chromatography (HPLC)

2.2.3. Purification of DHA-VE by flash chromatography

DHA-VE was purified on a system working with glass columns KONTES CHROMAFLEX packed with silica gel 60 (particle size of 40-63 µm) Figure 11. Gradient system was generated by 2 pumps Gilson model 306. Cyclohexane and ethyl acetate were used as mobile phase. The elution gradient was as follows: 0-40 min: 5-15% ethyl acetate, 40-60 min: 15- 40% ethyl acetate. The flow rate was 20 mL/min. Every minute, a sample of 20 mL was collected separately in a glass tube for proper identification. The presence of the ester in the tubes was detected by thin-layer chromatography on precoated silica gel $60F_{254}$ TLC plates, referring to the pure molecule as standard. The plates were visualized under UV light at 254 nm and then sprayed with a solution of sulphuric acid 20% in methanol. Fractions containing the ester were combined and the solvent was removed by evaporation under vacuum.

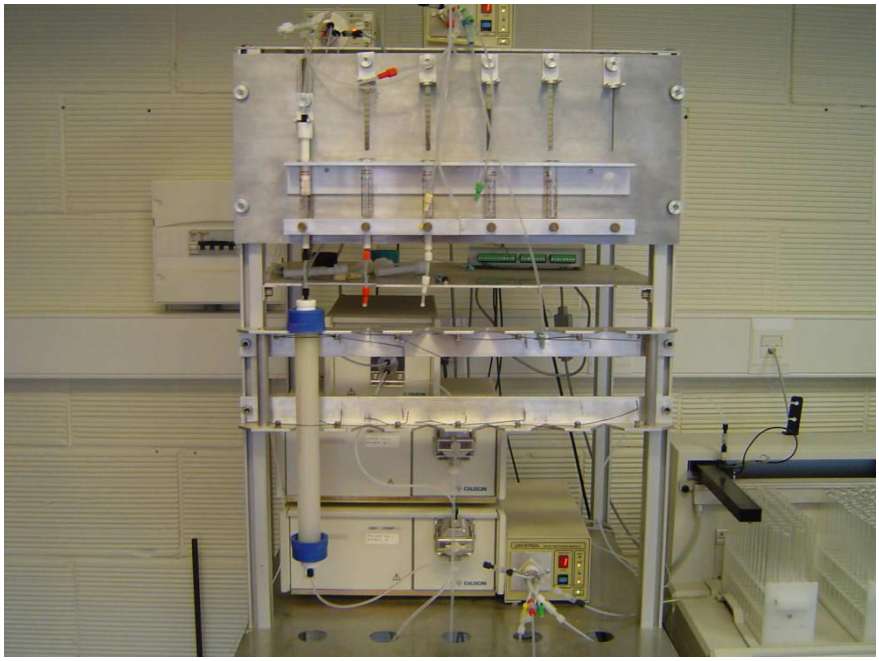

Figure 11. Flash Chromatography apparatus (robot).

2.2.4. Structural analyses

2.2.4.1. Liquid chromatography–mass spectrometry (LC–MS)

The structure of the product was determined by HPLC-MS Figure 12. The mass spectra were obtained using a binary solvent delivery pump and a linear ion trap mass spectrometer (LTQ-MS, Thermo Scientific, San Jose, CA, USA) equipped with an atmospheric pressure ionization interface operating in ESI mode. Control of equipment and data processing were realized using Xcalibur software (version 2.1).. The operational parameters of the mass spectrometer were as follows: the spray voltage was 4.5 kV and the temperature of heated capillary was set at 200°C. Flow rates of sheath gas, auxiliary gas, and sweep gas were set to 20.5 and 4 (in arbitrary units/min), respectively. Capillary voltage was -18 V, tube lens was -80 V, split lens was 11 V, and the front lens was 6.25 V. MS parameters were optimized by infusing a standard solution of DHA-VE in mobile phase (methanol/water: 70/30) at a flow rate of 5 µL/min.

Figure 12. Liquid chromatography–mass spectrometry (LC–MS)

2.2.4.2. Nuclear magnetic resonance (NMR)

The pure product was identified and characterized by NMR analyses Figure 13. NMR experiments were performed in $CDCl_3$ with a Bruker Avance DRX-400 instrument operating at a proton frequency of 400.13 MHz and equipped with a 5 mm broadband inverse detection z-gradient probe tuned to ^{13}C (100.61 MHz). For all 1D and 2D NMR experiments, pulse sequences provided by the spectrometer manufacturer were used.

Figure 13. Nuclear magnetic resonance (NMR)

2.2.5. Evaluation of antioxidant activity

2.2.5.1. Radical scavenging activity

2.2.5.1.1. DPPH• Radical Scavenging Activity method

The capacity of the compounds to scavenge the DPPH• radical (1,1-diphenyl-2-picrylhydrazyl radical) was determined as described by (Brand-Williams *et al.*, 1995) with some modifications. Briefly, 100 µl of a methanolic solution of the compound (0.5 or 1 or 2 mM) was added to 3.9 mL of a methanolic solution of the DPPH• radical (0.1 mM). Then, the total volume was adjusted to 4mL with methanol.

After 30 min of incubation at 30°C in the dark, the absorbance of the mixture was measured at 517 nm against methanol. The free radical scavenging activity (% FRSA) was evaluated by comparison with a control (3.9 mL of $DPPH^{\bullet}$ radical solution and 0.1 mL of methanol). Each measure was done in triplicate, and an average value was calculated. The free radical scavenging activity (FRSA) was expressed as the percentage of inhibition of the $DPPH^{\bullet}$ radical referring to the control (formula 1).

$$FRSA\ \% = [(Ac - As)/Ac] \times 100 \qquad (1)$$

where Ac and As are the absorbance of the control and the absorbance the sample, respectively

Antioxidant activity can also be expressed as EC_{50} value, corresponding to the concentration that leads to 50% of inhibition of the $DPPH^{\bullet}$ radical. In that case, the higher the EC_{50} value, the lesser the efficiency of the compound.

2.2.5.1.2. $ABTS^{+\bullet}$ method

The method was described by. $ABTS^{+\bullet}$ species were produced by (Re R. *et al.*, 1999a) reacting ABTS (2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) aqueous solution (7 mM) with potassium per-sulphate (2.45 mM final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h. The radical was stable under this form for more than two days when stored in the dark at room temperature. Before use, the $ABTS^{+\bullet}$ solution was diluted with ethanol aiming to obtain an absorbance of 0.7 at 734 nm and 30°C. 10µL of an ethanolic solution of the compound under study (0.5, 1, and 2 mM) were allowed to react with 1mL of diluted $ABTS^{\bullet+}$ solution for 15 min in the dark. Then the absorbance was measured at 734 nm. Ethanol was used as control. All determinations were carried out in triplicate. The FRSA was calculated using the formula 1. Just like before, the antioxidant activity can be expressed as EC_{50} value.

Free radical scavenging activities were expressed as Trolox Equivalent Antioxidant Capacity (TEAC) values. TEAC value is defined as the concentration of standard Trolox that exhibited the same antioxidant capacity as a 1-mM solution of the compound under investigation.

2.2.5.2. Inhibition of DNA scission

Inhibition activity of biomolecules towards DNA scission was determined as described by (Hiramoto Kazuyuki *et al.*, 1996b). Briefly, H_2O_2 (0.2 mM) and $FeSO_4$ (0.1 mM) were used to generate hydroxyl radicals that induced plasmid DNA strand breaking (10 µg/mL). DNA scission after 1 h was monitored by agarose gel electrophoresis. A blank (DNA only), a positive control (DNA + hydroxyl radicals) were prepared in parallel of reactions carried out in the presence of DHA and VA used separately or together. The bands were visualized under UV light and intensity of the band corresponding to the native supercoiled plasmid was measured by densitometry.

2.2.6. Study the oxidative stability of DHA phenolic esters (DHA-VE)

The main objective of these tests to study the effect of acylation between DHA-EE and VA on the oxidative stability under different storage conditions.

2.2.6.1. Accelerated oxidation test

5 g of each sample (DHA-EE, DHA-VE and reaction mixture containing 45% DHA-VE) were weighed in screw-capped glass tubes (10 ml) and stored at 100°C, 20°C and 4°C, in darkness. Oxidative stability was monitored throughout storage until significant differences between the treated samples were observed. Samples were periodically taken for further analysis.

2.2.6.2 Determination of conjugated dienes

Conjugated dienes (CD) are considered as primary products of oxidation. For each sample, the conjugated diene value was determined as previously described

(Weber *et al.*, 2008). A 0.5 mg sample was dissolved in 10 mL of n-hexane, then diluted or concentrated to obtain an absorbance between 0.1 and 0.8. The solution must be perfectly clear. The absorbance was measured at 234 nm, using n-hexane as a blank. Temperature effect on oxidation rate was illustrated by means of Arrhenius equation:

$$\ln (k) = \ln A - Ea/RT$$

where k is the reaction rate, A is the kinetic constant, Ea is the activation energy (KJ mol^{-1}), R is the molar gas constant (8.3145 J K^{-1} mol^{-1}), and T is the absolute temperature (K). Activation energy and kinetic constants were determined respectively from the slopes and the intercepts of the lines generated by regressing ln (k) vs. 1/T linear regression.

2.2.6.3 FTIR Instrumentation

Infrared spectra were recorded on a Tensor 27 mid-FTIR spectrometer (Bruker, Karlsruhe, Germany) with a deuterated triglycine sulphate (DTGS) detector, operating under Opus software (Figure 14). A ZnSe ATR sampling accessory from Spectra Tech (Shelton, CT) was used for Total Attenuated Reflection measurements. The diaphragm was set to 4 mm and the scanning rate was fixed to 10 kHz. 256 scans were performed for both reference and samples.

Figure 14. Fourier transform infrared spectroscopy (FTIR) Bruker Tensor 27.

2.2.6.4 Spectral Acquisition

A small amount of each sample, approximately 30 µL, was deposited in the attenuated total reflectance (ATR) ZnSe crystal, with avoiding the presence of air by circulating nitrogen. All spectra were recorded from 4000 to 800 cm^{-1}. To avoid high noise levels the spectra were collected with a resolution of 4 cm^{-1} to give a data point spacing of approximately 1.9 cm^{-1}, after Fourier transform and zero-filling. Spectra recorded with higher resolution gave similar frequency data for all samples but higher noise level and their registration took more time. Assignment of bands was made from comparison with literature spectral data and with reference compounds spectra included in the software spectral library. Height and area of each band were measured and calculated by using the essential FTIR software. This procedure avoided experimental errors associated with the subjectivity of external operators. After each operation, the crystal was thoroughly cleaned up, washed with ethanol and water, and then dried.

2.2.7. Biological activities and bioavailability

2.2.7.1. Primary cell cultures and treatments

Cell culture media and materials were from Invitrogen. Brain cortical neurons were taken from Wistar rat fetuses at embryonic day 16–17 and cultured in serum-free medium as described by (Pillot *et al.*, 1999). Neurons were treated with 1 µM of soluble β-amyloid peptide (Aβ) for 24 h prior monitoring cell viability using the MTT reduction assay, as previously described (Florent *et al.*, 2006). Alternatively, indicated concentrations of DHA or DHA-VE were mixed with fatty acid-free bovine albumin (0.1%, w/v) and added to the medium 24 h before treatment with Aβ.

2.2.7.2. Animals and diets

Pregnant female Wister rats were provided at gestation day 14 from Janvier (Le Genest-St-Isle, France) and arrived in our facility 3 days before dissection. Male C57BL/6J mice (10-week old) also came from Janvier breeding and accustomed for 2 weeks before starting the study. The animals were housed as described by (Grasso *et al.*, 2007) Figure 15. The animal facilities and all animal procedures were approved (# DDSV/54/04/ENV/065) by the Animal Care and Veterinary Committee of Meurthe-et-Moselle (Nancy, France). After adaptation, Mice were randomly assigned to three groups of 6 mice each and fed diets of comparable caloric density. Control mice received standard food (Harlan 2080S), while DHA-supplemented mice were fed diets prepared after incorporating either DHA-EE or DHA-VE at the concentration of 0.3% (w/w) into standard food.

Figure 15. Animals housed

2.2.7.3. Fatty-acid analysis

Retro-orbital blood samples (100 µL) were collected after 3-h fasting period in lightly anesthetized mice at baseline. These samples were immediately centrifuged to separate the erythrocytes from the plasma and frozen at -20°C until extraction and analysis. Blood samples were also collected in the same mice at the end of the 4-week dietary supplementation study. Immediately after sacrifice of anesthetized mice, hippocampal and cortical tissues were taken and frozen. FA were extracted from all samples in a methanol: dichloromethane (3:1) mix prior to transesterification, purification of methylated derivatives and separation by gas chromatography (Lepage andRoy, 1986).

2.2.8. Applied the esterification method with salmon oil

2.2.8.1. Enzymatic extraction of oil from salmon heads

Salmon heads routinely preserved at -20°C were removed from the freezer. Enzymatic hydrolysis was performed in a stirred thermostated reactor (2 L) where

raw material (15 kg) was suspended in distilled water. The adjustment of pH with 4 M NaOH was done for 15 min under mixing. The enzyme solution was then added and the reaction allowed proceeding for 2 h under nitrogen. The pH was kept constant by automatically adding 4 M NaOH during hydrolysis according to the pH-stat method. The medium was coarsely filtered to retain bones, whereas the liquid phase was subjected to subsequent centrifugation to separate oil and emulsified fractions from the underlying aqueous phase. Enzymes were inactivated in hydrolysates by heat treatment with live steam injection (95°C, 10 min).

2.2.8.2. Preparation and analysis of fatty acid methyl esters by GC

Fatty acid methyl esters (FAME) were obtained by transmethylation of lipid aliquots (100 mg) according to Ackman (1998). Samples were dissolved in 1.5mL hexane and 1.5mL borontrifluoride in methanol (8%, w/vol), and heated at 100°C for 1 h. After cooling, the FAME was extracted in hexane under nitrogen. FAME was analyzed by gas chromatography on a Perichrom 2000 system (Saulx-les-Chartreux, France), equipped with a flame ionization detector (FID) and a fused-silica capillary column (25m * 0.25mm * 0.5 mm; BPX70 SGE Australia Pty Ltd.) Figure 16. The temperature was set as follows: 2 min initial period at 70°C, then increasing at 40°c/min to reach a second step at 180°C during 8 min, and flowing out at 3°c/min to the final period (220°C, 45 min). Injection and detector ports were maintained at 230 and 260, respectively. Fatty acids were identified by comparison of their relative retention times with appropriate standard mixtures (PUFA 1 from marine source and PUFA 2 from animal source; Supelco, Bellefonte, PA, USA) and an internal standard (C23:0). The results were displayed as percentage of total identified fatty acids.

Figure 16. Gas chromatography (GC)

2.2.8.3. Lipid class analysis by thin-layer chromatography

The lipid classes were determined by Iatroscan TLC-FID MK V (Iatron Laboratories Inc., Tokyo, Japan) Figure 17. The ten silica gel chromarods-SIII held in a frame were first scanned twice immediately before sample application to remove impurities. Samples (10 mg/mL chloroform/methanol 2:1) were spotted onto the chromarods using a 1µL glass minicap pipette (Hirschmann Laborgeräte, Germany) and were submitted to migration in the following solvent systems at 20°C: hexane/diethyl ether/formic acid (80 : 20 : 2, vol/vol/vol) to separate neutral lipids, free fatty acids, and ketone traces. The air and hydrogen flow rates were set at 200 and 160 mL/min, respectively. The scan speed was set at 30 s/scan. The FID results were expressed as the mean of three separate samples. The following standards were used to identify the sample components: 1-monostearoyl-rac-glycerol, 1,2-

dipalmitoyl-sn-glycerol, tripalmitin and cholesterol. All standards were purchased from Sigma (Sigma-Aldrich Chemie St. Louis, MO, USA).

Figure 17. Iatroscan TLC-FID MK V for lipid classes

2.2.8.4. Synthesis enzymatic reaction

Before the reaction was started vanillyl alcohol and Lipase were pre-equilibrated in dissector at 4°C to establish fixed water activities for esterification. Vanillyl alcohol (VA) concentration 50g/L was dissolved in salmon oil (SO) overnight in rotary evaporator flask where the temperature was maintained at 40°C with an agitation of 200 rpm and 500 mbar pressures. The reaction was started by adding 20g/L of immobilized lipase (Novozym 435). Samples were withdrawn from the reaction mixture at relevant time intervals for the analytical monitoring of the reaction. The reaction was stopped (after 28h of incubation) by removing the enzyme. Calibration curve for vanillyl alcohol and PUFA-VE were obtained using standards and purified samples in acetonitrile. If Y = Peak area (AU) and X = concentration of detected ester (g/L), standard equations are $Y=1.27489*10^7$ X for VA, $Y=3.27756*10^6$ X for linolenic vanillyl ester, $Y=2.42107*10^6$ X for DHA-VE, $Y=4.65921*10^6$ X for

linoleic vanillyl ester and $Y=4.18232*10^6$ X for oleic vanillyl ester. Yields were calculated from the amount of substrate having reacted compared to the initial quantity of substrate.

2.2.8.5. Analysis and monitoring of reaction mixtures by HPLC and LC-MS

The extent of the reaction was monitored by HPLC analysis. HPLC analysis was done using a C18 Alltima reverse phase column (150*2.1 mm, 5 µm porosity – Grace/Alltech, Darmstadt, Germany) equipped with a C18 Alltima pre-column (7.5*2.1 mm, 5 µm porosity – Grace/Alltech). The different components were separated using a gradient of water containing 0.1% acetic acid (A) and acetonitrile containing 0.1% acetic acid (B), at a flow rate of 0.2µL/min, in the following gradient elution: 78-100% B over 50 min, 100% B for 5 min, followed by 10 min 78-100%. The column temperature was 35°C, the detection was carried out in UV at 280 nm and ELSD using evaporator and nebulizer temperatures of 35 and 45°C, respectively, and a gas flow rate was 1.5 standard liters for minute.

Liquid chromatography coupled to mass spectrometry (LC-MS) was performed using a system equipped with a Linear Trap Quadripole (LTQ) as mass analyzer (Thermo Fisher Scientific, San Jose, CA, USA). Chromatographic separation was performed on a C18 Alltima reverse phase column (150*2.1 mm, 5 µm porosity – Grace/Alltech, Darmstadt, Germany) equipped with a C18 Alltima pre-column (7.5*2.1 mm, 5 µm porosity – Grace/Alltech) at 25 °C and mobile phases consisted in water modified with acetic acid (0.1%) for A and pure acetonitrile for B. Fatty acids vanillyl esters were eluted using a linear gradient from 78% to 100% of B phase for 50 min at a flow rate of 0.2 mL min^{-1}. Photodiode array (PDA) and mass spectrometry (MS) detections were performed during the time of the run. Mass spectrometry operating parameters were as follows: Electrospray positive ionization mode (ESI^+)

was used; spray voltage was set at 4.5 kV; source gases were set (in arbitrary units min^{-1}) for sheath gas, auxiliary gas and sweep gas at 25, 10 and 10, respectively; capillary temperature was set at 250 °C; capillary voltage was set at 1 V; tube lens, split lens and front lens voltages were at 55 V, -38 V and -4.00 V, respectively. The ion optics parameters were optimized by automatic tuning using a standard solution of fatty acid esters 1 gL^{-1} infused in mobile phase (B) at a flow rate of 2 µL min^{-1}. Full scan MS spectra were acquired from 100 to 2000 m/z and manual MS^2 mode was also carried out in the way to obtain additional structural informations.

2.2.9. Oxidative stability of esterified oil

5 g of each sample (salmon oil, salmon oil + VA and esterified salmon oil) were weighed in screw-capped glass tubes (10 ml) and stored at 100°C and 20°C in darkness. Oxidative stability was monitored throughout storage period by measurement of conjugated diene (CD) and thoibarbituric acid (TBARS) according the AOCS standard method (AOCS., 1998).

2.2.10. Application of synthesis phenolic lipids in food emulsion

2.2.10.1. Oil in water (O/W) emulsions preparation

Oil in water emulsions was prepared from 10% (w/w) salmon oil, 1% (w/w) emulsifier (Tween 80) and 89% ultrapure water. Antioxidants compounds were added to salmon oil to obtain 200 mg/kg final concentration in the emulsion. A coarse emulsion was prepared using a Ultra-Turrax T25 basic (2 min, 11.400 rpm, IKA-Werke, Staufen, Germany) that was sonicated as follow by sonication (pulses 1s on/ 1s off, time 4 min, amplification 40%, *Vibra Cell 75115*, *Bioblock Scientific*, Illkirch, France*)* and circulated through a high pressure valve homogenizer Emulsiflex-C3

(Sodexim S.A, France) four times at 9.000 psi. The freshly prepared emulsions were placed in closed clear bottles and incubated at 4, 20 and 60 ºC.

2.2.10.2. Oxidative stability experiments

Lipid samples containing various inhibitors of the oxidation were prepared directly before use. 50 mg/kg and 200 mg/kg of the tested antioxidant compounds were added to margarine and salmon oil in water emulsions, respectively. Accelerated condition was used to study the antioxidative effect of tested antioxidants in margarine and salmon oil emulsion samples. Margarine samples (100 g) containing antioxidant compounds were maintained at 100 ± 2 °C, 20 ± 2°C and 4 ± 1°C and emulsion samples were stored at 37°C ± 2°C and 20± 2°C. Primary oxidation products were determined by peroxide measurements (PV and CD) and secondary oxidation products was measured using the *p*-anisidine value and TBA methods.

2.2.10.2.1. Peroxide value (PV)

The peroxide value corresponds to the amount of peroxide present in the sample, expressed in number of milli-equivalents of active oxygen per kilogram of product and oxidizing potassium iodide with liberation of iodine. This reaction is between a solution of potassium iodide and the fat dissolved in chloroform. The liberated iodine in acidic solution is titrated with sodium thiosulfate. A mixture of acetic acid - chloroform (3-2, v/v, 25 ml) was added to a sample from 1 to 5 g to solubilise the fat. One ml of a saturated solution of KI (5 g/5 ml water) was added and the mixture was stirred for 1 min, then let stand for 5 minutes protected from light. Thirty ml of distilled water were added (to stop the reaction), then 1 ml of starch solution 1% that serve as an indicator. The liberated iodine was titrated with stirring

with solution of 0.01 N sodium thiosulfate. A blank test was performed without fat. The results were expressed as follows

$$\text{Peroxide value (meq kg-1)} = \frac{(V_S - V_B) \times N \times 1000}{W}$$

Where V_S is the titre value (ml) of sodium thiosulphate solution for sample, VB is the titre value (ml) of sodium thiosulphate solution for blank; N is the normality of sodium thiosulphate solution and W is the weight of sample in gram.

2.2.10.2.2. Conjugated diene value (CD)

The conjugated diene value (CD) was determined as described by (Weber *et al.*, 2008). Briefly, the sample was dissolved in 25 ml *n*-hexane and recorded the optical density (1 cm light path) at 234 nm against *n*-hexane blank. The conjugated diene value was calculated according to the equation:

$$\text{CD value} = [C_{CD} \times (2.5 \times 10^4)] / W$$

$$C_{CD} = A\ 234 / (E \times L)$$

Where CCD is the CD concentration in mmol/ml, A 234 is the absorbance of the lipid solution at 234 nm, E molar absorption coefficient, 26 000 L/mol, L is the path length of the cuvette in cm (1 cm) and 2.5 x 104 is a factor that encompasses the volume of *n*-hexane (25 ml) used to dissolve the oil sample.

2.2.10.2.3. Anisidine value (p-An.v)

The *p*-anisidine value (AV) was determined according to Cd 18-90 method suggested by (AOCS, 1995). This method depends on spectrophotometric determination of products formed between aldehyde compounds and *p*-anisidine. Oil samples (0.5-2.0 g) were dissolved in 25 ml iso-octane and absorbance of this fat solution was measured at 350 nm using a spectrophotometer (Shimadzu UV-1605). Five millilitres of the above mixture was mixed with 1 ml 0.25% *p*-anisidine in glacial

acetic acid (w/v) and after 10 min standing, the *p*-anisidine value (AV) was calculated according to the equation:

$$AV = 25 \times (1.2\ A_S - A_B) / m$$

Where A_S is the absorbance of the fat solution after reaction with the *p*-anisidine reagent; A_B is the absorbance of the fat solution and m is the mass of oil sample (g).

Measurements of *p*-anisidine value are commonly used together with peroxide value measurements in describing the total extent of oxidation by the Totox value, which equals the sum of the *p*-anisidine value plus twice the peroxide value. However, the Totox value is an empirical parameter since it corresponds to the addition of two parameters with different units (Fereidoon andUdaya, 2008).

2.2.10.2.4. Thoibarbituric acid reactive substances (TBARS) assay

TBARS method was determined according to the method of Tong et al (2000) with some modifications. One hundred microlitres of emulsion was mixed with 0.9 mL of water and 1 mL of TBARS reagent (15% (w/v) TCA and 0.375% (w/v) TBA in 0.25 M HCl), then vortexed. The reaction mixture was heated at 90°C in water bath for 20 min. The samples were cooled to room temperature and centrifuged at 2000 x g for 10 min (MiniSpin Eppendorf). The absorbance of the supernatant was then measured at 532 nm (Shimadzu UV-1605). Concentrations of TBARS were determined from a standard curve prepared using 1, 1, 3, 3-tetraethoxypropane (TEP) in concentrations ranging between 0.6 µM and 40 µM. The results were expressed as µM of TBARS.

2.2.11. Statistical analysis

Statview analysis program was used for the statistical analysis. Data in vitro were obtained from three to four separate experiments with four determinations each. Normal data distribution was verified using Kolmogorov-Smirnov's test. Differences between groups and effects of treatments were analyzed using parametrical statistic tests (paired and non-paired Student's t-tests and ANOVA). All reported values represent the mean ± SEM. Significance was considered for p values lower than or equal to 0.05.

Results and Discussion

Chapter 3: Results and Discussion

Partie 1: Enzymatic production of bioactive docosahexaenoic acid phenolic ester

1.1. Introduction

Dans la littérature, les esters phénoliques d'acides gras sont de préférence produits par des bioprocédés enzymatiques qui présentent une sélectivité élevée envers des substrats polyfonctionnels et des conditions réactionnelles douces en comparaison avec les voies de synthèse chimique classiques. Le plus souvent, les réactions d'estérification enzymatiques sont catalysées par des lipases dans des milieux organiques pauvres en eau. Toutefois, quelques articles rapportent la possibilité de travailler en l'absence de solvant, l'un des substrats de la réaction étant utilisé en large excès et permettant la solubilisation du deuxième substrat (Hong, Ma, Kim, Seo, et Kim, 2012; Weitkamp, Weber, et Vosmann, 2008). On peut noter en outre, que la plupart des études publiées jusqu'ici sont principalement destinées à démontrer la faisabilité des synthèses et ne comportent pas d'éléments d'optimisation des procédés permettant d'envisager une production des esters à plus grande échelle.

L'objectif de ce travail était de développer un procédé sans solvant, efficace, pour la synthèse de l'ester associant le DHA et l'alcool vanillique (DHA-VE), dans des quantités suffisantes permettant la mise en œuvre d'études *in vitro*, *in vivo* et de formulation. Le DHA a été choisi comme acide gras polyinsaturé modèle en raison de ses propriétés nutritionnelles intéressantes ; l'alcool vanillique (VA), quant à lui, est un composé phénolique naturellement présent dans les denrées alimentaires et dont le pouvoir antioxydant a été démontré.

Le développement du procédé s'est déroulé en plusieurs étapes :

Dans un premier temps, une étude préliminaire a été menée pour vérifier la faisabilité de la synthèse de l'ester en milieu solvant (méthyl-2-butanol-2 et acétonitrile), en présence d'un léger excès de donneur d'acyle (ester éthylique de DHA). Les cinétiques obtenues ont montré que ces conditions nécessitent en moyenne 8 h de réaction pour atteindre un équilibre et obtenir une production maximale d'ester. Dans ces conditions, un pourcentage de conversion de l'alcool vanillique de 60% a pu être obtenu.

Bien que classiques, ces procédés en milieu solvant sont aujourd'hui de plus en plus décriés en raison du coût et de l'impact environnemental des solvants mis en œuvre. Les procédés sans solvant, appelés aussi procédés en milieu fondu, constituent une alternative intéressante. C'est l'approche qui a été retenue pour la suite de l'étude. Dans ces conditions, l'un des substrats de la réaction est introduit en large excès et sert à solubiliser (au moins partiellement) le second substrat. Les principaux avantages sont d'éviter l'utilisation de solvants organiques et de déplacer l'équilibre de la réaction d'estérification en faveur de la synthèse de l'ester (lois thermodynamiques de Le Chatelier). L'effet de cet excès de substrat peut être renforcé par des conditions de température et pression favorisant l'élimination en continu du sous-produit réactionnel (de l'eau dans le cas d'une estérification directe ; de l'alcool dans le cas d'une transestérification). Ainsi, dans le but d'optimiser le rendement de la synthèse et la production de l'ester DHA-VE, l'effet de la concentration et du mode d'alimentation du réacteur en alcool vanillique a été étudié (milieux dilués, milieux saturés, mode semi-continu). En outre, différentes conditions de température et pression ont été appliquées de manière à étudier l'effet de l'élimination du sous-produit réactionnel (dans notre cas l'éthanol issu de l'alcoolyse de

l'ester éthylique de DHA) sur les performances de la réaction. Une attention particulière a également été portée aux conditions réactionnelles permettant de limiter la dégradation oxydative du milieu au cours de la synthèse.

La dernière partie de cette étude porte sur la purification et la caractérisation de la molécule produite ainsi que sur l'évaluation de ses activités biologiques. L'alcool vanillique présentant 2 fonctions hydroxyle sur sa structure, il s'agissait notamment de vérifier la position du greffage de la chaîne DHA et de conclure sur la régio-sélectivité de la réaction. Au vu des propriétés intrinsèques des deux molécules associées, l'activité antioxydante et l'activité neuroprotectrice de l'ester ont plus particulièrement été étudiées *in vitro*. Des expériences ont en outre été menées *in vivo*, pour déterminer l'effet du greffage du composé phénolique sur la biodisponibilité du DHA. Ces études biologiques ont été réalisées en collaboration avec l'équipe Biodisponibilité et Fonctionnalités des Lipides Alimentaires de l'URAFPA. L'équipe Sucres du SRSMC a quant à elle contribué à la mise au point d'un procédé de purification de l'ester ainsi qu'à sa caractérisation structurale.

Ces travaux ont fait l'objet d'un article accepté dans Food Chemistry.

1.2. Enzymatic production of bioactive docosahexaenoic acid phenolic ester

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Abbreviations: Aβ, amyloid-β; ABTS, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid); BHT, butylated hydroxytoluene; CALB, *Candida antarctica* lipase B; DHA, docosahexaenoic acid; DHA-EE, DHA-ethyl ester; DHA-VE, DHA-vanillyl ester; DPPH, 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl; EPA, eicosapentaenoic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TEAC, Trolox equivalent antioxidant capacity; VA, vanillyl alcohol.

Keywords: Antioxidant, DHA, enzymatic esterification, neuroprotection, vanillyl alcohol

ABSTRACT

Docosahexaenoic acid (DHA) is increasingly considered for its health benefits. However, its use as functional food ingredient is still limited by its instability. In this work, we developed an efficient and solvent-free bioprocess for the synthesis of a phenolic ester of DHA. A fed-batch process catalyzed by *Candida antarctica* lipase B was optimized, leading to the production of 440 g/L vanillyl ester (DHA-VE). Structural characterization of the purified product indicated acylation of the primary OH group of vanillyl alcohol. DHA-VE exhibited a high radical scavenging activity in acellular systems. *In vivo* experiments showed increased DHA levels in erythrocytes and brain tissues of mice fed DHA-VE-supplemented diet. Moreover, *in vitro* neuroprotective properties of DHA-VE were demonstrated in rat primary neurons exposed to amyloid-β oligomers. In conclusion, DHA-VE synergized the main beneficial effects of two common natural biomolecules and therefore appears a promising functionalized ingredient for food applications.

1. INTRODUCTION

Omega-3 polyunsaturated fatty acids from fish oils promote well-established health and anti-aging benefits that justify their use as functional ingredients in dietary supplements, healthy foods and nutraceutical products (Swanson *et al.*, 2012b). Among them, eicosapentaenoic (EPA, C20:5) and docosahexaenoic acids (DHA, C22:6) continue to receive particular attention because of their numerous biological properties and positive effects on human health (Lorente-Cebrian *et al.*, 2013). In addition to recognized benefits for the prevention of cardiovascular diseases, EPA and DHA were reported to protect from inflammation-mediated disorders including obesity and diabetes, Alzheimer's and related neurodegenerative diseases (Calder, 2013 ; Farooqui, 2012).

Human metabolism exhibits limited ability to synthesize ω3 PUFAs. Dietary supply of preformed compounds therefore appears an essential alternative. However, the practical use of such lipids as food ingredients is often limited by their high susceptibility to oxidation, which is responsible for the undesirable off-flavor and odor of rancid oils, associated with a loss of nutritional value (Albert Benjamin B. *et al.*, 2013c). Various solutions can be implemented to minimize these degradation pathways and the most commonly used by manufacturers is addition of antioxidants (Wang H. *et al.*, 2011b). Intensive research has been pursued on natural phenolic antioxidants issued from plants. Many studies reported their high efficiency to protect ω3-enriched food products from oxidation (Huber G. M. *et al.*, 2009a ; Sekhon-Loodu S. *et al.*, 2013). In addition, some works pointed out the interest of formulations mixing ω3 PUFAs and phenolic compounds for the prevention of Alzheimer's disease and the treatment of obesity through lipid-lowering effects (Cole *et al.*, 2005 ; Radler *et al.*, 2011). Another approach consists in bringing together ω3 lipids and phenolic compounds into a single entity as described by few authors (Mbatia B. *et al.*, 2011 ; Torres de Pinedo A. T. *et al.*, 2005b ; Zhong andShahidi, 2011). Such an association was shown to improve the stability of highly oxidizable fatty acids while facilitating the solubilization of phenolic compounds in lipid phases. Additional effects could be an increased bioavailability of phenols as well as cumulative and even synergistic biological activities (Tan *et al.*, 2012 ; Wahle *et al.*, 2010). In addition, PUFA phenolic esters were reported to exhibit higher antimicrobial, antioxidant and anti-inflammatory activities than native phenols (Mellou *et al.*, 2005 ; Zhong *et al.*, 2012).

Fatty-acid phenolic esters are preferentially produced by enzymatic bioprocesses that exhibit high selectivity towards polyfunctionnal substrates and mild reaction conditions comparing with chemical synthesis pathways. Most often, esterification reactions were carried out in dry organic solvent, but few studies reported the possibility to process without any solvent (Hong *et al.*, 2012 ; Weitkamp *et al.*, 2008). Moreover, most of these studies only intended to make the proof of concept, but ignored upscale potential.

The objective of the present work was to develop an efficient and environment-friendly solvent-free bioprocess for the synthesis of DHA vanillyl ester (DHA-VE) in sufficient quantities to allow further *in vitro* and *in vivo* experiments as well as potential applications. DHA was chosen because of its major protective potential, while vanillyl alcohol (VA) is a phenolic compound commonly found as antioxidant in foodstuffs. DHA-VE bioavailability was evaluated in mice, as well as its neuroprotective properties *in vitro*, in comparison with those of DHA and VA used alone or in combination.

2. MATERIALS AND METHODS

2.1. Chemicals and enzyme

Candida antarctica lipase B (CALB) immobilized on a macroporous acrylic resin (Novozym 435®, Novo Industry) was used to catalyze acylation reactions. Docosahexaenoic-acid ethyl ester (DHA-EE, 95% pure) was supplied by KD-Pharma (Bexbach, Germany). Solvents of analytic or HPLC grade were from Merck. Vanillyl alcohol (4-hydroxy-3-methoxybenzyl alcohol, VA) and all other chemicals were purchased from Sigma-Aldrich Chemicals.

2.2. Enzymatic synthesis of DHA vanillyl ester (DHA-VE)

2.2.1. Enzymatic synthesis of DHA-VE in organic medium

Enzymatic acylation reactions were achieved in organic solvent, under atmospheric pressure. Reaction media were prepared by solubilizing VA (100 mM, 15.4 g/L) and DHA-EE (200 mM) in 2 mL of acetonitrile. The solvent was predried on 4-Å molecular sieves before use, aiming to low water activity below 0.1. Reactions were performed in 10-mL amber tubes submitted to orbital shaking (300 rpm) and initiated by adding 20 g/L of Novozym 435®. This protocol is further referred as solvent system.

2.2.2. Synthesis of DHA-VE in molten media

Reactions were performed under either atmospheric or reduced pressure. For syntheses achieved under atmospheric pressure, reaction media were prepared by solubilizing VA (200 mM, 30.8 g/L that corresponds to the maximal solubility of the substrate at 50°C) in a large excess of DHA-EE as acyl donor (2 mL), at 50°C. Reactions were performed in 10-mL amber tubes and initiated by adding 20 g/L of Novozym 435®. After 72 h of reaction, shaking was stopped, allowing enzyme particles to decant prior taking the supernatant. This protocol led to reaction system A.

Syntheses achieved under reduced pressure were performed in the sample flask of a rotary evaporator at 250-rpm rotation speed. Temperature and pressure conditions were set to 37°C and 500 mbar, so that the by-product of the reaction, *i.e.* ethanol, could be continuously eliminated while avoiding VA evaporation. Reaction media were prepared by solubilizing VA (162 mM, 25 g/L that corresponds to the substrate solubility at 37°C) in 10 mL of DHA-EE. Reactions were started by adding 20 g/L of Novozym 435®. After 72 h, the enzyme was eliminated by filtration. This protocol led to reaction system B.

2.2.3. Process intensification

The production of DHA-VE was intensified by increasing VA intakes. VA (50 g/L) was introduced under a nitrogen stream (fed batch process) in 10 mL of DHA-EE. Syntheses were performed in the flask of a rotary evaporator, at 37°C, under a 500-mbar pressure and at 250 rpm. After the first 4 h of synthesis, the reaction medium was filtered and transferred to another flask. A new supply of VA (50 g/L) was then carried out. Once the phenolic compound was totally solubilized, the reaction was restarted by adding fresh enzymatic preparation, thereby maintaining optimal activity. This protocol was repeated twice. This protocol led to reaction system C.

2.2.4. Kinetic following of the syntheses

Kinetic following of the reactions was performed by HPLC, using a Shimadzu Class-VP system equipped with a computer-controlled system (Class-VP 6.1 software). Separations were carried out on a reversed-phase Altima C18 column (150×2.1 mm, 5 µm, Grace-Alltech). VA and DHA-VE were detected at 280 nm on a multichannel photodiode-array detector (SPD-M10A VP). DHA-EE and free DHA were followed by evaporative light scattering detection (ELSD), using nebulizer and evaporator temperatures of 35°C and 45°C, respectively. Analyses were carried out with

compressed air as ELSD gas at a pressure of 1.5 bars. Elution was performed using a gradient of solvent A [methanol/water 70/30 (v/v)] and B [methanol (100%)], at a flow rate of 0.2 mL/min. Elution protocol was as follows: 0-5 min: 100-0% A, 5-25 min: 100% B, 25-35 min: 100-0% B, 35-45 min: 100% A. Calibrations were made using analytical standard compounds. An aliquot from each reactor was withdrawn at predetermined times during synthesis, and then diluted 100 times in solvent A. All samples were filtered through a 0.2-µm membrane before injection.

2.3. Purification of DHA-VE by flash chromatography

DHA-VE was purified on a system working with glass columns KONTES CHROMAFLEX packed with silica gel 60 (particle size of 40-63 µm). Gradient system was generated by 2 pumps Gilson model 306. Cyclohexane and ethyl acetate were used as mobile phase. The elution gradient was as follows: 0-40 min: 5-15% ethyl acetate, 40-60 min: 15-40% ethyl acetate. The flow rate was 20 mL/min. Every minute, a sample of 20 mL was collected separately in a glass tube for proper identification. The presence of the ester in the tubes was detected by thin-layer chromatography on precoated silica gel $60F_{254}$ TLC plates, referring to the pure molecule as standard. The plates were visualized under UV light at 254 nm and then sprayed with a solution of sulfuric acid 20% in methanol. Fractions containing the ester were combined and the solvent was removed by evaporation under vacuum.

2.4. Structural analyses

2.4.1. Liquid chromatography–mass spectrometry (LC–MS)

The structure of the product was determined by HPLC-MS. The mass spectra were obtained using a binary solvent delivery pump and a linear ion trap mass spectrometer (LTQ-MS, Thermo Scientific, San Jose, CA, USA) equipped with an atmospheric pressure ionization interface operating in ESI mode. Control of equipment and data processing were realized using Xcalibur software (version 2.1).. The operational parameters of the mass spectrometer were as follows: the spray voltage was 4.5 kV and the temperature of heated capillary was set at 200°C. Flow rates of sheath gas, auxiliary gas, and sweep gas were set to 20.5 and 4 (in arbitrary units/min), respectively. Capillary voltage was -18 V, tube lens was -80 V, split lens was 11 V, and the front lens was 6.25 V. MS parameters were optimized by infusing a standard solution of DHA-VE in mobile phase (methanol/water: 70/30) at a flow rate of 5 µL/min.

2.4.2. Nuclear magnetic resonance (NMR)

The pure product was identified and characterized by NMR analyses. NMR experiments were performed in $CDCl_3$ with a Bruker Avance DRX-400 instrument operating at a proton frequency of 400.13 MHz and equipped with a 5 mm broadband inverse detection z-gradient probe tuned to ^{13}C (100.61 MHz). For all 1D and 2D NMR experiments, pulse sequences provided by the spectrometer manufacturer were used.

2.5. Evaluation of antioxidant activity

2.5.1. Inhibition of DNA scission

Inhibition activity of biomolecules towards DNA scission was determined by using H_2O_2 (0.2 mM) and $FeSO_4$ (0.1 mM) to generate hydroxyl radicals that induced strand breaks in plasmid DNA (10 µg/mL) (Hiramoto Kazuyuki *et al.*, 1996b). DNA scission was monitored after 1 h by agarose gel electrophoresis. Negative (DNA only) and positive controls (DNA + hydroxyl radicals) were compared to reactions carried out in the presence of DHA and VA used separately or together. The bands were visualized under UV light and intensity of the band corresponding to the native supercoiled plasmid was measured by densitometry.

2.5.2. Radical scavenging activity

The capacity of the compounds to scavenge radical species was determined by DPPH and ABTS methods (Brand-Williams *et al.*, 1995 ; Re R. *et al.*, 1999a). Concentration range was 0.5-2 mM for VA and DHA-VE. Free radical scavenging activity was expressed as Trolox equivalent antioxidant capacity (TEAC) value, defined as the concentration of standard Trolox that exhibited the same antioxidant capacity as a 1-mM solution of the investigated compound.

2.6. Biological activities and bioavailability

2.6.1 Primary cell cultures and treatments

Cell culture media and materials were from Invitrogen. Brain cortical neurons were taken from Wistar rat fetuses at embryonic day 16–17 and cultured in serum-free medium (Florent *et al.*, 2006). Neurons were treated with 1 µM of amyloid-β (Aβ) oligomers for 24 h prior monitoring cell viability using the MTT reduction assay (Florent *et al.*, 2006). Alternatively, indicated concentrations of DHA or DHA-VE were mixed

with fatty acid-free BSA (0.1%, w/v) and added to the medium 24 h before Aβ treatment.

2.6.2. Animals and diets

Male C57BL/6J mice (10-week old) came from Janvier (Le Genest-St-Isle, France) and accustomed for 2 weeks before the study. Animals were housed in individual cages with access to food and water *ad libitum* and were kept in a controlled environment (22±1°C, 50±5% humidity) with a 12 h light/dark cycle. The animal facilities and all procedures were approved (# DDSV/54/04/ENV/065) by the Animal Care and Veterinary Committee of Meurthe-et-Moselle (Nancy, France). Mice were randomly assigned to three groups of 6 mice each and fed diets of comparable caloric density. Control mice received standard food (Harlan 2080S), while DHA-supplemented mice were fed specific diet consisting of standard chow containing either 0.3% (w/w) DHA-EE or DHA-VE.

2.6.3. Fatty-acid analysis

Retro-orbital blood samples (100 µL) were collected after 3-h fasting period in lightly anesthetized mice and the cellular fractions were immediately frozen at -20°C until extraction and analysis. Hippocampal and cortical tissues were taken from anesthetized mice shortly after sacrifice and immediately frozen. FA were extracted from all samples in a methanol/dichloromethane (3/1) mix prior to transesterification, purification of methylated derivatives and separation by GC (Lepage andRoy, 1986).

2.6.4. Statistical analysis

Stat View software was used for the statistical analysis. Data *in vitro* were obtained from three to four separate experiments with four determinations each. Normal data distribution was verified using Kolmogorov-Smirnov's test. Differences between groups and effects of treatments were analyzed using parametrical statistic tests (paired and non-paired Student's *t*-tests and ANOVA). Differences were considered significant for $p \leq 0.05$.

3. Results and discussion

3.1. Enzymatic synthesis of DHA-VE

The present work intended to develop an efficient and environment-friendly process to synthesize DHA-VE without any solvent. Various operating conditions were applied to produce high concentrations of ester while limiting oxidative degradation. An alcoholysis reaction between DHA-EE and VA was applied, leading to DHA-VE and ethanol as by-product. The reaction feasibility was verified in an organic solvent. The synthesis was then intensified in solvent-free media by shifting the thermodynamic equilibrium of the reaction.

3.1.1. Synthesis of DHA-VE in organic solvent

Lipase-catalyzed acylation reactions are classically carried out in dry organic solvents that allow a good substrate solubilization, while avoiding the denaturation of the enzyme. In the present study, experiments were achieved in acetonitrile where VA solubility was 15.8 g/L. The DHA-EE/VA molar ratio was adjusted to 2:1. HPLC analyses showed the synthesis of two products, along with VA and DHA-EE (respective retention times of 2.7 and 17.8 min) consumption. The first product (retention time of 16.5 min) was detected by DEDL and UV equipment, and identified as a monoester of DHA and VA by LC-MS analyses ($M+H^+ = 465.08$ g/mol) (Figure 1A). The second product (retention time of 15.4 min) was detected by DEDL only, and identified as free DHA resulting from the competitive hydrolysis of DHA-EE. At the end of the reaction, the ester production was 25 g/L, corresponding to conversion yields of 54% and 27% for VA and DHA-EE, respectively.

This conversion yield appeared somewhat worse than that obtained for the enzymatic synthesis of vanillyl nonanoate, also known as capsinoid, in dioxane (Kobata *et al.*, 2002a). However, this result was supported by other studies on VA acylation with long-chain acyl donors, showing that the longer the chain, the smaller the conversion yield (Reddy *et al.*, 2011). This tendency is even more pronounced in CALB-catalyzed acylation reactions between di-*ortho*-phenolic compounds and DHA-EE (Torres de Pinedo A. T. *et al.*, 2005b). Competitive DHA-EE hydrolysis was held responsible for these results.

3.1.2. Purification and structural characterization of DHA-VE

VA structure exhibits two hydroxyl groups corresponding to one primary alcohol and one phenolic function. According to the literature on CALB specificity properties, the reaction was likely to occur exclusively on the primary hydroxyl group of the substrate (Parmar *et al.*, 1999). In order to verify this assumption, the ester was purified (purification yield > 85%) and its structure was elucidated by NMR and FTIR analyses (Figures 1B and 1C). The polyunsaturated chain was grafted on the VA primary hydroxyl group, whereas the phenolic hydroxyl group remained unaffected, consistently with CALB selectivity towards polyhydroxylated phenolic compounds (Chebil *et al.*, 2006). For such substrates, this lipase was shown to catalyze primary or secondary hydroxyl group acylation only. Most significant IR bands were observed in the regions 3600–3100 cm^{-1}, 3013 cm^{-1} and 1737 cm^{-1}, respectively corresponding to phenol, *cis* double-bond and ester carbonyl stretching vibrations. Moreover, the purity of the ester was 98%, referring to the integration of ^{1}H-NMR signals of both terminal methyl (0.98 ppm) and methoxyphenyl (3.91 ppm) groups.

3.1.3. Synthesis intensification in melted media

This study also intended to develop a bioprocess able to produce DHA-VE in sufficient quantities to allow *in vitro* and *in vivo* experiments. This was achieved without any solvent in melted media, using acyl-donor substrate in large excess allowing VA solubilization. According to Le Chatelier's thermodynamic principle, this system tended to reduce the excess of acyl donor by shifting the reaction equilibrium in favor of ester synthesis. Syntheses were performed at 50°C under atmospheric pressure (reaction system A) or at 37°C under reduced pressure in either diluted (reaction system B) or saturated (reaction system C) VA solutions. These conditions led to ethanol elimination without VA distillation. VA solubility at 37°C and 50°C was 25 g/L and 30.8 g/L, respectively. Corresponding DHA-EE/VA molar ratios were 16.5 and 13.4.

Molten media increased VA conversion yields as compared with solvent medium (Table 1). The performance of the process was further improved by applying reduced pressure that allowed continuous elimination of ethanol throughout the reaction in systems B and C while limiting exposure to oxygen. VA conversion was completed after 2 h, independently of its initial concentration. 150 g/L of ester were obtained starting from VA-saturated media (reaction system C). Yellowing of the reaction

medium was observed in syntheses achieved at 50°C under atmospheric pressure, indicating an oxidative degradation. In contrast, no oxidation was noticed at 37°C under reduced pressure. DHA-EE conversion yields were 7.5%, 6%, and 12% for reaction systems A, B, C, respectively.

These results are consistent with data on lipase-catalyzed reactions combining vacuum and molten media, showing total VA conversion with conjugated linoleic acid in the presence of *Rhizomucor miehei* lipase (Hong *et al.*, 2012). However, for a given phenolic alcohol, the conversion yield seems to depend on the acyl-donor substrate, ethyl eicosapentaenate and docosahexaenate leading to lower performances than shorter acylating agents (Torres de Pinedo A. T. *et al.*, 2005b).

3.1.4. Synthesis of DHA-VE in fed batch mode

We developed a fed-batch bioprocess where the reactor was fed with dry VA, aimed to improve ester synthesis and concomitant DHA-EE consumption. Reaction kinetics was defined by subtracting the time required for VA dissolution from the total reaction time.

Figure 2 illustrates the production of DHA-VE along the reaction when feeding the reactor with VA (50 g/L) three times. VA was totally converted at the end of each step, except for the last feeding that led to a conversion yield of 91%. The reaction rate was observed to decrease progressively with time. 440 g/L of DHA-VE were ultimately produced, corresponding to VA and DHA-EE conversion yields of 97% and 31%, respectively. Additional feedings were attempted to further improve the process, but both reaction rate and VA conversion yield were observed to decrease along with oxidative degradation (data not shown).

3.2. Biological properties of DHA-VE

3.2.1. *Antioxidant activity*

Numerous studies showed that antioxidant activities of biomolecules are closely related to their chemical structure. Here, the antioxidant properties of VA and DHA-VE were determined and compared by using different *in vitro* assays.

3.2.1.1. *Evaluation by DPPH$^{\bullet}$ method*

DPPH$^{\bullet}$ is a stable lipophilic radical characterized by a deep violet color. The capacity of compounds to quench the DPPH$^{\bullet}$ radical can be evaluated from decrease

in absorption at 517 nm (Xu J. Z. *et al.*, 2004). The scavenging activity of DHA-VE and VA was compared with reference molecule, butylated hydroxytoluene (BHT) (Figure 3B). BHT, VA and DHA-VE exhibited similar scavenging potential towards $DPPH^{\bullet}$ radicals, suggesting that acylation did not affect this activity, as supported by *(Grasso et al., 2007)*. Acylation with DHA was even shown to increase the $DPPH^{\bullet}$ scavenging capacity of epigallocatechin gallate (Zhong andShahidi, 2011). Conversely, long-chain phenolic esters were reported to be less active towards $DPPH^{\bullet}$ radicals than short-chain esters or than native phenols, probably due to higher hydrophobicity (Mbatia B. *et al.*, 2011 ; Reddy *et al.*, 2011 ; Salem *et al.*, 2010).

3.2.1.2. *Evaluation by $ABTS^{\bullet+}$ method*

$ABTS^{\bullet+}$ is a blue/green-colored radical that exhibits maximal absorbance at 734 nm and allows measuring antioxidant activity of both hydrophilic and lipophilic biomolecules (Re R. *et al.*, 1999a). BHT exhibited the highest activities, (Figure 3B). VA acylation did not affect its capacity to inhibit $ABTS^{\bullet+}$ radicals. A similar observation was reported when acylating isoquercitrin with fatty acids of various chain length, showing no effect of acylation for acyl chain of more than 10 carbon atoms (Salem *et al.*, 2010).

3.2.1.3. *Inhibition of DNA scission*

Many studies reported the deleterious effect of oxygen radicals on DNA. The capacity of DHA-VE to protect plasmid DNA against oxidative damages induced by hydroxyl radicals was evaluated and compared to that of VA, DHA-EE and free DHA (Figure 3A). DNA scission appeared in absence of any antioxidant compound as well as with free DHA. Addition of DHA-VE or VA led to retention rates of 96 and 92% of native supercoiled DNA, respectively. Since DHA-EE only retained 29%, the antioxidant properties of DHA-VE clearly pointed out the crucial role of the phenolic moiety. The ester seemed even more protective than VA, suggesting that VA monoacylation with DHA may improve the antioxidant protection against DNAA scission, likely based on hydroxyl radical scavenging activity and capacity to bind to iron (Perron *et al.*, 2008). Such an effect was also observed for epigallocatechin gallate DHA tetraester (Zhong andShahidi, 2012), whereas negative outcome of acylation was reported with hydroxytyrosol, homovanillyl alcohol and their saturated esters (Grasso *et al.*, 2007). These results suggest a significant influence of the fatty acid chain on the intrinsic antioxidant properties of phenolic esters.

3.2.2 Bioavailability of DHA-VE in mice

DHA is the major PUFA in neurons, where it is expected to provide protection in various stress conditions. We compared the bioavailability of dietary DHA in three groups of 3-month old mice fed control, DHA-EE or DHA-VE diet during 4 weeks. Body weight remained unchanged in these groups, although DHA-VE food intake was increased by 2.2% ($p<0.05$) and 6.0% ($p<0.001$) as compared to control and DHA-EE, respectively (data not shown). This suggested that VA could render food more appetizing to mice.

The FA profiles were then measured in brain and in blood cell membranes, mainly erythrocytes, known to store and transport DHA in the periphery and to the central nervous system (Hashimoto *et al.*, 2005 ; Spector, 2001). After the 4-week study, a significant increase in relative DHA content accompanied with decreased ARA levels was observed in blood cell membranes of mice from DHA-EE and DHA-VE groups as compared to control mice as well as to levels at baseline (Figure 4A). These findings agreed with previous studies on DHA-EE (Arterburn *et al.*, 2006 ; Hashimoto *et al.*, 2002 ; Hashimoto *et al.*, 2005).

Similar enrichment in DHA was measured in hippocampal membranes in DHA supplemented mice *vs.* control group, along with decreased ARA levels and ARA/DHA ratio (Figure 4B). These antagonistic effects upon DHA supplementation likely resulted from competitive incorporation of DHA and ARA at the *sn*-2 position of membrane phospholipids, as already suggested (Smink *et al.*, 2012). Such changes could provide benefits to DHA-enriched cells in preventing inflammation. Interestingly, ARA/DHA ratio was even significantly lower in both tissues of DHA-VE mice *vs.* DHA-EE mice. Altogether, our results clearly indicated that the phenolic moiety of DHA-VE tended to improve DHA bioavailability for target tissues. This also suggests that these esters were comparably metabolized by lipase/esterase activities and none of them induced apparent hepatotoxicity in supplemented mice as deduced from unchanged procaspase-3 profiles (data not shown).

3.2.3 Effect of DHA-VE on neuronal cell viability upon Aβ exposure in vitro

Free DHA was reported to protect neurons from amyloid stress *in vitro* (Florent *et al.*, 2006). Using the MTT mitochondrial activity test, we observed that DHA-VE (up to 10 µM) did not induce any deleterious effects on primary neurons, in contrast with

DHA-EE whose metabolism led to cytotoxic ethanol release (data not shown). Cell viability was lower when neurons were treated with free VA, but significantly increased with DHA and DHA-VE (Figure 5A). Interestingly, a higher MTT activity was measured in neurons treated with DHA-VE than with DHA alone or in equimolar [DHA+VA] mixture. This suggests that free VA could be toxic in neuronal cultures, independently of DHA presence in the medium. Conversely, cumulative protective effects with DHA could be provided by VA upon its intracellular release from DHA-VE.

Next, we studied the influence of these treatments on neuronal resistance to Aβ insult known to induce oxidative synaptic impairment and neuronal apoptosis (Sponne *et al.*, 2003). Expectedly, Aβ cytotoxicity led to a residual 80% MTT activity as compared with control cells. In contrast, neurons pretreated with 1 µM VA, DHA, [DHA+VA] or DHA-VE showed significant resistance (Figure 5B). Upon Aβ exposure, viability of AV- and DHA-pretreated neurons was improved by 30 and 55%, respectively, which is in agreement with our previous data (Florent *et al.*, 2006). DHA-VE offered the highest protection level in cultures as compared with the equimolar [DHA+VA] mixture (60% *vs.* 40% of Aβ-exposed neurons). Additional experiments should elucidate the beneficial pathways specifically activated by DHA-VE. Linkage of DHA to VA might prevent oxidation of DHA and promote its beneficial effects on neuronal membranes, thereby preventing Aβ-induced pro-apoptotic effects as widely demonstrated (Cao *et al.*, 2009 ; Florent *et al.*, 2006).

4. Conclusions

This paper presented the operating conditions that allowed efficient enzymatic acylation between two common natural food ingredients, *i.e.* vanillyl alcohol and DHA. The solvent-free protocol developed here led to high-level production of an ester that carries interesting potential for food industry and nutrition: (i) improved organoleptic qualities of DHA-VE-supplemented diet; (ii) elevated antioxidant activity that should stabilize DHA as well as various food matrices such as oils, fats and emulsions; (iii) increased bioavailability of DHA leading to higher DHA levels in erythrocytes and neurons; (iv) combined beneficial effects of phenols and ω3 PUFAs; (v) increased neuroprotection against amyloid stress; (vi) no visible toxicity. DHA-VE and analogous esters can be considered stable bioactive ingredients that should broaden the scope of nutritional applications of ω3 PUFAs whose health benefits are increasingly sought.

ACKNOWLEDGEMENTS

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Tables and Figures:

Table 1. Effects of enzymatic reaction conditions on substrate conversion yields and ester production

Reaction system	DHA-EE conversion yield (%)	VA conversion yield (%)	DHA-VE production (g/L)
System A	7.5	90	84
System B	6	100	75
System C	12	100	150
Solvent system	27	54	25

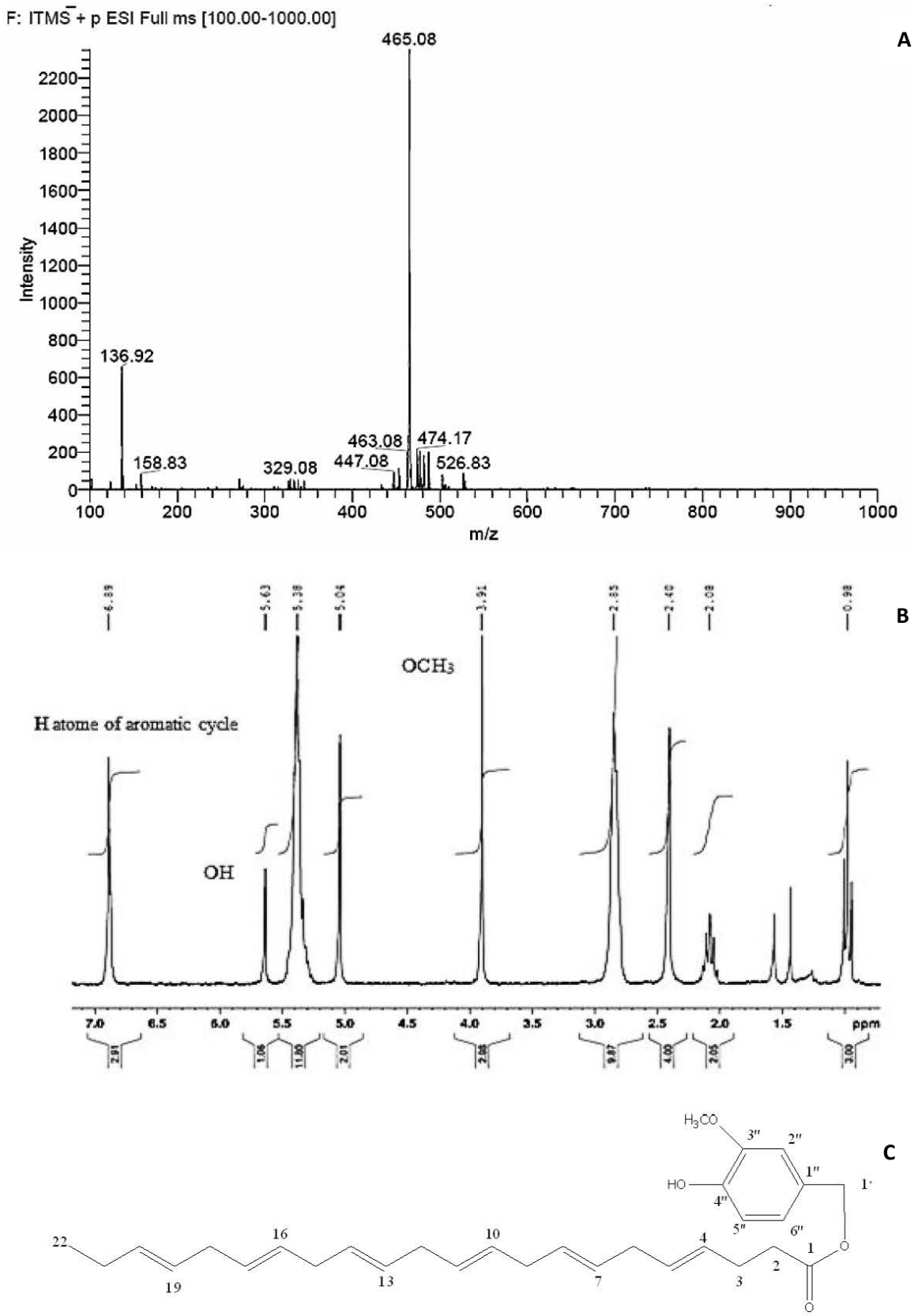

Figure 1: **Structural characterization of DHA-VE.** (A) MS analysis. (B) ^{1}H NMR analysis. (C) Deduced chemical structure.

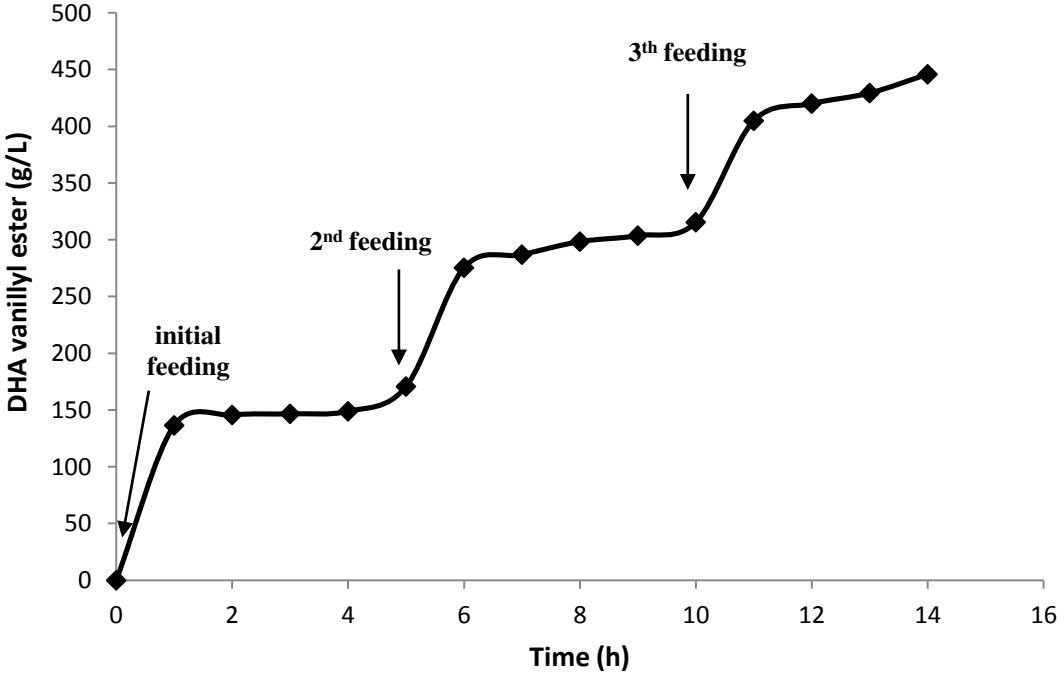

Figure 2: Kinetic curve relative to enzymatic acylation of VA with DHA-EE. The CALB-catalyzed reaction was performed at 37°C, under a 500-mbar pressure. The reaction medium was fed three times (arrows) with VA.

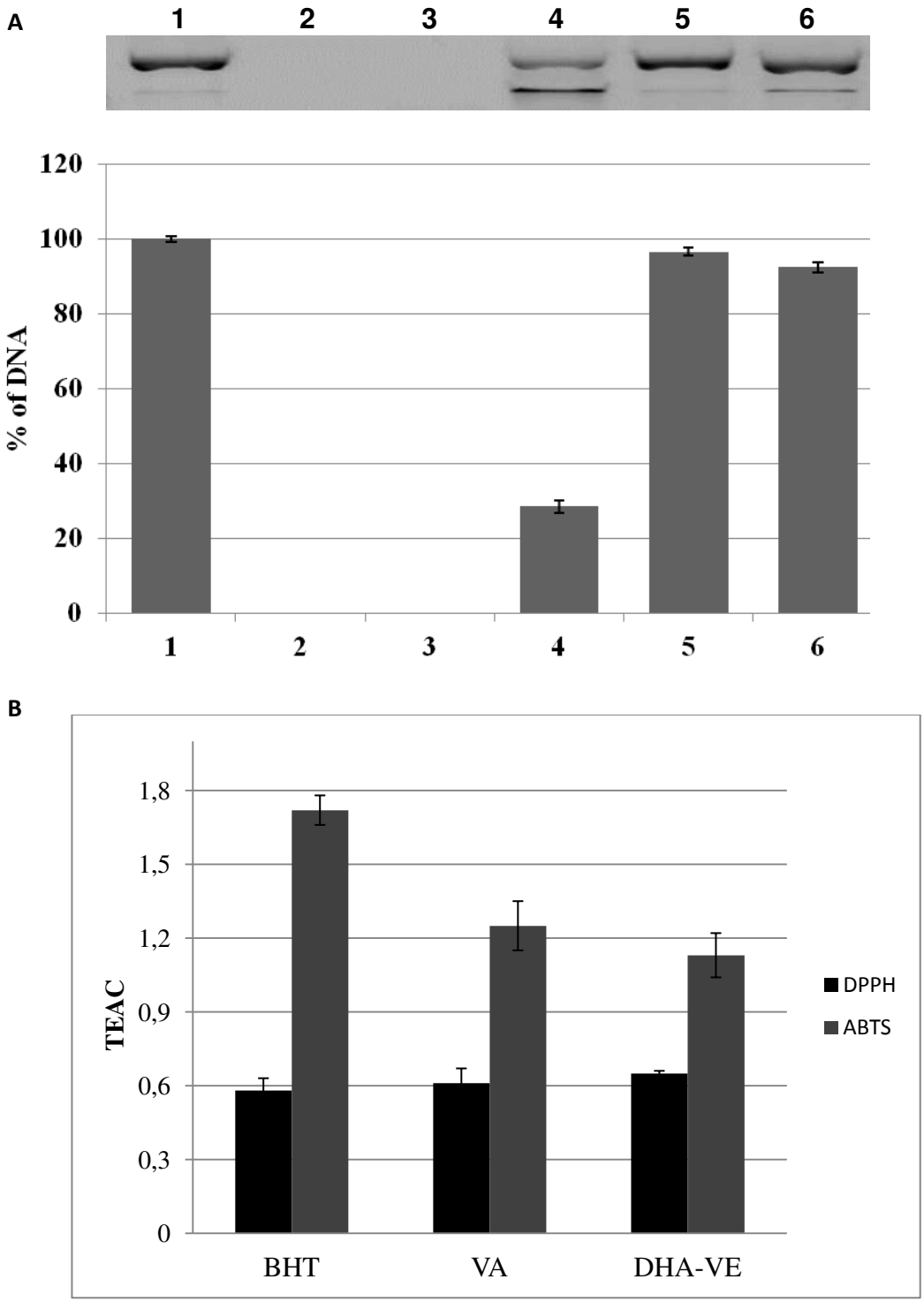

Figure 3: *In vitro* antioxidant activity of DHA-VE. (A) DNA scission by hydroxyl radicals. Reactions were incubated at 37°C for 1 h prior analysis in agarose gel electrophoresis. After estimation of band intensity, proportion of native supercoiled DNA was calculated and expressed in % of total DNA in each lane. Lane 1, native plasmid DNA; 2, plasmid DNA + [H_2O_2+$FeSO_4$]; 3, plasmid DNA + [H_2O_2+$FeSO_4$] +

free DHA; 4, plasmid DNA + [H_2O_2+$FeSO_4$] + DHA-EE; 5, plasmid DNA + [H_2O_2+$FeSO_4$] + DHA-VE; 6, plasmid DNA + [H_2O_2+$FeSO_4$] + VA. **(B)** Scavenging capacity towards $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radicals. Activities were compared to BHT as reference molecule. Data were expressed as TEAC values. Data are expressed as mean ± SEM, n=3.

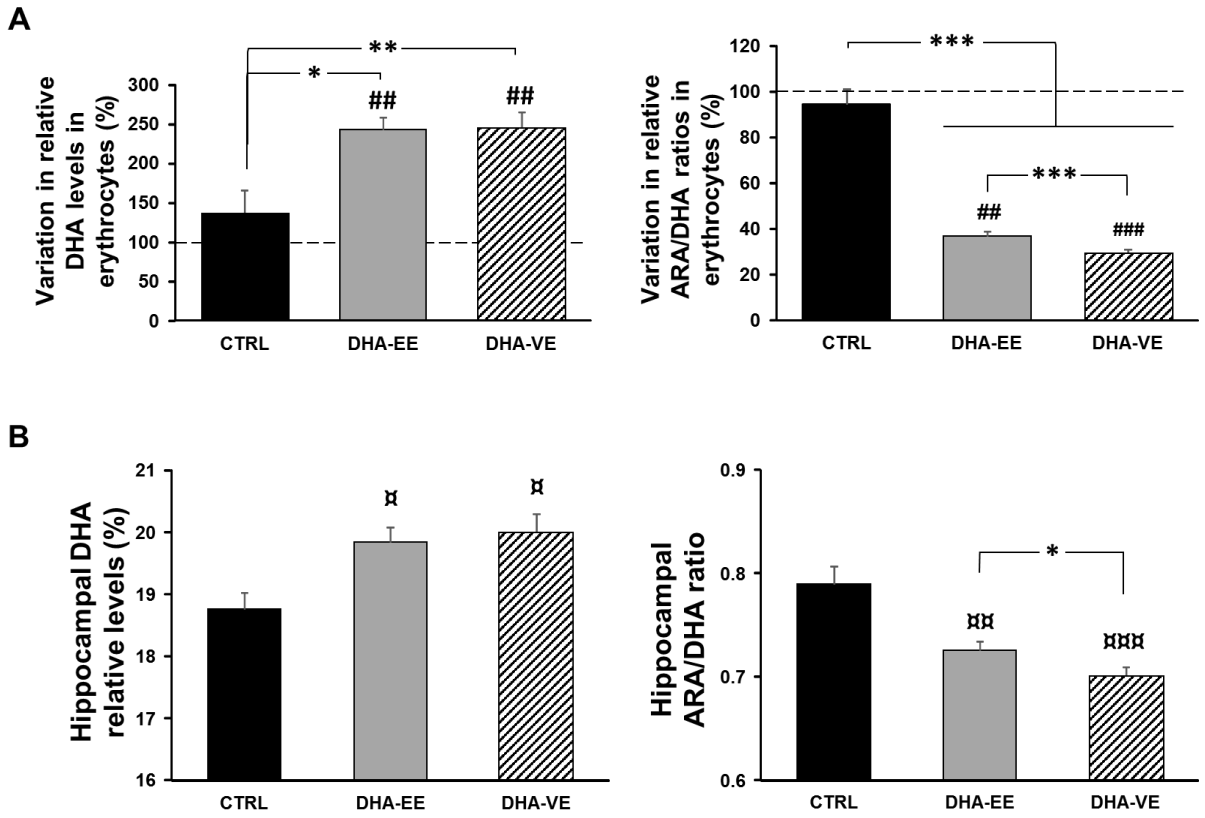

Figure 4: Effects of DHA-VE-supplemented diet on FA relative contents. Mice (n=6 per group) were fed specific diet for 30 days. (A) FA relative content in mice erythrocytes. FA concentrations after diet administration were compared for each individual and normalized to levels at baseline. (B) FA relative levels in hippocampus. Data are expressed as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001: significant difference between indicated groups. ##p<0.01, ###p<0.001: significant difference after 30 days *vs.* baseline on indicated diet. ¤p<0.05, ¤¤p<0.01, ¤¤¤p<0.001: significant difference *vs.* CTRL diet.

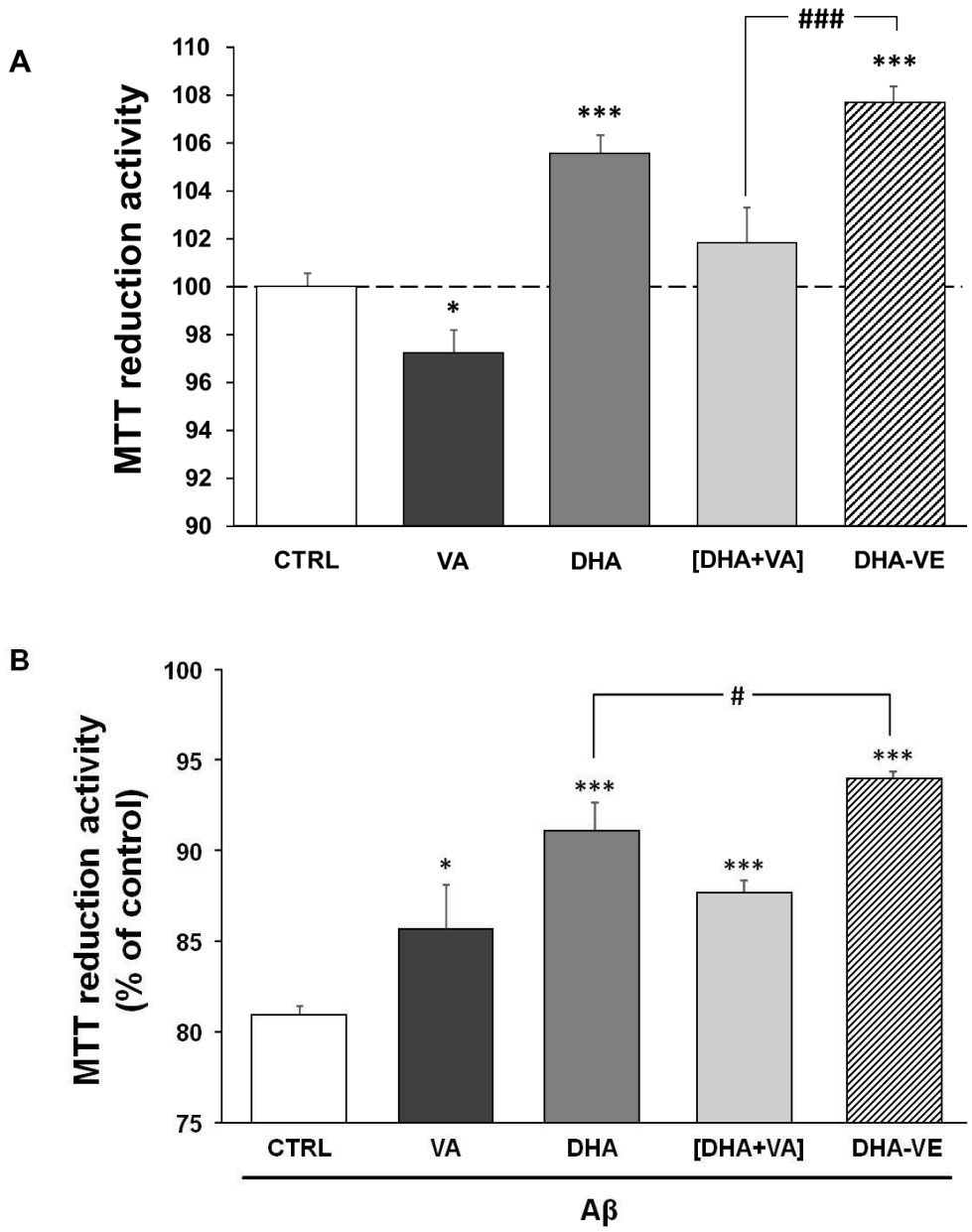

Figure 5. Effects of DHA-VE on primary neurons *in vitro.* Cell viability was assayed by measuring mitochondrial MTT reduction activity. (A) Beneficial effects of DHA-VE. Neurons were cultured for 24 h in standard medium (CTRL) or supplemented with 1 µM VA, DHA, [DHA+VA] (equimolar mixture), or DHA-VE. (B) Neuroprotective effects of DHA-VE on Aβ-induced neurotoxicity. Neurons were pre-incubated for 24 h with VA, DHA, [DHA+VA] and DHA-VE, prior to exposure to Aβ (1 µM, 24 h). Data were normalized to the activity in CTRL cells exposed to vehicle (no Aβ) designated as 100%. Data are expressed as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001: significant difference *vs.* CTRL. #p<0.05, ###p<0.001: significant difference between indicated groups.

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1.3. Contribution de l'article

Cette partie est consacrée à la mise au point des conditions réactionnelles permettant la synthèse enzymatique de l'ester phénolique de DHA à partir de l'ester éthylique de l'acide docosahexaénoïque (DHA-EE) et de l'alcool vanillique (VA). Le travail présenté avait pour objectif majeur de développer un bioprocédé performant, pour une production sûre et respectueuse de l'environnement. Une démarche d'intensification du procédé dans des milieux exempts de solvant a été menée dans le but de produire des quantités d'esters suffisantes pour la réalisation d'études *in vitro* et *in vivo* et des premiers essais d'application. Ainsi, une production d'ester de 440 g/L a pu être obtenue, correspondant à un rendement de conversion de l'alcool vanillique de 91%. Ces performances ont été atteintes en travaillant sous pression réduite, de manière à éliminer en continu l'éthanol produit, et grâce à une alimentation semi-continue du réacteur en alcool vanillique. Dans ces conditions, aucun signe de dégradation oxydative du milieu n'a été observé au cours des synthèses.

Les conditions de purification de l'ester ont été optimisées, conduisant à un ester pur à 98%. Les analyses structurales par spectrométrie de masse et résonnance magnétique nucléaire ont permis d'élucider sa structure et de montrer que le greffage du DHA a lieu au niveau de l'hydroxyle benzylique de l'alcool vanillique. Ceci est en accord avec les propriétés de sélectivité de la lipase B de *C. antarctica* qui tend à favoriser l'acylation des hydroxyles primaires. Les activités biologiques de la nouvelle molécule ont été étudiées par des tests *in vitro* et *in vivo* afin de préciser son potentiel applicatif. L'activité inhibitrice de l'ester vis-à-vis de diverses espèces radicalaires a été montrée. L'acylation de l'alcool vanillique par le DHA ne semble pas affecter son potentiel antioxydant. Les résultats obtenus montrent par ailleurs que la

biodisponibilité du DHA est améliorée dans le cas d'une supplémentation en DHA-VE par rapport à une supplémentation en DHA-EE. Ceci se traduit par une teneur en DHA plus élevée dans les érythrocytes et les membranes neuronales de souris soumises à un régime enrichi en DHA-VE. De plus, les propriétés neuroprotectrices du DHA-VE ont été démontrées *in vitro*. Ceci se manifeste par une haute survie cellulaire des neurones primaires de rats exposés au stress β-amyloïde. Les travaux réalisés ont montré que le DHA-VE n'induit pas de toxicité visible. Au contraire, cette molécule semble combiner les effets bénéfiques des acides gras polyinsaturés ω3 (AGPI) et les propriétés antioxydantes des composés phénoliques.

En conclusion, l'ester DHA-VE exhibe les principaux effets bénéfiques des deux entités qui le constituent. Un tel composé fonctionnel devrait faciliter la préparation d'aliments enrichis en DHA et ainsi, élargir le champ d'application des acides gras polyinsaturés et des composés phénoliques, dont les bénéfices "santé" sont de plus en plus recherchés.

Partie 2: Oxidative stability of DHA phenolic ester

2.1. Introduction

L'incorporation des acides gras polyinsaturés oméga-3 dans des matrices alimentaires est souvent limitée par leur grande sensibilité à l'oxydation, conduisant à la formation d'hydroperoxydes et des aldéhydes responsables du phénomène de rancissement. Différentes stratégies ont été décrites dans la littérature et appliquées dans l'industrie pour réduire ce phénomène. La définition de la solution la plus adéquate au regard d'un système donné implique de bien connaître le mécanisme de l'oxydation et de pouvoir suivre sa progression par des analyses appropriées. Ces dernières permettront également de déterminer la durée de vie des produits, d'évaluer leur acceptabilité et leur qualité nutritionnelle. Le travail présenté dans ce chapitre visait à étudier l'effet du greffage de l'alcool vanillique sur la stabilité à l'oxydation du DHA à l'aide de différentes méthodes spectroscopiques, et notamment la spectroscopie infra-rouge.

Cette étude fait l'objet d'un article accepté dans Food Chemistry.

2.2. Oxidative stability of DHA phenolic ester

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Oxidative stability of DHA phenolic ester

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ABSTRACT

Docosahexaenoic acid vanillyl ester (DHA-VE) was synthesized from docosahexaenoic acid ethyl ester (DHA-EE) and vanillyl alcohol by a solvent-free alcoholysis process catalysed by *Candida antarctica* lipase B. Oxidative stability of pure DHA-VE and the crude reaction medium consisting of 45% DHA-VE and 55% DHA-EE were compared with that of DHA-EE under various storage conditions. Oxidation progress was followed by determination of conjugated dienes and FTIR measurements. Analyses showed that DHA-EE was rapidly oxidised under all storage conditions in comparison with DHA-VE and crude reaction medium, whatever the temperature and the storage time. The grafting of vanillyl alcohol appeared as a powerful way to stabilize DHA against oxidation. Thanks to their stability, both DHA-VE and the crude reaction medium, allowing the production of the ester, offer huge potential as functional ingredients.
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1. Introduction

DHA is required for brain and eyesight development of both foetus and infant (Swanson, Block, & Mousa, 2012). For adults, DHA is involved in maintaining the normal functioning of brain and nervous system (Domenichiello, Chen, Trepanier, Stavro, & Bazinet, 2014; Kuratko, Barrett, Nelson, & Salem, 2013; Picq, Bernoud-Hubac, & Lagarde, 2013). DHA deficiency has been found in several disorders, such as Alzheimer's disease, schizophrenia, dyslexia, and some cases of attention-deficit or hyperactivity disorder (Cunnane, Chouinard-Watkins, Castellano, & Barberger-Gateau, 2013; Wu et al., 2014). Because mammals have limited ability to synthesize polyunsaturated fatty acids (PUFA), these must be supplied in the diet. The American Heart Association generally recommends a daily intake of n-3 PUFA, up to 400–500 mg of EPA and DHA, which may be reached by consuming at least two servings of oily fish per week or cod liver fish oil or encapsulated n-3 PUFA ethyl esters (EEs) (Lichtenstein et al., 2006). However, the practical use of such lipids for a preventive purpose is often limited because of their high susceptibility to oxidation, producing hydroperoxides and aldehydes responsible for the undesirable rancidity off-flavour (Arab-Tehrany et al., 2012; St. Angelo, Vercellotti, Jacks, & Legendre, 1996). In addition to these organoleptic limitations, nutritional aspects also have to be considered, as the consumption of high amounts of oxidised products is suspected to cause oxidative stress, and then to induce diseases, such as cancer, diabetes and rheumatoid arthritis (Lin, Lai, Lin, & Chiang, 2000).

The oxidative degradation of unsaturated lipids has been the subject of many studies and still today remains one major scientific topic for both researchers and industries. Under mild conditions and according to the autoxidation mechanism, molecular oxygen reacts with unsaturated fatty acid radicals, leading to primary oxidation products, namely peroxides and hydroperoxides, and then to secondary oxidation products, such as ketone and aldehyde compounds responsible for rancidity. This degradation pathway is complex and variable from one oil to another due to its dependency on fatty acid composition and conditions causing oxidation. Different strategies have been described in the literature and applied in the industry to reduce this phenomenon. A common way consists in using synthetic or natural antioxidants (Roby, Sarhan, Selim, & Khalel, 2013b; Staszewski, Pizones Ruiz-Henestrosa, & Pilosof, 2014; Wang et al., 2011). For the past few

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Abstract

Docosahexaenoic acid vanillyl ester (DHA-VE) was synthesized from docosahexaenoic acid ethyl ester (DHA-EE) and vanillyl alcohol by a solvent-free alcoholysis process catalyzed by *Candida antarctica* lipase B. Oxidative stability of pure DHA-VE and the crude reaction medium consisting of 45% DHA-VE and 55% DHA-EE were compared with that of DHA-EE under various storage conditions. Oxidation progress was followed by determination of conjugated dienes and FTIR measurements. Analyses showed that DHA-EE was rapidly oxidized under all storage conditions in comparison with DHA-VE and crude reaction medium whatever the temperature and the storage time. The grafting of vanillyl alcohol appeared as a powerful way to stabilize DHA against oxidation. Thanks to their stability, both DHA-VE and the crude reaction medium allowing the production of the ester offer huge potential as functional ingredients.

Keywords

Enzymatic esterification, lipase, oxidative stability, oxidation, FTIR spectroscopy, omega-3, PUFA, DHA and vanillyl alcohol

1. Introduction

DHA is required for brain and eyesight development of both fetus and infant (Swanson *et al.*, 2012a). For adults, DHA is involved in maintaining the normal functioning of brain and nervous system (Domenichiello *et al.*, 2014 ; Kuratko *et al.*, 2013 ; Picq *et al.*, 2013). DHA deficiency has been found in several disorders such as Alzheimer's disease, schizophrenia, dyslexia, and some cases of attention-deficit or hyperactivity disorder (Cunnane *et al.*, 2013 ; Wu F. J. *et al.*, 2014). Because mammals have limited ability to synthesize polyunsaturated fatty acids (PUFA), these must be supplied in the diet. The American Heart Association generally recommends a daily intake of n-3 PUFA up to 400–500 mg EPA and DHA, which may be reached by consuming at least two servings of oily fish per week or cod liver fish oil or encapsulated n-3 PUFA ethyl esters (EEs) (Lichtenstein *et al.*, 2006). However, the practical use of such lipids for a preventive purpose is often limited because of their high susceptibility to oxidation producing hydroperoxides and aldehydes responsible for the undesirable rancidity off-flavor (Arab-Tehrany *et al.*, 2012 ; St. Angelo *et al.*, 1996). In addition to these organoleptic limitations, nutritional aspects have also to be considered as the consumption of high amounts of oxidized products is suspected to cause oxidative stress, and then to induce diseases as cancer, diabetes and rheumatoid arthritis (Lin *et al.*, 2000).

The oxidative degradation of unsaturated lipids has been the subject of many studies and remains still today one major scientific topic for both researchers and industries. Under mild conditions and according to the autoxidation mechanism, molecular oxygen reacts with unsaturated fatty acid radicals leading to primary oxidation products namely peroxides and hydroperoxides, and then to secondary oxidation products as ketone and aldehyde compounds responsible for rancidity. This degradation pathway is complex and variable from one oil to another due to its dependency on fatty acid composition and conditions causing oxidation. Different strategies have been described in the literature and applied in the industry to reduce this phenomenon. A common way consists in using synthetic or natural antioxidants (Roby *et al.*, 2013b ; Staszewski *et al.*, 2014 ; Wang Hua *et al.*, 2011a). For the past few years, natural antioxidants as phenolic compounds have been more and more suggested as interesting alternatives to synthetic materials (Kindleysides S. *et al.*, 2012b). The antioxidant activity of such compounds generates a lot of interest and many works have been carried out aiming to benefit from their properties in a wide

range of applications (Roby *et al.*, 2013a). Most often, phenols have been simply added in lipid phases in order to protect them against oxidation (Mosca *et al.*, 2013 ; Rubilar *et al.*, 2012). However, there are limits to this approach since the antioxidant capacity of phenols strongly depends on their location relatively to the lipid phase (Liu Lingyi *et al.*, 2014a). Another approach has been proposed that consists in combining lipids with phenols into a single structure by forming an ester bond (Kobata *et al.*, 2002b). The main objective was the production of lipophilic derivatives of phenols aiming to enhance their antioxidant activity in lipid systems. (Mbatia B. *et al.*, 2011) found that incorporating rutin or vanillyl alcohol into a PUFA-enriched extract through an esterification process improved the hydrophobicity of phenolic compounds and then enhanced their antioxidant activity in lipid phases. Similar results were obtained in emulsions and biological systems with esters combining epigallocatechin and stearic, eicosapentaenoic or docosahexaenoic acids (Zhong andShahidi, 2012).

For a given system, the choice of the right antioxidant solution requires the capacity to make a qualitative and quantitative assessment of oxidation progress. To this end, many methods have been developed based on the determination of primary and secondary oxidation products (Barriuso *et al.*, 2013 ; Shahidi Fereidoon andZhong, 2005). The most commonly used consists in measuring the concentration of hydroperoxide (peroxide value, PV); nevertheless PV is not necessarily a good indicator to follow PUFA oxidation because of the high instability of hydroperoxides issued from their degradation (Cho *et al.*, 1987). Another method based on the measure of absorbance at specific wavelengths, namely 232 nm and 270 nm, allows evaluating the content of conjugated dienes (CD) and trienes (CT) (Frankel, 2005). The above-mentioned methods have found wide spread applications as routine tests to determine oxidative deterioration of lipids. However they provide a single index, which does not give information on actual chemical composition of the product, and then provides limited insight into the problem. Few years ago the determination of epoxy compounds in thermo-oxidized oils has been reported (Velasco *et al.*, 2002). Epoxy compounds are sensitive to detect and allow characterizing low oxidation levels in various oils and food lipids; they are nevertheless time-consuming. In this context, vibrational spectroscopy has been considered as a useful tool for fast measurement of lipid oxidation because of its high capacity to provide detailed information about molecular systems. FTIR has been successfully used to monitor oil oxidation under moderate and accelerated conditions (Vandevoort *et al.*, 1994). Main changes in FTIR

bands were interpreted and related to oxidation mechanism (Brys *et al.*, 2013). Parameters related to oil oxidation were quantified based on the determination of specific compounds as peroxides (Guillen andCabo, 2000 ; Ruiz *et al.*, 2001), anisidine (Dubois *et al.*, 1996), volatile compounds (Ahro *et al.*, 2002) and malonaldehyde (Mirghani *et al.*, 2002).

This work aimed: (i) to study the effect of enzymatic esterification of DHA with vanillyl alcohol on the oxidative stability of DHA using different spectroscopic methods, and (ii) model oxidation kinetics under different storage conditions.

2. Materials and methods

2.1. Chemicals

Lipase B from Candida Antarctica immobilized on a macro-porous acrylic resin (Novozym 435®, Novo Industry) was used to catalyze acylation reactions. DHA ethyl ester (docosahexaenoic acid ethyl ester, abbreviated as DHA-EE) and vanillyl alcohol (4-hydroxy-3-methoxybenzyl alcohol) were purchased respectively from KD-Pharma (Bexbach, Germany, purity 95%) and Sigma-Aldrich Chemicals (St. Louis, MO, purity 98%). All solvents were of analytical grade.

2.2. Sample preparation

DHA vanillyl ester (DHA-VE) was obtained from an enzymatic esterification between DHA-EE (sample 1) and vanillyl alcohol. Vanillyl alcohol is a powerful aroma constituent of many food products. This phenol has been proved to be as intense as vanillin, the most abundant component in vanilla (*Vanilla planifolia*) (Perez-Silva *et al.*, 2006). In addition to this interesting olfactory property, vanillyl alcohol exhibits a high radical scavenging capacity that was suggested to be responsible for its anticonvulsive and antioxidant properties (Shyamala *et al.*, 2007).

The reaction was achieved in the evaporation flask of a rotary evaporator, at 37 °C, under a 500 mbar pressure, with a 250 rpm rotation speed. Vanillyl alcohol (0.5 g) was added to 10 ml DHA-EE three times, every 24 h (fed batch process). The ethanol formed as a by-product of the reaction was continuously eliminated from the medium, moving the reaction equilibrium in favor of DHA-VE production. To stop the reaction, the enzyme was separated from the reaction medium by filtration. At the end of the synthesis, the reaction medium (sample 3) contained 45% DHA-VE. Flash chromatography was applied to get pure DHA-VE (sample 2).

2.3. Accelerated oxidation test

5 g of each sample (1, 2 and 3) were weighed in screw-capped glass tubes (10 ml) and stored at 100°C, 20°C and 4°C, in darkness. Oxidative stability was monitored throughout storage until significant differences between the treated samples were observed. Samples were periodically taken for further analysis.

2.4. Determination of conjugated dienes

Conjugated dienes (CD) are considered as primary products of oxidation. For each sample, the conjugated diene value was determined as previously described (Weber *et al.*, 2008). A 0.5 mg sample was dissolved in 10 mL of n-hexane, then diluted or concentrated to obtain an absorbance between 0.1 and 0.8. The solution must be perfectly clear. The absorbance was measured at 234 nm, using n-hexane as a blank. Temperature effect on oxidation rate was illustrated by means of Arrhenius equation:

$$\ln (k) = \ln A - Ea/RT$$

where k is the reaction rate, A is the kinetic constant, Ea is the activation energy (KJ mol^{-1}), R is the molar gas constant (8.3145 J K^{-1} mol^{-1}), and T is the absolute temperature (K). Activation energy and kinetic constants were determined respectively from the slopes and the intercepts of the lines generated by regressing ln (k) vs. 1/T linear regression.

2.5. FTIR Instrumentation

Infrared spectra were recorded on a Tensor 27 mid-FTIR spectrometer (Bruker, Karlsruhe, Germany) with a deuterated triglycine sulphate (DTGS) detector, operating under Opus software. A ZnSe ATR sampling accessory from Spectra Tech (Shelton, CT) was used for Total Attenuated Reflection measurements. The diaphragm was set to 4 mm and the scanning rate was fixed to 10 kHz. 256 scans were performed for both reference and samples.

2.6. Spectral Acquisition

A small amount of each sample, approximately 30 µL, was deposited in the attenuated total reflectance (ATR) ZnSe crystal, with avoiding the presence of air by circulating nitrogen. All spectra were recorded from 4000 to 800 cm^{-1}. To avoid high noise levels the spectra were collected with a resolution of 4 cm^{-1} to give a data point spacing of approximately 1.9 cm^{-1}, after Fourier transform and zero-filling. Spectra recorded with higher resolution gave similar frequency data for all samples but higher noise level and their registration took more time. Assignment of bands was made from

comparison with literature spectral data and with reference compounds spectra included in the software spectral library. Height and area of each band were measured and calculated by using the essential FTIR software. This procedure avoided experimental errors associated with the subjectivity of external operators. After each operation, the crystal was thoroughly cleaned up, washed with ethanol and water, and then dried.

2.7. Statistical Analyses

All analytical values represent the means ± SD of triplicate analyses. Two results were considered to be statistically significant when $p < 0.05$. All data were processed using the Microsoft Excel package.

3. Results and discussion

3.1. Conjugated dienes (CD)

Conjugated dienes are primary oxidation products, resulting from the rearrangement of polyunsaturated lipid double bonds. CD determination is a measure of oxidation state making it a good indicator of oil stability and antioxidant effectiveness (Iqbal andBhanger, 2007). An increase of absorbance at 234 nm is correlated to lipid degradation due to CD formation during the first stage of oxidation.

CD within DHA-EE, DHA-VE and crude reaction medium were followed during storage at different temperatures. Results are shown on Fig.1. As expected, CD contents increased with time during the storage whatever the sample and the temperature, indicating an oxidative degradation. CD values were globally higher for DHA-EE than for DHA-VE and the crude reaction medium. At 20°C and 4°C DHA-VE and crude reaction medium remained almost stable during few weeks whereas DHA-EE get oxidized during the first 2 weeks of storage. DHA-VE appeared as the most stable compound whereas the crude reaction medium exhibited a midway behavior between pure DHA-VE and pure DHA-EE. For any storage temperature, two stages were observed in the degradation process. In the first stage, CD values slowly increased, and then this trend accelerated in the second stage. CD values obtained at 4°C and 20°C were not significantly different. For all samples, the second stage of oxidation seemed to start after 8 weeks of storage at 20°C, in comparison with 10 weeks of storage at 4°C, suggesting a higher stability of the samples at low

temperature. At 100°C, oxidation was observed to start in the early hours of storage, and then rapidly progressed with time. These results are in accordance with previous studies demonstrating that the increase of temperature speed up oxidative degradation (Iqbal andBhanger, 2007 ; Wang Hua *et al.*, 2011a). Furthermore, the high stability of DHA-VE comparing with DHA-EE was demonstrated, showing the interest of combining highly oxidizable lipids with phenolic compound *via* esterification process.

Arrhenius model describes the relationship between reaction rate and temperature. Sample oxidation at low and high temperatures may pass through different steps or reaction pathways depending on activation energy (Nagy andTuranyi, 2012). Moreover, temperature affects reaction kinetic but also oxygen diffusivity and solubility. The apparent reaction kinetic is dependent of multiparametric system and Arrhenius model integrates all these factors to produce a global model. In Fig 1, two linear steps of oxidation kinetic appear and Fig.2 provides parameters for Arrhenius equation (kinetic constant and activation energy) for each of them. At any studied temperature, the reaction rates (k1 and K2) were the lowest for DHA-VE compared with DHA-EE and reaction medium; so, oxidation of DHA-VE was the slowest at any temperature. DHA-EE was highly sensitive to oxidation in comparison with DHA-VE.

The activation energy (E_a) is proportional to the slope of Arrhenius equation (Slope = -Ea/R). E_a for DHA-EE was lower than those of DHA-VE for both first and second step of oxidation. It means that DHA-VE oxidation kinetic was more activated by increasing temperature than DHA-EE oxidation. So, at ambient temperature and below, DHA-VE was much more stable than DHA-EE. As temperature increase up to 100°C, oxidation kinetic became closer and the protective effect of the phenol is less efficient.

3.2. Fourier transforms infrared spectroscopy (FTIR)

Samples were analyzed during storage by infrared spectroscopy. Both intensity and frequency of the bands were assessed aiming to point out structural changes due to oxidation. FTIR spectra of fresh samples (DHA-EE, DHA-VE and reaction medium) are shown in Fig.3. DHA-EE and DHA-VE spectra comprised the characteristic bands of the two pure compounds: 3600 – 3100 cm^{-1} due to O-H bond stretching of vanillyl

alcohol hydroxyl groups, 3013 cm^{-1} due to =C-H stretching of DHA *cis*-double bonds and 1738 cm^{-1} due to C=O stretching of DHA-EE and DHA-VE ester groups. Reaction medium spectrum was characteristic of a mixture of residual DHA-EE and DHA-VE resulting from esterification (Belhaj *et al.*, 2010 ; Vongsvivut *et al.*, 2012).

During storage some changes in FTIR spectra were observed indicating the oxidative degradation of the samples. Main changes were the emergence of a broad band near 3445cm^{-1} in the case of DHA-EE and the broadening of bands from 3600 to 3100 cm^{-1} in the case of DHA-VE and crude reaction medium, indicating the formation of hydroperoxide oxidation products. A slight decrease of the band around 3013 cm^{-1} was observed due to disappearance of DHA *cis*-double bonds. In addition the frequency of the C=O band around 1737cm^{-1} was observed to decrease with time. All these changes were indicative of the oxidative degradation of lipids and are detailed just below (Ahro *et al.*, 2002 ; Akhtar *et al.*, 2014 ; Belhaj *et al.*, 2010).

3.2.1. Changes in the spectral region 3100 - 3600 cm^{-1}

Significant changes occurred during storage in the 3600 - 3100 cm^{-1} spectral region whatever the sample (Fig. 4). In the case of DHA-EE, a band appeared and increased at 3457 cm^{-1}, from the first month of storage at 20°C and 4°C and from the first two hours of storage at 100°C. This was indicative of the presence of hydroxyl groups newly formed resulting from the formation of hydroperoxides. A similar trend was observed by (Belhaj *et al.*, 2010) during the storage of salmon oil and was attributed to oxidative degradation of polyunsaturated lipids. In the case of pure DHA-VE, intensification and broadening of the bands were observed with time from the second month of storage at 4°C and 20°C. These changes were significant during the first two hours of storage at 100°C and were explained by overlapping of original bands of DHA-VE and new absorption bands due to hydroperoxides generated during oxidation process. Just as before, FTIR spectra of the crude reaction medium evolved intermediately between DHA-EE and pure DHA-VE. Whatever the storage temperature, DHA-VE and DHA-EE appeared respectively as the most and the less stable samples towards oxidation. The crude reaction medium exhibited a midway stability. As expected, for all samples, a storage temperature of 4°C was shown to

decrease oxidation rate in comparison with 20°C. All these results were in agreement with above mentioned CD results (see Fig.1).

3.2.2. Changes in the band at 3013 cm^{-1}

In addition to changes observed in the region related to OH bond vibrations, FTIR spectra showed a significant decrease in the intensity of the band at 3013 cm^{-1} whereas bands around 2850 cm^{-1} intensified. These bands relate respectively to =C-H bond stretching vibration of *cis*-double bonds and symmetric stretching vibration of saturated CH_2 groups. From a practical point of view the time until the band at 3013 cm^{-1} begins to decrease could be considered as an indicator of oxidation (Guillen andCabo, 2002). This trend was clearly observed in DHA-EE sample stored at 100°C whereas no significant change was noticed for DHA-VE sample (Fig.5.A). At storage temperatures of 4°C and 20°C, the ratio between the absorbance of the band at 2853 cm^{-1} and that of the band at 3013 cm^{-1} (A2854/A3013) was followed with time as an indicator of oxidation progress (Fig.5.B.). In fact the increase of this ratio was due to disappearance of *cis*-double bonds of lipids during oxidation favoring the formation of saturated bonds (Van de Voort et al, 1994). No significant change was observed for DHA-VE and crude reaction medium samples stored at 4°C. At this temperature, DHA-EE became oxidized after 10 weeks of storage. Storage at 20°C was shown to increase oxidation rate leading to the degradation of DHA-VE, crude reaction medium and DHA-EE after 9 weeks, 4 weeks and 3 weeks, respectively. Unsurprisingly, high storage temperature was shown to favor oxidation.

3.2.3. Changes in the spectral region 1800 - 1600 cm^{-1}

The spectral region 1600 – 1800 cm^{-1} provides information about secondary oxidation products, especially aldehydes responsible for rancidity. More specifically, a shift of the band relative to ester carbonyl bond towards lower wavenumbers is indicative of lipid oxidation due to the formation of aldehydes (Muik *et al.*, 2005 ; Rohman andMan, 2013). Fresh samples exhibited a characteristic band around 1738 cm^{-1} corresponding to ester carbonyl bond stretching. The frequency of this band was followed during storage as shown in Fig.6. For all samples and storage conditions, a shift of the C=O stretching band was observed with time. Main shifts were obtained for DHA-EE whereas similar trends were noticed for DHA-VE and the crude reaction medium. Once again the phenomenon was accentuated by high temperatures. The

frequency remained almost stable in the case of DHA-VE and the crude reaction medium stored at 4°C or 20°C. On the contrary, the frequency significantly decreased with time in the case of DHA-EE even at low temperature. Besides these changes, other authors reported the appearance of a band at 1654 cm^{-1} that was assigned to α,β-unsaturated aldehydes and ketones as secondary oxidation products (Vandevoort *et al.*, 1994). In the present study no significant change was observed at this wavenumber.

4. Conclusions

In this study, different spectroscopic methods were used to monitor the oxidative stability of DHA-based esters: DHA ethyl ester is a commercially available form of DHA; DHA vanillyl ester results from the enzymatic alcoholysis reaction between DHA-EE and vanillyl alcohol. Conjugated dienes determination is a widespread and inexpensive technique providing information about the first stage of oxidation that leads to primary oxidation products. In the present work, CD determination was particularly effective to study the thermal and temporal stability of DHA-based compounds. Furthermore, FTIR is now well-known for its high efficiency to follow structural changes in complex evolving systems. The different regions of FTIR spectra provide useful information about functional groups and their chemical environment. On a practical point of view, this rapid and nondestructive method does not require any sample or chemical preparation, and then allows significant time- and cost-savings in comparison with classical analyses. In this work, FTIR was shown to be an efficient tool to follow lipid oxidation thanks to significant changes in the frequency and the magnitude of characteristic bands. More specifically, the intensity of the band related to =C-H bond stretching vibration of *cis*-double bonds at 3013 cm^{-1} depended on the degree of unsaturation of the samples, and then was used as a marker for DHA oxidation. Another sensitive indicator was the ratio between the absorbance at 3013 cm^{-1} and the absorbance at 2853 cm^{-1} that corresponded to the vibration of saturated C-H bonds.

Unsurprisingly, the oxidative stability of the compounds was negatively affected by increasing temperature and storage time. All results indicated a higher stability of DHA-VE in comparison with DHA-EE, showing the interest of combining this highly oxidizable lipid with vanillyl alcohol in a single structure. According to FTIR data,

oxidation was delayed till 8 weeks in the case of pure DHA-VE stored at 20°C against 2 weeks in the case of pure DHA-EE. A midway stability was determined for the crude reaction medium made of 45% DHA-VE and 55% DHA-EE. Main advantages of such a medium are, firstly, a high stability despite a significant content in DHA, and secondly, easy preparation and use that do not require any purification step. Phenolic esters of DHA undoubtedly appear as promising derivatives that could make easier the use of polyunsaturated lipids in food preparations.

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Figures:

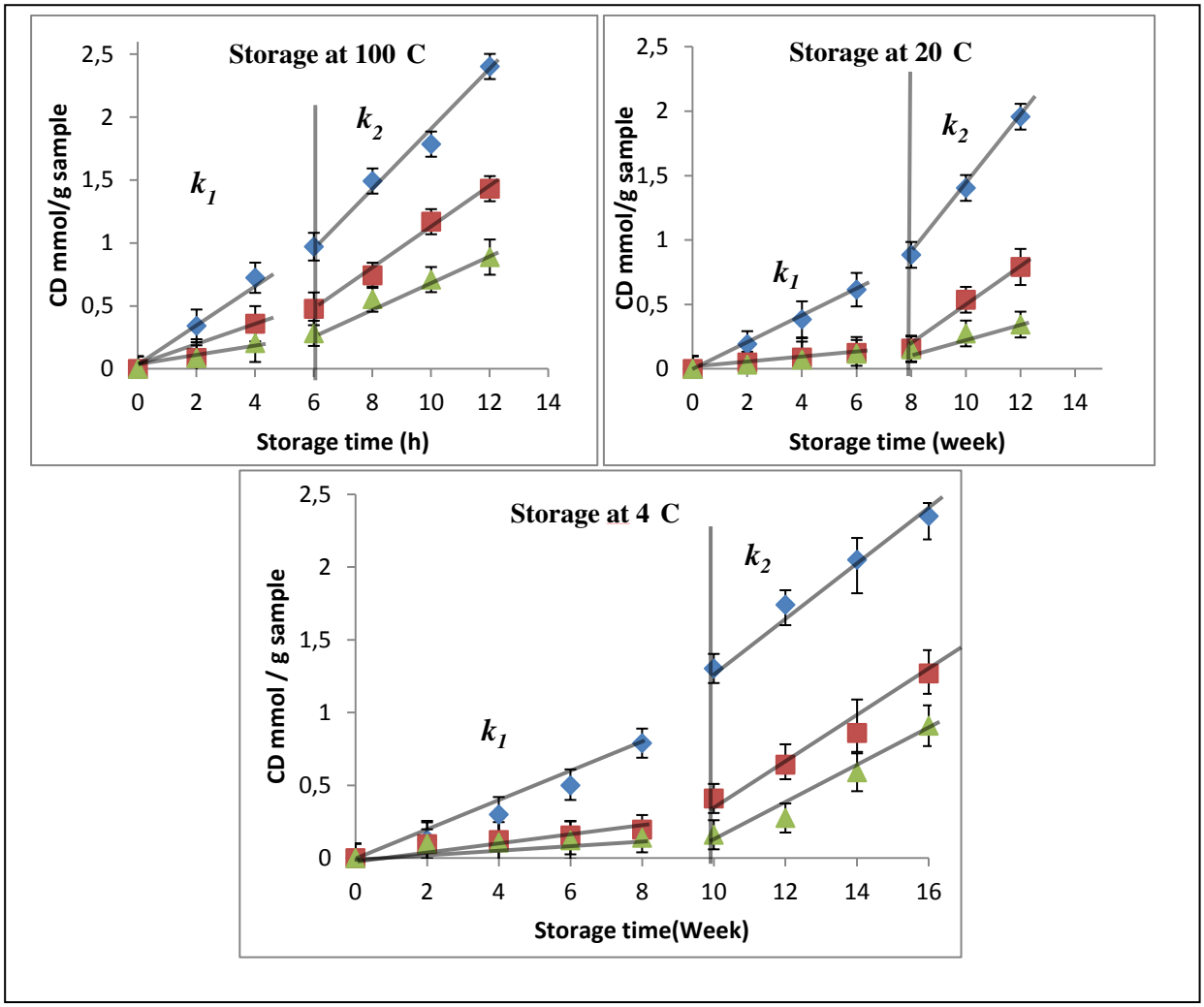
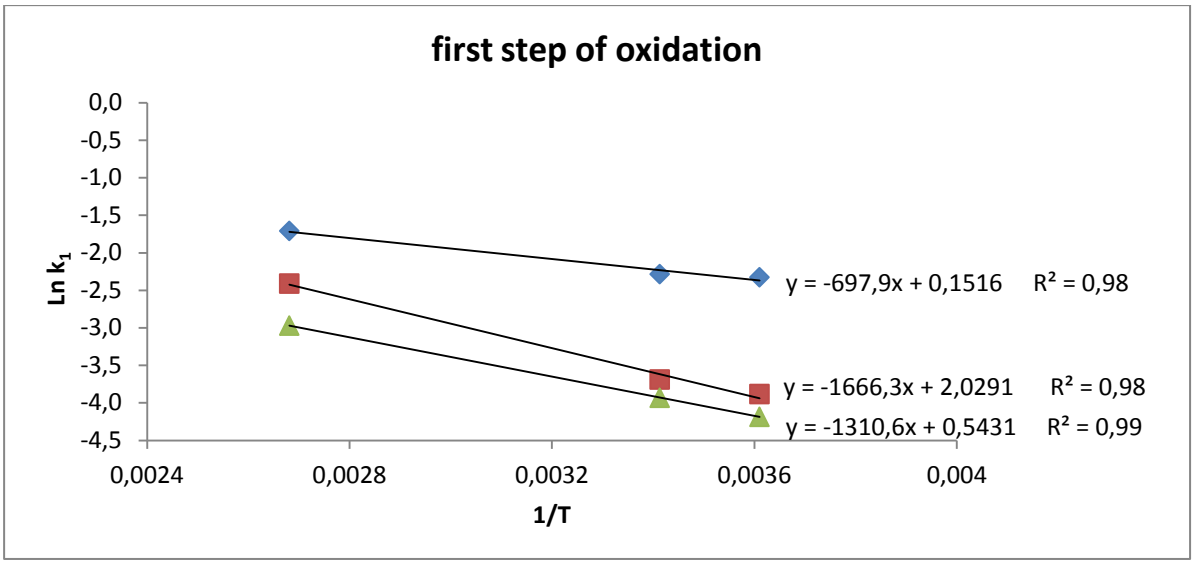


Fig.1. Conjugated dienes at 234 nm of samples oxidation at different conditions using UV-Visible spectrometry analysis. Values obtained are the average ± standard deviation of 3 replicates. DHA-EE ♦, Synthesis reaction medium ■ and DHA-VE ▲.



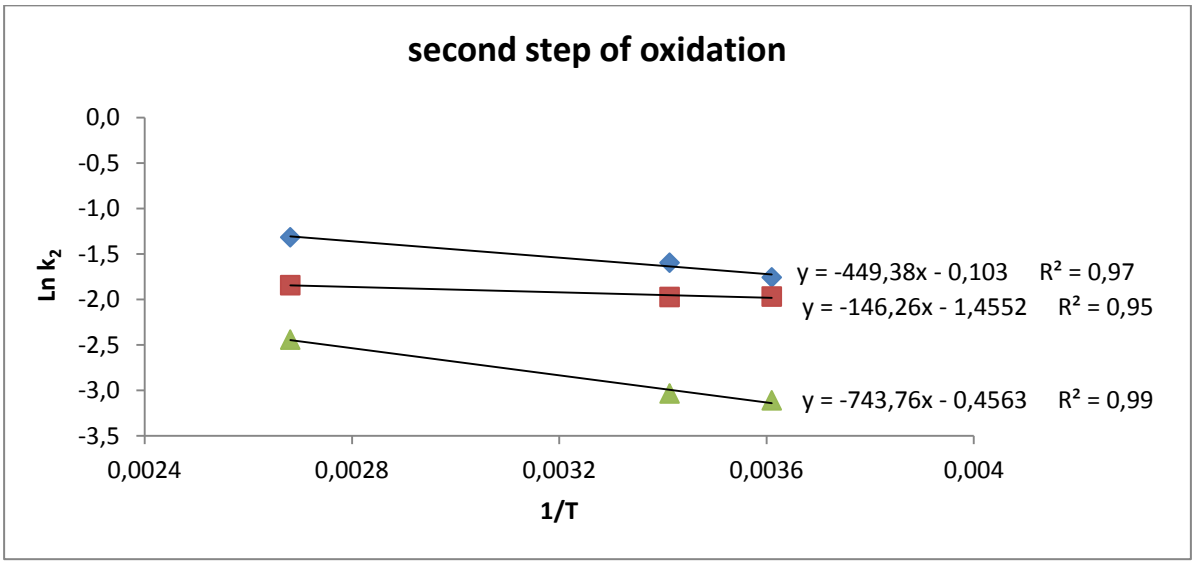


Fig.2. Regression parameters for Arrhenius relationships between the reaction rate and temperature for various samples. DHA-EE ♦, synthesis reaction medium ■ and DHA-VE ▲.

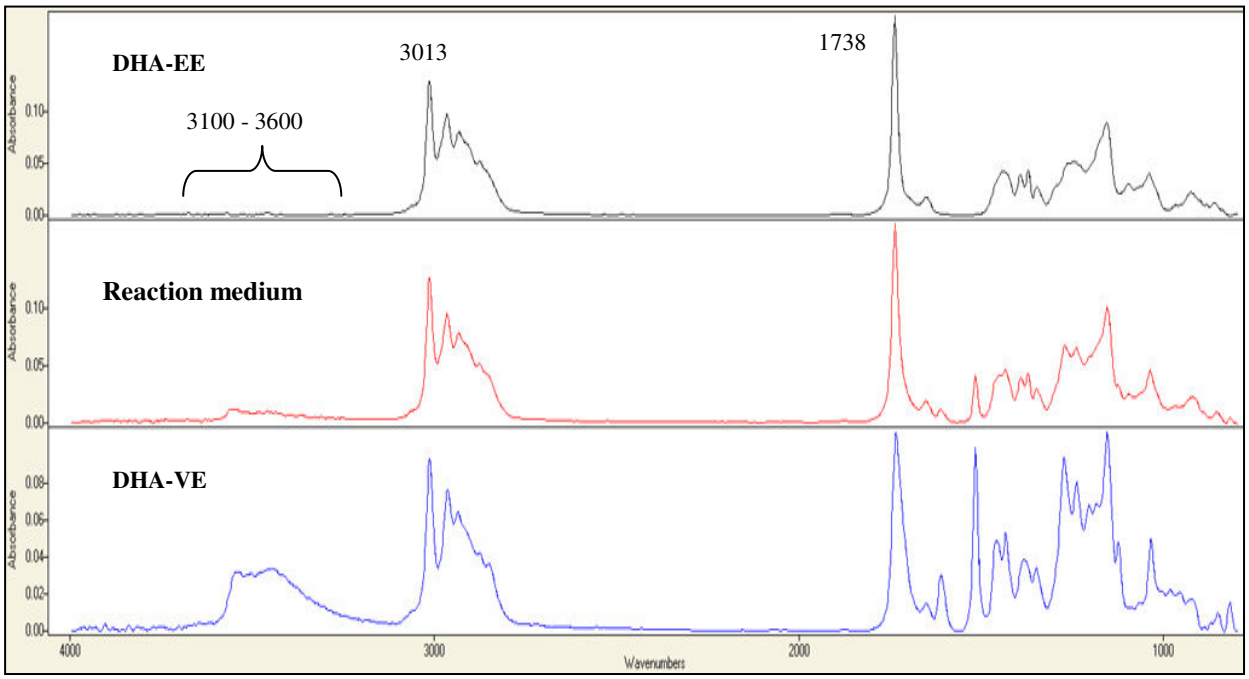


Fig.3. FTIR spectra of fresh sample of DHA-EE, DHA-VE and synthesis reaction medium.

Fig. 4 Changes produced in the region between 3650 – 3100 cm^{-1} of the infrared spectrum of samples stored during 3 months. t=0 month ——, t=1 month ....., t=2 months — — and t=3 months — · · at 4°C & 20°C and of samples stored at 100°C t=0h ——, t=4h ....., t=8h — — and t=10h — · ·

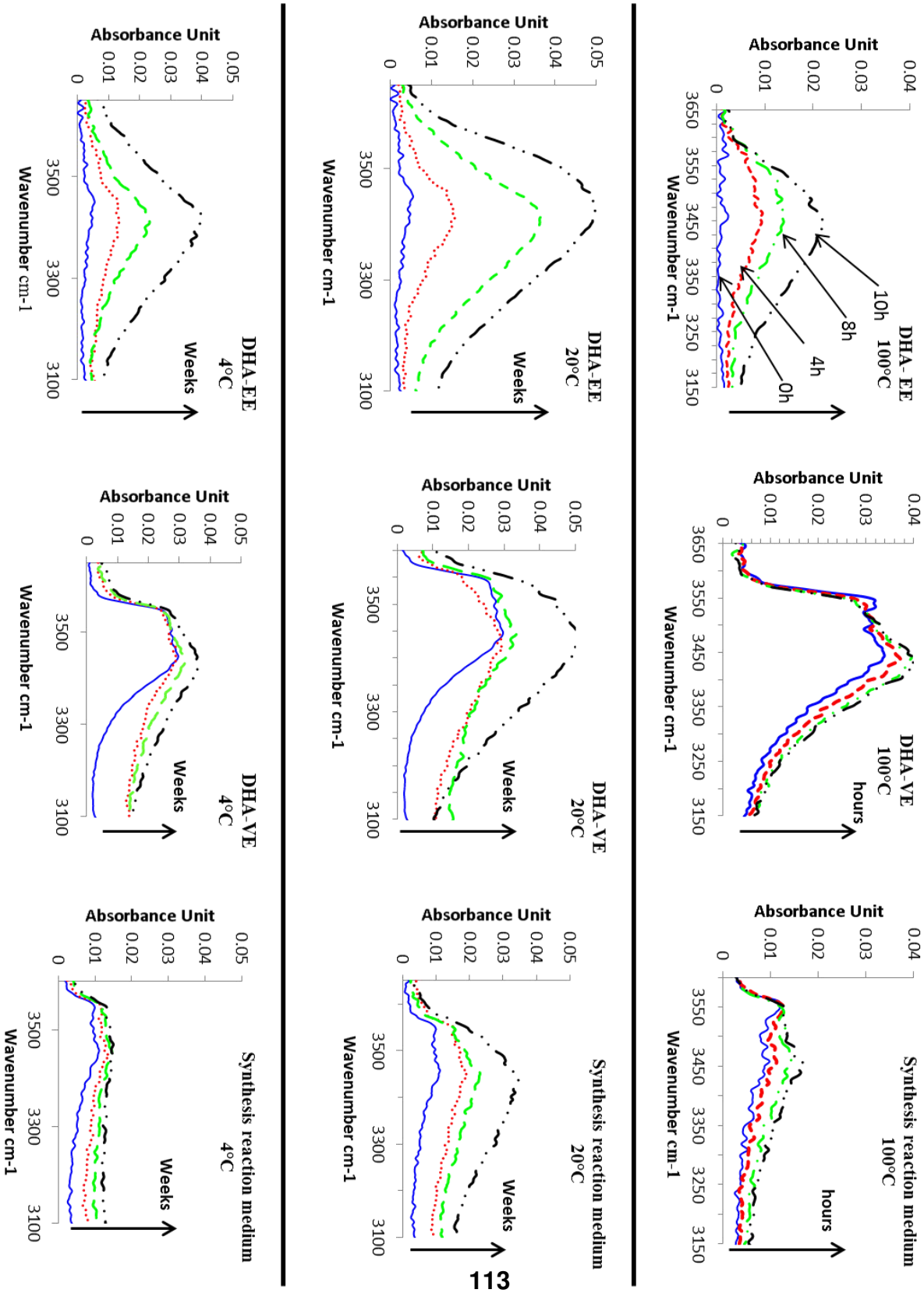
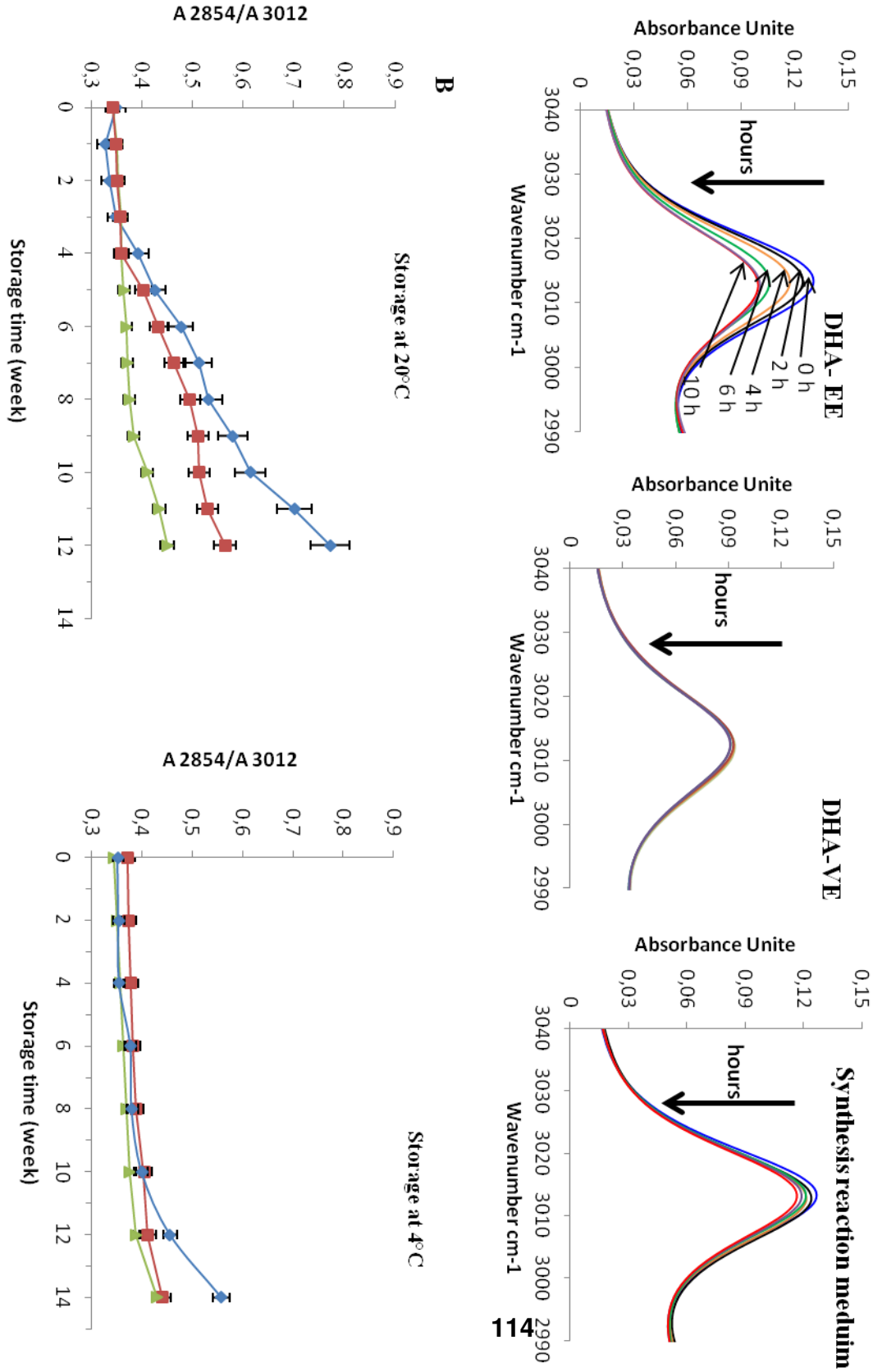


Fig.5. (A) Changes produced in the band at 3013cm^{-1} of the infrared spectrum of samples stored at 100°C. **(B)** The variation in A2853/A3012 ratio during the oxidation process at 4°C and 20°C of samples DHA-EE ♦, Synthesis reaction medium ■ and DHA-VE ▲.



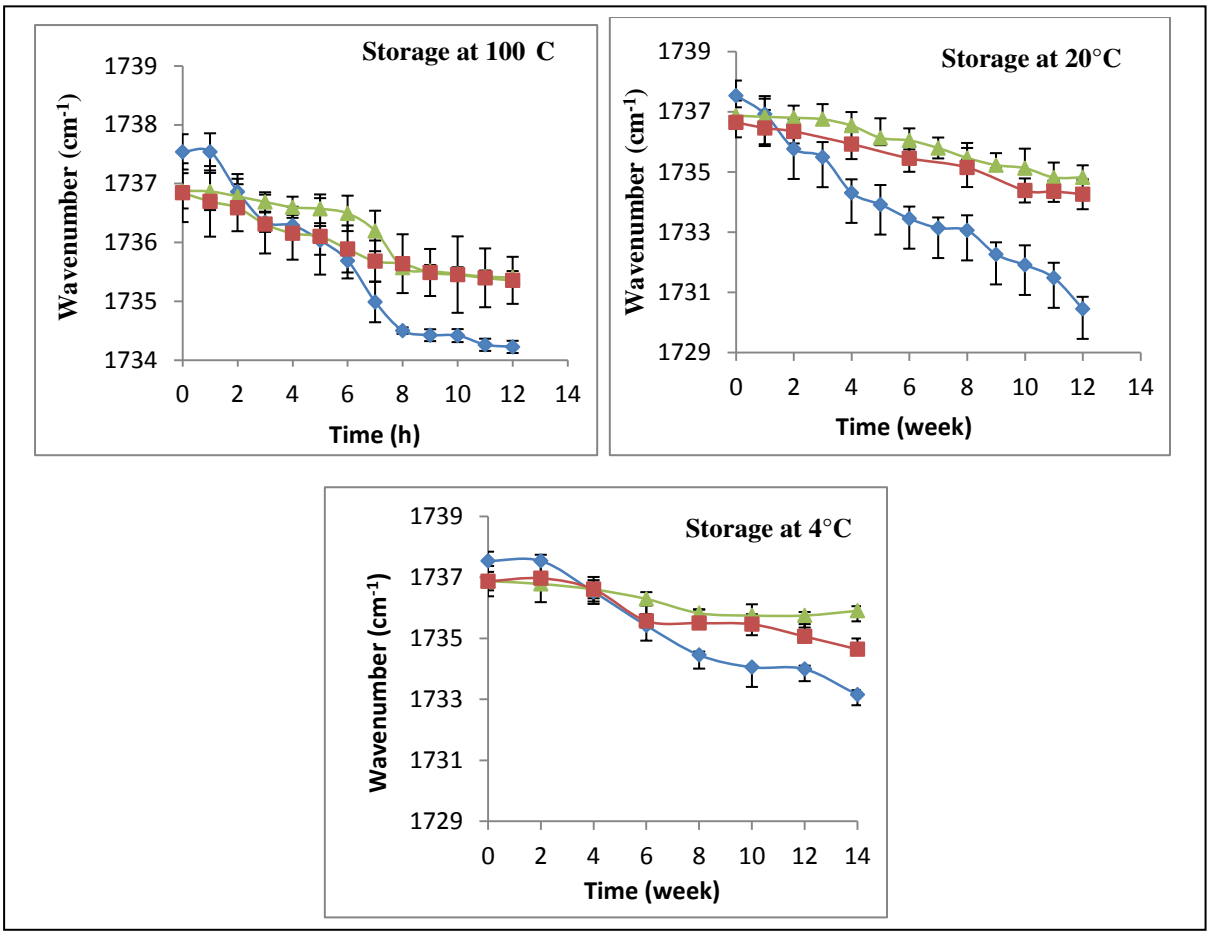


Fig.6. Evolution of wavenumber values of C=O band of DHA-EE ♦, Synthesis reaction medium ■ and DHA-VE ▲ under different oxidative conditions.

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3.3. Contribution de l'article

Ce travail démontre l'intérêt de greffer une entité phénolique antioxydante sur le DHA pour le stabiliser vis-à-vis de l'oxydation. Différentes méthodes spectroscopiques ont été utilisées pour étudier la stabilité à l'oxydation de l'ester DHA-VE dans diverses conditions de stockage. Dans ce travail, le suivi de la formation des diènes conjugués est apparu comme une méthode efficace pour étudier la stabilité thermique et temporelle des composés à base de DHA. Les résultats obtenus montrent que, quelles que soient les conditions de conservation, le DHA s'oxyde plus rapidement sous forme d'ester éthylique que l'ester DHA–VE ou le milieu de synthèse dont il est issu. Le greffage de l'alcool vanillique apparaît comme un puissant moyen de stabiliser le DHA vis-à-vis de l'oxydation. De par leur stabilité, le DHA-VE et le milieu réactionnel brut permettant son obtention présentent un fort potentiel d'application en tant qu'ingrédients fonctionnels. Nous avons montré que la méthode des diènes conjugués et la spectroscopie IR sont efficaces pour suivre l'oxydation des échantillons. Un élément important est qu'il ne semble pas nécessaire de purifier l'ester pour profiter de ses propriétés. La présence d'une faible concentration d'ester au sein de l'ester éthylique de DHA suffit à stabiliser l'ensemble. Ceci montre que non seulement l'ester est stable mais qu'en plus, il a un effet protecteur sur son environnement.

Partie 3: Enzymatic synthesis of vanillyl fatty acid esters from salmon oil in solvent-free medium.

3.1. Introduction

Les huiles de poisson présentent un grand intérêt nutritionnel en raison de leur teneur élevée en acides gras polyinsaturés (AGPI) de la série oméga 3, reconnus pour leurs effets positifs sur la santé humaine (Cunnane *et al.*, 2013 ; Kuratko *et al.*, 2013 ; Nicholson *et al.*, 2013). Les mammifères ayant une capacité limitée à synthétiser de tels acides gras, ceux-ci doivent être apportés en quantité suffisante par le biais de l'alimentation. Dans un tel contexte, les huiles de poisson apparaissent comme des sources d'AGPI intéressantes pour la supplémentation de produits alimentaires. Toutefois, leur mise en œuvre à l'échelle industrielle est difficile en raison de leur haute sensibilité à l'oxydation (Nowak, 2013 ; Ren *et al.*, 2013). Pour résoudre ce problème, de nombreuses études rapportent l'utilisation d'antioxydants phénoliques et montrent l'intérêt de ces composés pour stabiliser des huiles et des émulsions (Acosta-Estrada *et al.*, 2014 ; Staszewski *et al.*, 2014 ; Velderrain-Rodriguez *et al.*, 2014). Plus récemment, quelques auteurs ont proposé une autre stratégie consistant à assembler des composés phénoliques et des lipides riches en AGPI au sein d'une même structure. À cette fin, deux approches principales ont été proposées. La première est une estérification des AGPI d'intérêt issus de l'hydrolyse d'huiles par un alcool phénolique (procédés d'estérification directe ou d'alcoolyse) (Liu Lingyi *et al.*, 2014a ; Udomrati and Gohtani, 2014 ; Yang K.Z. *et al.*, 2014), tandis que la deuxième est basée sur l'incorporation directe d'acides phénoliques par un procédé d'acidolyse (Aouf *et al.*, 2014 ; Ray *et al.*, 2013 ; Wang J. K. and Shahidi, 2014). Les produits dérivés obtenus sont susceptibles d'allier les propriétés des AGPI et des composés phénoliques, à savoir une haute valeur nutritionnelle et des propriétés antioxydantes

intéressantes. Quelques travaux rapportent même des propriétés accrues des dérivés par rapport aux molécules natives.

Les principaux objectifs de cette étude étaient la mise au point d'un procédé sans solvant permettant la production d'esters phénoliques d'acides gras. L'huile de saumon a été utilisée comme source d'acides gras ; de même que précédemment, l'alcool phénolique choisi est l'alcool vanillique. Les esters majoritairement produits ont été identifiés et quantifiés au cours de la réaction. Leurs proportions relatives ont été déterminées et comparées à la composition de l'huile en acide gras, de manière à étudier les propriétés de spécificité de l'enzyme. La stabilité et la capacité antioxydante du milieu réactionnel brut ont été déterminées et comparées à celles de l'huile de saumon non transformée. Les résultats escomptés étaient l'obtention d'extraits prêts à l'emploi, combinant une haute valeur nutritionnelle, une grande stabilité et une activité antioxydante élevée.

Cette étude fait l'objet d'un article qui sera prochainement soumis dans Journal of Biotechnology.

3.2. Enzymatic synthesis of vanillyl fatty acid esters from salmon oil in solvent-free medium.

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Abstract

A solvent-free alcoholysis reaction of the oil recovered from salmon heads was performed with vanillyl alcohol (VA), using immobilized lipase B from *Candida antarctica* (Novozyme435®). Almost total conversion of VA was achieved after 24h of reaction, leading to the production of a wide variety of esters that mirror the initial composition of the oil. The chemical structure of majority esters was determined by mass spectrometry and the production of specific compounds including oleoyl, linoleoyl, linolenoyl and docosahexaenoyl esters was more particularly followed. The synthesis of some esters like oleoyl and linolenoyl esters was shown to be favored compared to that of other products like docosahexaenoyl and linoleoyl esters due to fatty acid distribution in the oil combined with enzyme selectivity properties and potential intra-molecular acyl transfer isomerization. Alcoholysis crude reaction medium exhibited high stability and antioxidant properties, pointing out the interest of vanillyl esters compared to a simple supplementation of the oil with vanillyl alcohol. In conclusion, fish oil alcoholysis with phenolic alcohol appeared to be an interesting way to produce stable and ready-to-use extracts with high nutritional value and promising antioxidant potential.

Keywords

PUFA, fish oil, vanillyl alcohol, alcoholysis, antioxidant activity, oxidative stability

1-Introduction

Heads from *Salmo Salar* resulting from the filleting operation have the potential to be used as raw material for the production of salmon oil. They contain a large amount of lipids (14–18%, w/w), especially PUFA including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Wu Ted H. *et al.*, 2011). Many studies reported the interest of these fatty acids for the prevention of a variety of human diseases and disorders (Nicholson et al., 2013). The health benefits of dietary PUFA include reduced susceptibility to malignant tumors, reduced risk of cardiovascular disease (Arab-Tehrany *et al.*, 2012 ; Swanson *et al.*, 2012a) and improved development of brain and nervous tissue in the infant (Kuratko et al., 2013). PUFA are also known for their anti-inflammatory properties including the reduction of rheumatoid arthritis symptoms and Crohn's disease (Igarashi *et al.*, 2013 ; Prostek *et al.*, 2014 ; Wu F. J. *et al.*, 2014). High intake of PUFA has been associated with lower risk of developing Alzheimer's disease and depressions (Cunnane *et al.*, 2013 ; Hardman, 2002).

Since a few years, many food manufacturers have shown real interest in using PUFA as functional ingredients to improve the nutritional profile of food products. However, one major obstacle is still the high oxidizability of these lipids, thus causing flavor deterioration, loss of nutrients and formation of potentially toxic compounds (Albert B. B. *et al.*, 2013b ; Tong *et al.*, 2000). This constitutes without doubt the major limitation in incorporating PUFA-rich oils in foods. To solve this problem, many studies reported the use of phenolic antioxidants and pointed out the great interest of these radical scavengers to stabilize bulk oils and emulsions (Huber Gwendolyn M. *et al.*, 2009b ; Kindleysides Sophie *et al.*, 2012a ; Sekhon-Loodu Satvir *et al.*, ; Wang Hua *et al.*, 2011a)

More recently, some authors proposed another strategy consisting in bringing phenolic compounds and PUFA-rich lipids together within a single structure. To this end, two main approaches were proposed. The first one refers to esterification reaction between PUFA and phenolic alcohols whereas the second one is based on transesterification reaction between oil and phenolic acids aiming to produce structured triacylglycerides (acidolysis reaction) (Aziz *et al.*, 2012 ; Mbatia B. *et al.*, 2011 ; Nicholson *et al.*, 2013 ; Shahidi F. andZhong, 2010 ; Sorour N. *et al.*, 2012b ; Zhong *et al.*, 2011); (Mellou *et al.*, 2006 ; Speranza andMacedo, 2012 ; Torres de Pinedo A. *et al.*, 2005a)

Expected outcomes are: an increased solubility of phenols within lipidic phases, a higher stability of polyunsaturated lipids, and even cumulative and synergistic activities. These reactions were carried out preferably using an enzymatic process due to high regioselectivity and mild reaction conditions that avoid substrate alteration. Most often, lipases were used as biocatalysts either in dry organic solvent or in molten media. In addition to catalyzing esterification and acidolysis reactions, these enzymes are able to catalyze alcoholysis reactions between triacylglycerides and alcohols. To our knowledge, no study has ever been reported about the synthesis of PUFA phenolic esters basing on an alcoholysis process.

The present study aimed to develop a solvent-free alcoholysis process for the production of lipophilic phenolic esters. Salmon oil was used as a source of fatty acids while vanillyl alcohol was chosen as phenolic alcohol. In addition to aromatic properties, vanillyl alcohol exhibits antioxidant, antimicrobial and anti-inflammatory activities that justify its use in food, cosmetics and pharmaceutical products (Shyamala et al., 2007). Several works reported the enzymatic synthesis of lipophilic derivatives of vanillyl alcohol basing on esterification process catalyzed by a lipase. That is the way to produce capsinoids acting as capsiate analogs (Hong *et al.*, 2012 ; Kobata *et al.*, 2002a ; Reddy *et al.*, 2011).

In the present work, salmon oil was characterized and then submitted to an alcoholysis reaction with vanillyl alcohol. Major esters were identified and quantified throughout the reaction. The stability and the antioxidant capacity of the crude reaction medium were investigated. Expected results were the production of ready-to-use extracts that combine stability, antioxidant activity and high nutritional interest.

2- Materials and methods

2.1. Materials

Salmon heads (*Salmo salar*) were supplied by a local company (Les Salaisons d'Orly, Orly, France). Lipase B from *C. antartica*, immobilized on a macroporous acrylic resin (Novozym 435, Novo Industry, Germany) was used as biocatalyst. Protease from *Bacillus Licheniformis* used for the extraction of oil from salmon heads and vanillyl alcohol (VA) (4-hydroxy-3-methoxybenzyl alcohol) purity 98% were from Sigma Chemical Co. (St. Louis, MO, USA). Organic solvents of high-performance liquid chromatography (HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). All solvents used were of analytical grade.

2.2. Enzymatic extraction of oil from salmon heads

Salmon heads routinely preserved at -20°C were removed from the freezer. Enzymatic hydrolysis was performed at 70°C in a stirred thermostated reactor (2 L) where raw material (15 kg) was suspended in distilled water. pH was adjusted to 7 with 4 M NaOH for 15 min under mixing. The enzymatic solution was then added and the reaction allowed proceeding for 2 h under nitrogen. The pH was kept constant by automatically adding 4 M NaOH during hydrolysis according to the pH-stat method. The medium was coarsely filtered to retain bones, whereas the liquid phase was subjected to subsequent centrifugation to separate oil and emulsified fractions from the underlying aqueous phase. Enzymes were inactivated in hydrolysate by heat treatment with live steam injection (95°C, 10 min).

2.3 Preparation and analysis of fatty acid methyl esters

Fatty acid methyl esters (FAME) were obtained by transmethylation of lipid aliquots (100 mg) according to (Ackman, 1998). Samples were dissolved in 1.5 mL hexane and 1.5 ml borontrifluoride in methanol (8%, w/vol), then heated at 100°C for 1h. After cooling, FAME were extracted in hexane under nitrogen. FAME were analyzed by gas chromatography on a Perichrom 2000 system (Saulx-les-Chartreux, France), equipped with a flame ionization detector (FID) and a fused-silica capillary column (25m * 0.25mm * 0.5 mm; BPX70 SGE Australia Pty Ltd.). The temperature was set as follows: 2 min initial period at 70°C, then increasing at 40°C/min to reach a second step at 180°C during 8 min, and flowing out at 3°C/min to the final period (220°C, 45 min). Injection and detector ports were maintained at 230°C and 260°C, respectively. Fatty acids were identified by comparison of their relative retention times with appropriate standard mixtures (PUFA 1 from marine source and PUFA 2 from animal source; Supelco, Bellefonte, PA, USA) and an internal standard (C23:0). The results were displayed as percentage of total identified fatty acids.

2.4 Lipid class analysis by thin-layer chromatography

The lipid classes were determined by Iatroscan TLC-FID MK V (Iatron Laboratories Inc., Tokyo, Japan). The ten silica gel chromarods-SIII held in a frame were first scanned twice immediately before sample application to remove impurities. Samples (10 mg/mL chloroform/methanol 2:1) were spotted onto the chromarods using a 1 µL glass minicap pipette (Hirschmann Laborgeräte, Germany) and then

submitted to migration in the following solvent system at 20°C: hexane/diethyl ether/formic acid (80:20:2, vol:vol:vol) to separate neutral lipids, free fatty acids, and ketone traces. The air and hydrogen flow rates were set at 200 and 160 mL/min, respectively. The scan speed was set at 30 s/scan. The results were expressed as the mean of three separate samples. The following standards were used to identify the sample components: 1-monostearoyl-*rac*-glycerol, 1, 2-dipalmitoyl-*sn*-glycerol, tripalmitin and cholesterol. All standards were purchased from Sigma (Sigma-Aldrich Chemie, GmbH, Germany).

2.6. Enzymatic alcoholysis of salmon oil

Before the reaction was started, VA and enzymatic preparation were pre-equilibrated in dissector at 4°C to lower water activity to 0.1. Then, VA (50 g/L) was dissolved in salmon oil (SO) overnight in the flask of a rotary evaporator, at 40°C, under reduced pressure (500 mbar) and stirring rate of 200 rpm. The flask was purged with nitrogen and kept in the dark aiming to limit oxidative degradation. The reaction was started by adding 20 g/L of immobilized lipase (Novozym 435). Samples were withdrawn from the reaction mixture at relevant time intervals for the analytical monitoring of the reaction. The reaction was stopped (after 28 h of incubation) by removing the enzyme. Calibration curve for vanillyl alcohol and PUFA-vanillyl esters (PUFA-VE) were obtained using purified samples in acetonitrile. If Y = Peak area (AU) and X = concentration (g/L) of detected ester, standard equations are $Y=1.27489*10^7$ X for VA, $Y=3.27756*10^6$ X for linolenic vanillyl ester, $Y=2.42107*10^6$ X for DHA-VE, $Y=4.65921*10^6$ X for linoleic vanillyl ester and $Y=4.18232*10^6$ X for oleic vanillyl ester.

2.7. Characterization of reaction mixtures by HPLC and LC-MS

The extent of the reaction was monitored by HPLC analyses of the reaction medium, using a C18 Alltima reverse phase column (150*2.1 mm, 5 µm porosity – Grace/Alltech, Darmstadt, Germany) equipped with a C18 Alltima pre-column (7.5*2.1 mm, 5 µm porosity – Grace/Alltech). The column was kept at 35°C. UV (280 nm) and evaporating light scattering detector (ELSD) were used with evaporator and nebulizer temperatures of 35°C and 45°C, respectively, and a gas flow rate of 1.5 standard liters per minute. Water, 0.1% acetic acid (A) / acetonitrile, 0.1% acetic acid (B) elution system and a flow rate of 0.2 µL/min were applied. The components were separated

using the following elution gradient: 78-100% B over 50 min, 100% B for 5 min, followed by 10 min 78-100% B.

Liquid chromatography coupled to mass spectrometry (LC-MS) analyses was performed using a system equipped with a Linear Trap Quadripole (LTQ) as mass analyzer (Thermo Fisher Scientific, San Jose, CA, USA). Chromatographic separation was performed at 25°C on the same column and with the same elution system as those previously described. PUFA-VE were eluted using a linear gradient from 78% to 100% of B phase for 50 min, at a flow rate of 0.2 mL/min. Photodiode array (PDA) and mass spectrometry (MS) detections were performed during the time of the run. Mass spectrometry operating parameters were as follows: Electrospray positive ionization mode (ESI^+) was used; spray voltage was set at 4.5 kV; source gases were set (in arbitrary units min^{-1}) for sheath gas, auxiliary gas and sweep gas at 25, 10 and 10, respectively; capillary temperature was set at 250 °C; capillary voltage was set at 1 V; tube lens, split lens and front lens voltages were fixed to 55 V, -38 V and -4.00 V, respectively. The ion optics parameters were optimized by automatic tuning using a standard solution of fatty acid esters (1 gL^{-1}) infused in the mobile phase (B) at a flow rate of 2 µL/min. Full scan MS spectra were acquired from 100 to 2000 m/z and manual MS^2 mode was also carried out aiming to obtain additional structural information.

2.7. Evaluation of antioxidant activity

2.7.1. DPPH• Radical Scavenging Activity method

The capacity of the compounds to scavenge the DPPH• radical was determined as described by (Brand-Williams et al., 1995) with some modifications. Briefly, 100 µl of a methanolic solution containing the esterified oil (0.5, 1.25, 2 and 2.5 mg/ml) was added to 3.9 mL of DPPH• radical methanolic solution of (0.1 mM). Then, the total volume was adjusted to 4mL with methanol. After 30 min of incubation at 30°C in the dark, the absorbance of the mixture was measured at 517 nm against methanol. The free radical scavenging activity (% FRSA) was evaluated by comparison with a control (3.9 mL of DPPH• radical solution and 0.1 mL of methanol). Each measure was done in triplicate, and an average value was calculated. The free radical scavenging activity (FRSA) was expressed as the percentage of inhibition of the DPPH• radical referring to the control (equation 1).

$$FRSA\ \% = [(Ac - As)/Ac] \times 100 \qquad (1)$$

Where Ac and As are absorbance's of control and sample, respectively.

2.7.2. $ABTS^{+\bullet}$ method

The method was described by (Re Roberta *et al.*, 1999b). $ABTS^{+\bullet}$ species were produced by reacting ABTS aqueous solution (7mM) with potassium per-sulphate (2.45 mM final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h. The radical was stable under this form for more than two days when stored in the dark at room temperature. $ABTS^{+\bullet}$ solution was diluted with ethanol aiming to obtain an absorbance of 0.7 at 734 nm and 30°C. 10µL of the solution containing (0.6, 1.25, 1.9 and 2.5 mg/ml) of esterified salmon oil were allowed to react with 1mL of diluted $ABTS^{\bullet+}$ solution for 15 min in the dark. Then the absorbance was measured at 734 nm. Ethanol was used as control. All determinations were carried out in triplicate. Antioxidant activity was expressed as the percentage of inhibition of the ABTS radical referring to the control (equation 2).

$$FRSA\ \% = [(Ac - As)/Ac] \times 100 \qquad (2)$$

Where Ac and As are absorbance values for the control and the sample, respectively.

2.7.3. Inhibition against DNA scission

Antioxidant activity of esterified and non-esterified salmon oil against DNA scission was determined as described by (Hiramoto K. *et al.*, 1996a). Briefly, H_2O_2 (0.2 mM) and $FeSO_4$ (0.1mM) were used to generate hydroxyl radicals after incubation 2h at 37°C that induced DNA strand breaking (100 µg/mL). DNA scission was monitored by agarose gel electrophoresis. A blank (DNA only) and a positive control (DNA + Fenton's reagent) were prepared. DNA bands were visualized under UV light; the intensity of the band corresponding to the native supercoiled plasmid was measured by densitometry.

2.8. Oxidative stability of esterified oil

5 g of each sample (salmon oil, salmon oil + VA (50 g/L) and extract recovered from alcoholysis reaction) were weighed in screw-capped glass tubes (10 ml) and stored at 100°C or 20°C in darkness. Oxidative stability was monitored throughout storage period by measurement of conjugated diene (CD) and thiobarbituric acid (TBARS) according to the AOCS standard method (AOCS., 1998).

2.9. Statistical analysis

All analytical values represent the means ± SD of triplicate analyses. Main effects means indicating significant differences were tested using Duncan's multiple range tests using Statistical Package for the Social Sciences (SPSS) (2003).

3. Results and discussion

3.1. Fatty acid composition

Salmon oil is known for its interesting nutritional profile due to a high content of omega 3 essential fatty acids, a mineral content that can be up to 25 times more than that of any other food of animal origin and the presence of fully soluble vitamins that help to keep bones strong (Urwin et al., 2012). In the present study, fatty acid composition of salmon oil was determined by GC, as shown in Table 1. Salmon oil exhibited a high content of unsaturated fatty acids (76%) and a low content of saturated fatty acids like myristic, palmitic and stearic acids (7.3, 14.6 and 3.2 %, respectively). 50% unsaturated fatty acids are polyunsaturated lipids, like linolenic acid (2.3%), DHA (11.5%), and linoleic acid (5.1%). These results are consistent with those reported by other authors about the fatty acid composition of salmon oil, except for oleic acid content that was 15.8% in the present study *versus* more than 30% in some other works (Belhaj *et al.*, 2010 ; Kahveci andXu, 2011 ; Mbatia B. *et al.*, 2011). Lipids were separated and analyzed according to their class by thin-layer chromatography (Iatroscan), showing that salmon oil consisted in TAG exclusively (100%).

3.2. Enzymatic alcoholysis of salmon oil with vanillyl alcohol

Enzymatic alcoholysis of salmon oil TAG was performed with vanillyl alcohol (VA) to produce fatty acid vanillyl esters (FA-VE). Preference was given to an enzymatic solvent-free process that avoids subsequent treatments of the extracts before use. Temperature and pressure conditions were adjusted to avoiding vanillyl alcohol evaporation. The progress of the reaction was followed basing on vanillyl alcohol consumption and the production of vanillyl esters. Other indicators like water activity, oxidative degradation, lipid destructuration and competitive hydrolysis were also monitored throughout the reaction.

As shown on Fig. 1, vanillyl alcohol conversion yield was 90% after 8 h of reaction, and then stabilized around 94% after 24 h. In the same time, TAG disappeared while the concentration of free fatty acids increased in the reaction medium, indicating a competitive hydrolysis of the oil (Fig. 2 A). Particular attention was paid to the initial drying of all components constituting the reaction medium in order to limit hydrolytic phenomena. However, the water activity of the reaction medium was observed to slightly increase during the synthesis, probably due to little moisture regain during sample withdrawing (Fig. 2 A). As a direct consequence, the partial hydrolysis of TAG occurred, leading to 12% of lipids under FFA form at the end of the reaction. An indirect consequence of TAG hydrolysis was the partial oxidation of the reaction medium, as indicated by CD and TBARS analyses (Fig.2 B). Indeed, the propensity of lipids to oxidize is closely linked to their structuration, TAG being much more stable towards oxidation than the corresponding free fatty acids.

Due to the complex composition of salmon oil, numerous esters were produced as shown by the HPLC elution profile of the reaction medium (Fig.3). Previous works indicated that acylation took place on VA primary hydroxyl, due to high reactivity and accessibility combined with CALB selectivity properties. LC–MS analyses were carried out to determine the structure of the esters produced. More specifically, the esters were readily identified using post-acquisition data mining for specific neutral losses of the appropriate product ion. Confirmation of the assignments was then achieved by examination of the full scan and associated MS data for each indicated peak. Main FA-VE were identified as vanillyl stearidonate (stearidonate-VE), vanillyl eicosapentenoate (EPA-VE), vanillyl linolenate (linolenate-VE), vanillyl docosahexaenoate (DHA-VE), vanillyl docosapentaenoate (DPA-VE), vanillyl linoleate (linoleate-VE), and vanillyl oleate (oleate-VE), corresponding to the peaks 1, 2, 3, 4, 5, 6 and 7, respectively. Some minority products remained undefined. MS data for some esters produced during the reaction are shown on Fig. 4. The m/z values of the characterized FA-VE are listed in Table 2. Similar compounds were obtained by (Mbatia B. *et al.*, 2011) when performing the enzymatic esterification of a concentrate of PUFAs from salmon oil with phenolic alcohols. These authors reported a multi-step process where salmon oil TAGs were firstly hydrolyzed. Secondly, PUFAs were concentrated, and then esterified with vanillyl alcohol or rutin leading to mixtures of PUFA phenolic esters. Our approach mainly differs from this work by the fact that an alcoholysis reaction was

applied to the oil rather than successive hydrolysis and esterification reactions. A direct consequence is the production of a complex mixture of esters that mirrors the composition of salmon oil, including not only PUFA vanillyl esters but also MUFA and saturated FA esters.

The production of the esters linoleate-VE, linolenate-VE, oleate-VE and DHA-VE was followed specifically as the corresponding fatty acids are particularly sought for their nutritional properties (Fig.5 A). Whatever the ester, the concentration rapidly increased during the first 24 h of reaction, and then stabilized. Maximal concentrations of 73, 22, 16 and 16 mM were obtained respectively for oleate-VE, DHA-VE, linoleate-VE and linolenate-VE, starting from 0.32 M VA. Initial reaction rates were 7.585, 1.996, 0.989 and 1.272 mM/min for oleate-VE, DHA-VE, linoleate-VE and linolenate-VE, respectively. The relative concentrations of oleate-VE, DHA-VE, linoleate-VE and linolenate-VE were compared to that of the corresponding FA in salmon oil in order to reveal a potential selectivity of alcoholysis reaction. The relative proportions of oleic, DHA, linoleic and linolenic acids in salmon oil were 45%, 33%, 15% and 7%, respectively. At the end of the reaction, the relative proportions of the corresponding esters were 64%, 14%, 10% and 12% (Fig. 5B). These data showed in particular that the proportion of oleoyl ester was higher than that of oleic acid in the oil, indicating a preferential esterification of this fatty acid compared to other fatty acids. An opposite trend was observed in the case of DHA. Such result can be explained by the combination of two main factors: the preferential location of PUFAs like DHA on the *sn*-2 position and the selectivity of the lipase B of *C. Antarctica* that favors the alcoholysis reaction on the *sn*-1 and *sn*-3 positions (Irimescu *et al.*, 2002 ; Mbatia Betty *et al.*, 2010a). In addition, potential intramolecular acyl migration isomerizations were also likely to occur.

3.3. Oxidative stability of alcoholysis crude reaction medium

The incorporation of vanillyl alcohol within salmon oil was expected to decrease its sensitivity to auto-oxidation. The stability of the reaction medium recovered from salmon oil alcoholysis was investigated through accelerated oxidation tests and compared to that of the native oil. The stability of VA-enriched salmon oil was also determined aiming to check the beneficial effect of alcoholysis reaction in comparison with simple supplementation with vanillyl alcohol. Samples corresponding to salmon

oil, VA-enriched salmon oil and alcoholysis crude reaction medium were stored at 100°C and 20°C. CD as primary oxidation products and TBARS as secondary oxidation products were regularly monitored. Results are shown on Fig. 6. Whatever the sample and the storage conditions, CD and TBARS contents increased with time, indicating an oxidative degradation that was more pronounced at 100°C comparing with 20°C. These results are in accordance with general acceptance that an increase of temperature speeds up oxidative degradation (Iqbal andBhanger, 2007 ; Wang Hua *et al.*, 2011a). Whatever the storage conditions and the storage time, the incorporation of vanillyl alcohol in salmon oil by either simple mixing or alcoholysis process significantly improved the oxidative stability of PUFAs. The mixture recovered from salmon oil alcoholysis appeared more stable than VA-enriched salmon oil, regardless of the storage temperature. During storage at 20°C, alcoholysis reaction medium remained almost stable during the first two weeks whereas salmon oil got oxidized during the first 2 days of storage. These results were consistent with those reported by some authors about the transesterification of omega 3-rich oils with phenolic acids aiming to improve their oxidative stability and even provide them antioxidant properties (Aziz andKermasha, 2014a). Binding of phenolic compounds to polyunsaturated lipids appeared as an efficient method to provide stable ingredients, regardless of whether the phenolic entity was incorporated in TAG structure (case of phenolic acids) or under the form of FA esters (case of phenolic alcohols).

3.4. Antioxidant activity of alcoholysis crude reaction medium

3.4.1 Scavenging activity towards $ABTS^{+\cdot}$ and $DPPH^{\cdot}$ radicals

The incorporation of vanillyl alcohol within salmon oil was expected not only to decrease its propensity towards auto-oxidation but also to give additional properties like antioxidant activity. In the present study, the antioxidant activity of alcoholysis crude reaction medium was determined by the ABTS and DPPH methods, and then compared to that of salmon oil and pure vanillyl alcohol (Table 3). Unsurprisingly, the highest antioxidant activities were observed with pure vanillyl alcohol. A significant difference ($p < 0.05$) was observed between the antioxidant activities of the oil submitted to alcoholysis process and fresh salmon oil. More specifically, the incorporation of vanillyl alcohol into the oil was shown to provide high antioxidant potential in addition to high stability towards oxidation. The crude reaction medium

recovered from oil alcoholysis kept around 60% of vanillyl alcohol activity. All these findings are in perfect agreement with a previous study that compared the antioxidant activity of vanillyl alcohol to that of PUFA-vanillyl esters (Mbatia B. *et al.*, 2011). In view of this, extracts recovered from salmon alcoholysis appeared as promising radical scavenging systems that could be used to protect various matrices from oxidative damage.

3.4.2 Inhibition of DNA scission

Native DNA may take three forms: open circular, single supercoiled band and linear. Mutagens may cause oxidative induced breaks in DNA strands favoring open circular forms (Chen *et al.*, 2013 ; Negi *et al.*, 2014). Fig. 7 shows the partition of DNA under either the circular form or the linear form as an indicator of its oxidative degradation. The capacity of alcoholysis crude reaction medium to protect plasmid DNA against oxidative damage induced by hydroxyl radicals was evaluated and compared to that of VA (Fig. 7). DNA scission occurred in absence of any antioxidant compound and, to a lesser extent, in presence of salmon oil. Addition of VA or vanillyl esters led to supercoiled DNA retention rates of 80 and 95%, respectively, showing the crucial protective role of the phenolic entity. Another important observation was the greater effect of vanillyl esters than VA itself. A similar trend was previously observed in the case of some pure esters of phenols, suggesting that the hydrophobic/hydrophilic balance of the molecules may affect their interactions with radical species, and then their scavenging activity (Hiramoto K. *et al.*, 1996a).

Conclusion

Lipase-catalyzed alcoholysis of salmon oil with vanillyl alcohol was performed through an efficient solvent-free bioprocess, leading to a mixture of SFA, MUFA and PUFA vanillyl esters (FA-VE). 50 g/L vanillyl alcohol was almost totally converted after 24 h of reaction. The crude reaction medium recovered from alcoholysis exhibited a high stability during storage compared to salmon oil. Moreover, some interesting antioxidant activities were pointed out. In view of this, solvent-free alcoholysis appeared as a promising way to produce ready-to-use extracts that combine high stability and high antioxidant properties. This approach is particularly relevant in the case of salmon oil because of high content of MUFA and PUFA that makes it very attractive from a nutritional point of view but narrows its scope for industrial

applications due to high instability. Extracts recovered from alcoholysis with vanillyl alcohol appeared as promising systems that could help harness the positives and eliminate the negatives relative to the use of salmon oil in food and nutraceutical applications.

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Tables and Figures

Table 1. Major fatty acid composition of salmon oil

Fatty acid	Content (area%)
C14:0	7.3 ± 0.65
C16:0	14.6± 0.43
C16:1	8.5 ± 0.35
C18:0	3.2 ± 0.02
C18:1 n-9	15.8 ± 0.59
C18:2 n-6	5.1 ± 0.34
C18:3 n-3	2.3 ± 0.94
C18:4 n-3	1.4 ± 0.68
C20:1 n-9	6.9 ± 0.16
C20:4 n-6	9.6 ± 0.31
C20:4 n-3	1.5 ± 0.78
C20:5 n-3	3.8 ± 0.06
C22:5 n-3	3.3 ± 0.43
C22:6 n-3	11.5 ± 0.31
∑ SFA	25.1
∑ MUFA	31.1
∑ PUFA	38.4

All values are mean of three replicates ± standard deviation.

SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

Table 2. Molecular weights and retention times of synthesized FA-VE as observed in the LC-MS analysis.

FA-VE	Retention time (rt) min.	Molecular weight (MW)
Stearidonate -VE	11.1	413
EPA – VE	13.0	439
Linolenate – VE	14.6	415
DHA – VE	15.3	465
DPA – VE	18.2	467
Linoleate –VE	20.0	417
Oleate –VE	28.8	420

MW: M + H.

Table 3. Antioxidant activity of vanillyl alcohol and reaction medium recovered from salmon oil alcoholysis with vanillyl alcohol determined by DPPH and ABTS methods. Values relative to salmon oil were determined as a comparison.

Samples	DPPH method		ABTS method	
	IC50	ARP	IC50	ARP
Salmon oil	6.50 ± 0.35[a]	0.17 ± 0.09[a]	7.53 ± 0.41[a]	0.13 ± 0.10[a]
Vanillyl alcohol	0.36 ± 0.15[b]	2.78 ± 0.13[b]	0.30 ± 0.26[b]	3.33 ± 0.23[b]
Alcoholysis crude reaction medium	0.63 ± 0.23[c]	1.57 ± 0.16[c]	0.44 ± 0.31[c]	2.25 ± 0.26[c]

Different superscript letters were attributed to significantly different values (mean ± SD of three replicates). ARP: Antiradical power

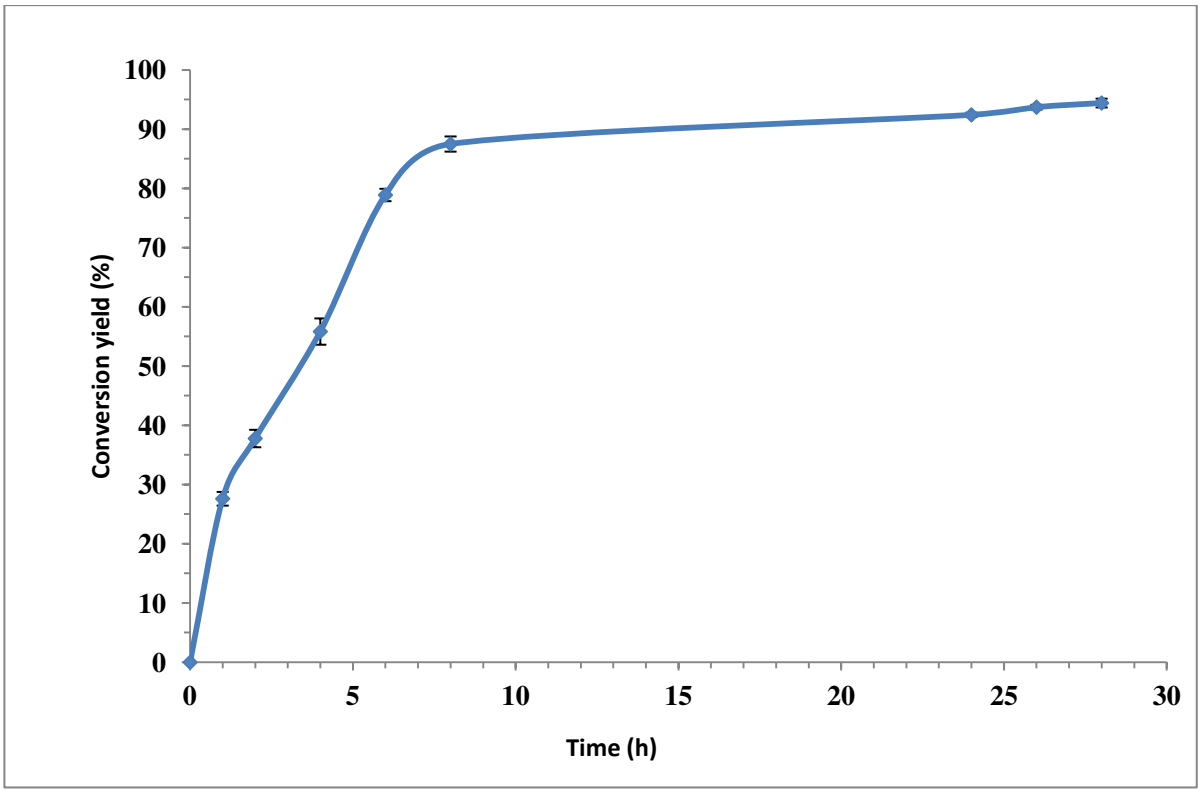

Fig.1. Yields of alcoholysis reaction between salmon oil and vanillyl alcohol vs time at 40°C

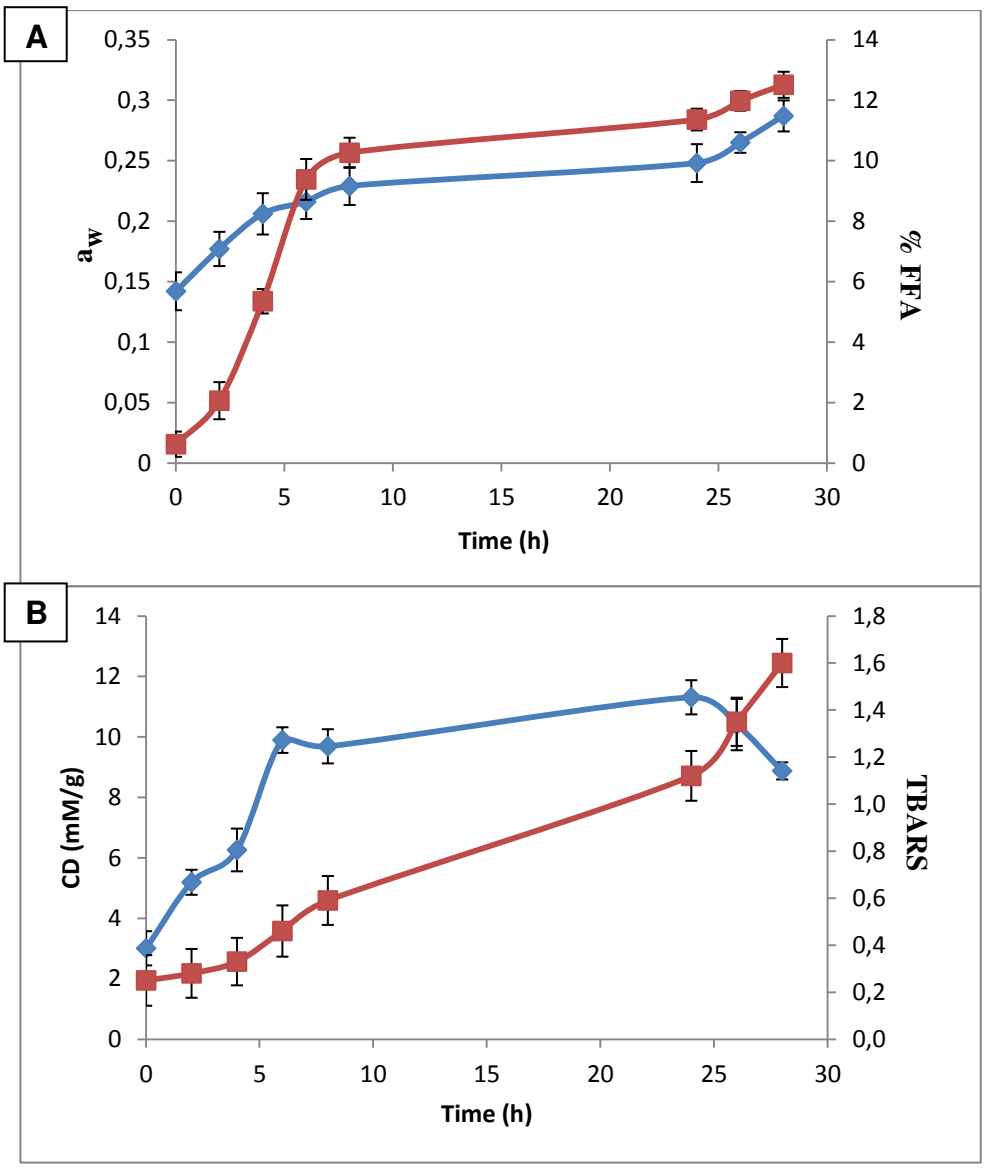

Fig. 2. **A-** Water activity (♦) and FFA (■) content in reaction medium during salmon oil alcoholysis with vanillyl alcohol. **B-** Monitoring of oxidation indicators throughout esterification process: CD (♦) and TBARS (■)

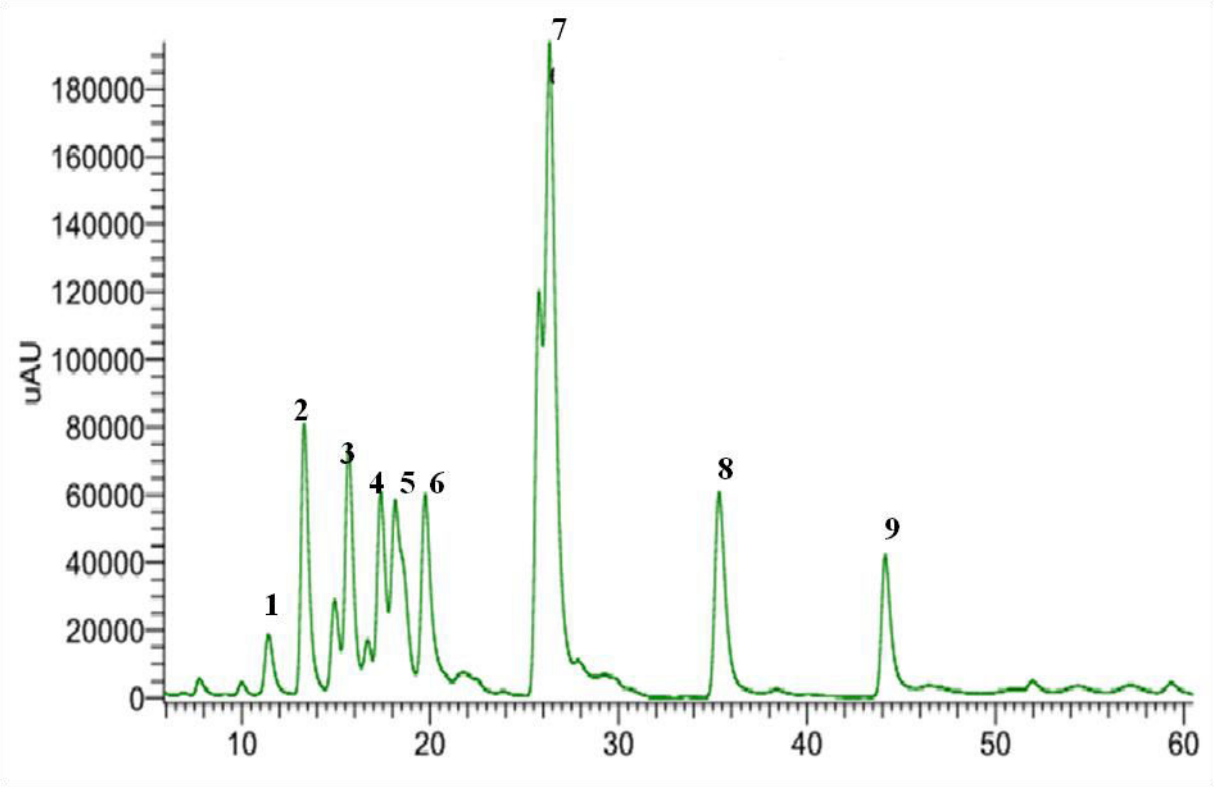

Fig. 3. HPLC profile of reaction medium after salmon oil alcoholysis process with vanillyl alcohol. Detection was performed at 280 nm. Esters were identified as 1: stearidonate-VE, 2: EPA–VE, 3: linolenate–VE, 4: DHA-VE, 5: DPA–VE, 6: linoleate–VE, 7: oleate–VE, 8: ND and 9: ND.

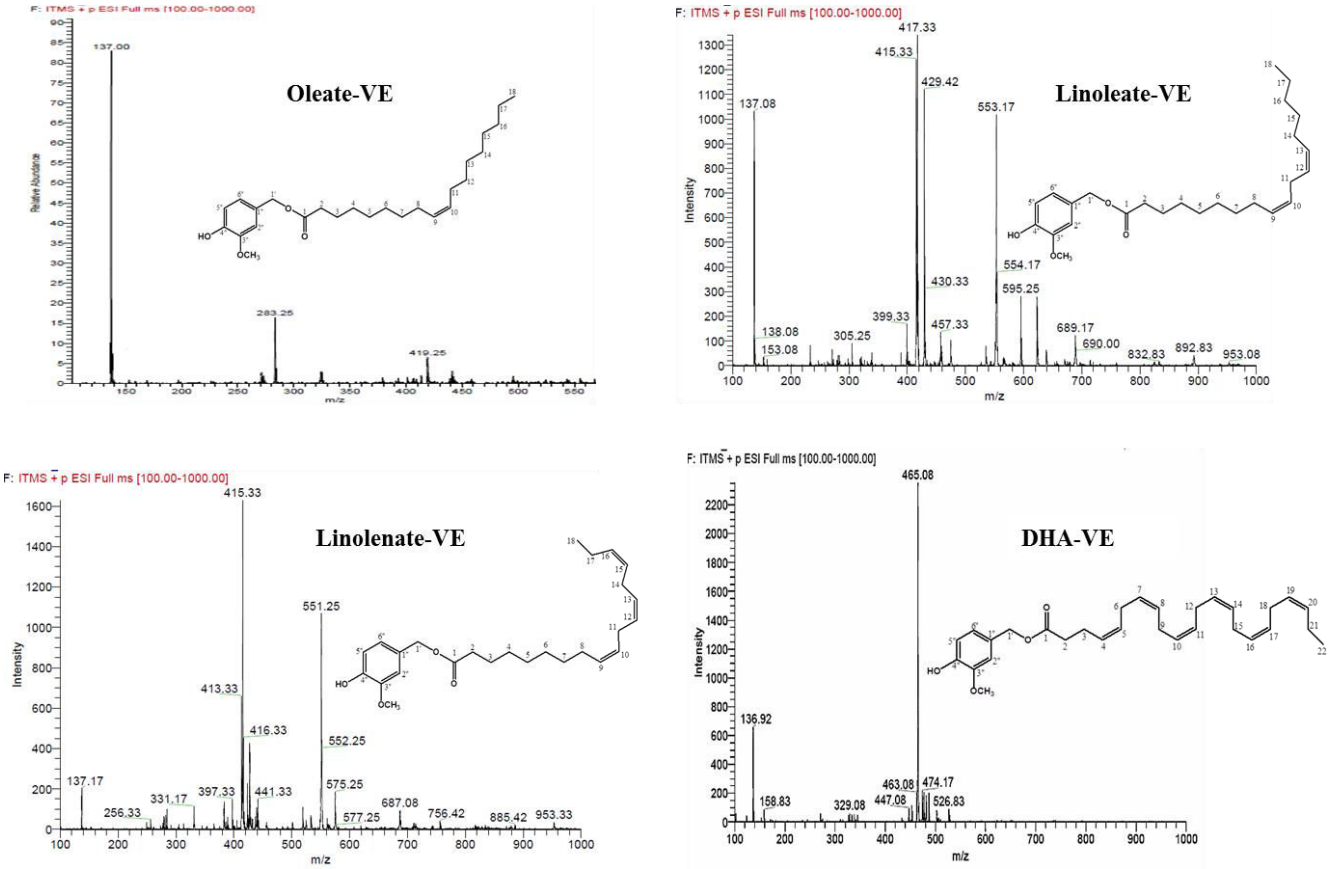

Fig. 4. LC-MS spectra of some FA-VE monitored at 280nm. Column oven: 35°C, Tray oven: 15°C, Mobile phase: A=(water + 0,1% acetic acid; B=acetonitrile 100%), Flow: 0,2 mL/min.

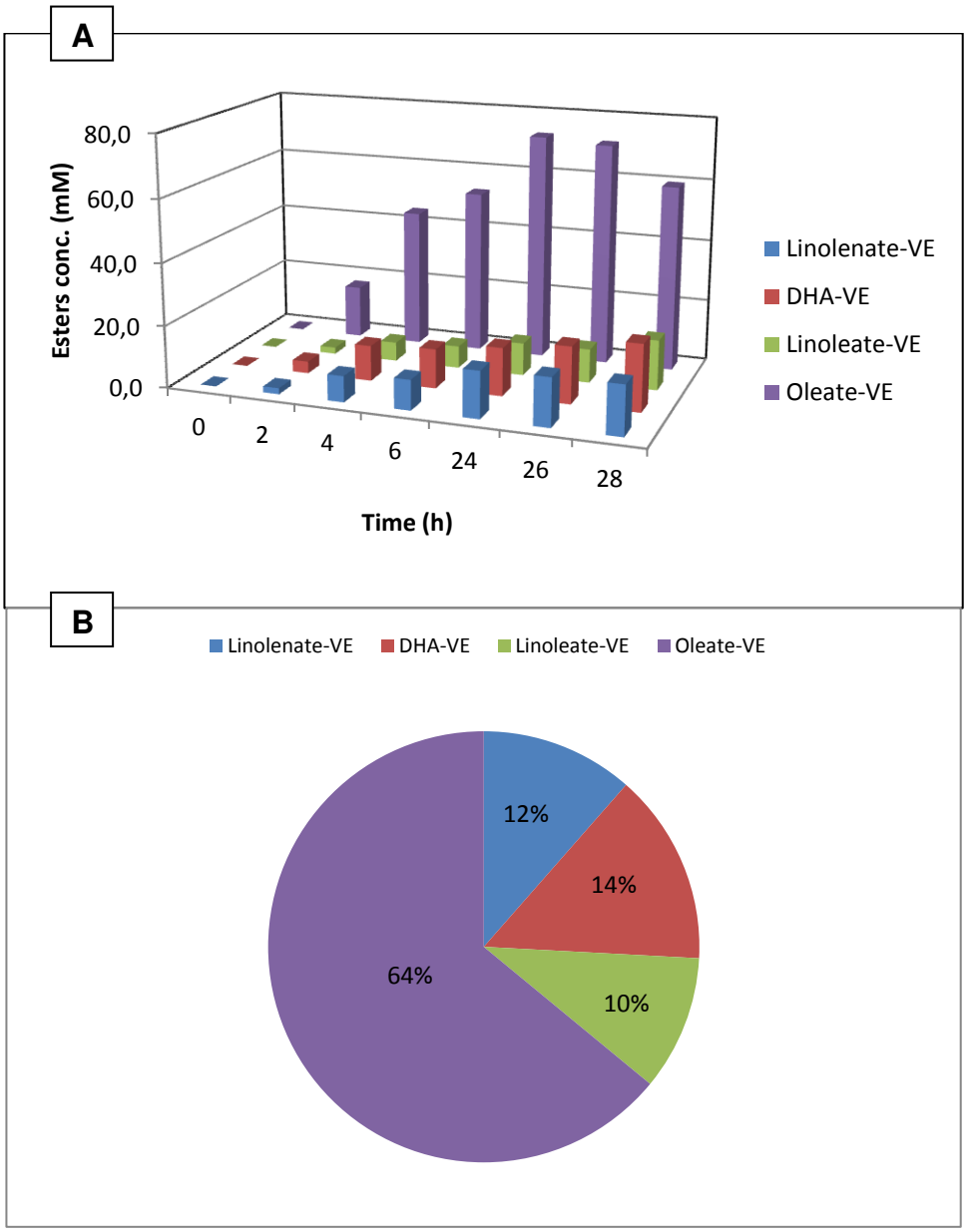

Fig. 5. A- Salmon oil alcoholysis process with vanillyl alcohol referring to the production of vanillyl esters. **B-** Relative proportions of esters (oleate-VE, DHA-VE, linoleate-VE and linolenate-VE) after esterification.

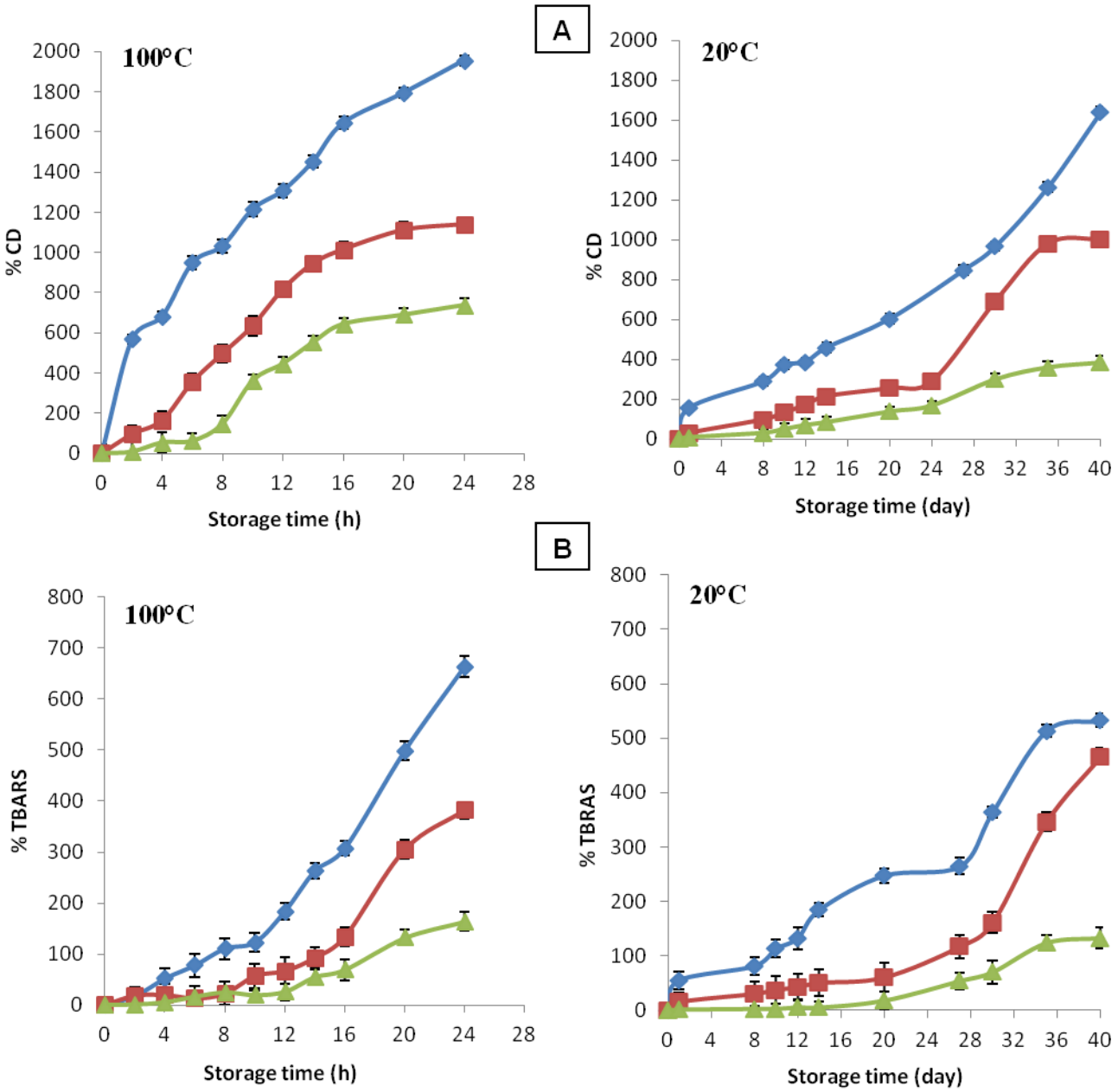

Fig. 6. Oxidative stability of salmon oil (♦), VA-enriched salmon oil (■) and reaction medium recovered from salmon oil alcoholysis (▲) at 20°C and 100°C, determined by conjugated dienes (A) and thiobarbituric acid (B) methods.

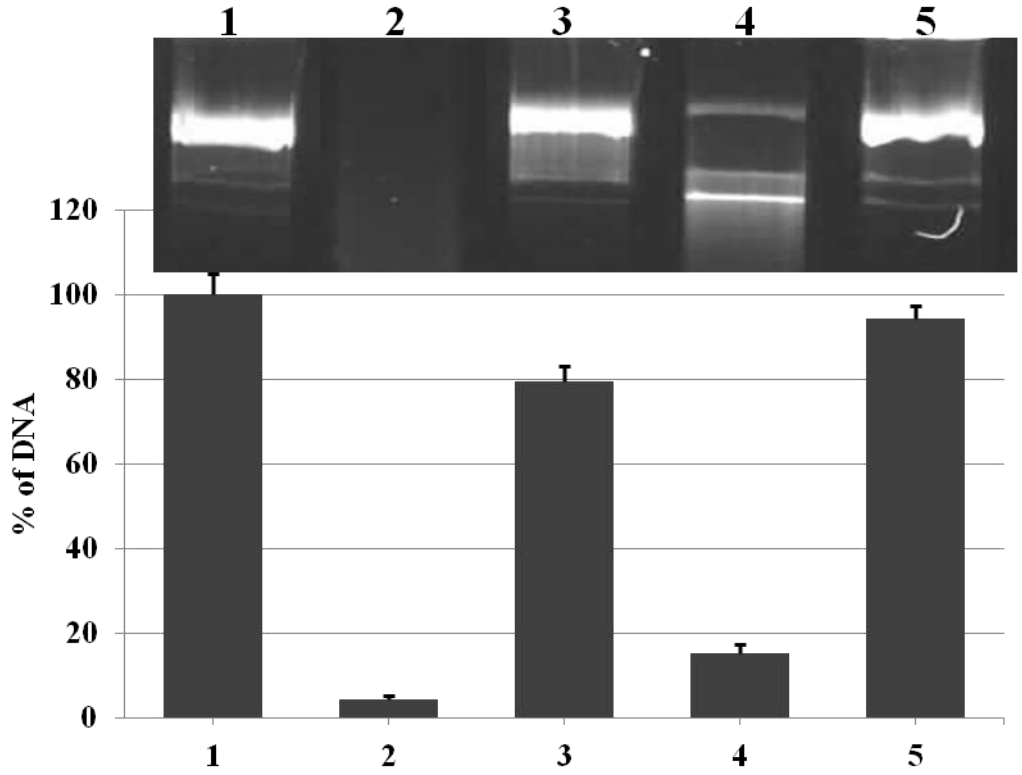

Fig. 7. Protective effect of vanillyl alcohol and crude reaction medium recovered from salmon oil alcoholysis towards DNA nicking caused by hydroxyl radical. Line 1: control (Distilled water + DNA), Line 2: DNA + Fenton's reagent, Line 3: DNA + Fenton's reagent+ VA, Line 4: DNA + Fenton's reagent + salmon oil and Line 5: DNA+ Fenton's reagent + alcoholysis crude reaction medium.

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3.3. Contribution de l'article

Un procédé d'alcoolyse sans solvant, catalysé par la lipase B de *C. antarctica*, a été appliqué à l'huile extraite de têtes de saumon pour produire des extraits riches en esters d'alcool vanillique et d'acides gras (FA-VE). 50 g/L d'alcool vanillique ont été incorporés dans l'huile après 24 h de réaction, dont 47 g/L sous forme d'ester d'acide gras. La structure chimique des principaux esters a été déterminée par spectrométrie de masse. La production de composés spécifiques, tels que les esters des acides oléique, linoléique, linolénique et docosahexaénoïque a été plus particulièrement suivie. Les résultats obtenus montrent que la synthèse des esters des acides oléique et linoléique est favorisée par rapport à celle d'autres produits tels que les esters des acides docosahexaénoïque et linoléique. Ceci est probablement dû à plusieurs facteurs comme la composition initiale de l'huile en acides gras, la localisation de ces acides gras au niveau des positions sn1, sn2 et sn3 associée aux propriétés de sélectivité de l'enzyme, ou encore des transferts d'acyle intramoléculaire. Le milieu réactionnel brut issu de l'alcoolyse de l'huile présente une stabilité élevée par rapport à l'huile de saumon native, quelles que soient les conditions de stockage. En outre, des activités antioxydantes intéressantes ont été mises en évidence. L'ensemble de cette étude montre que l'alcoolyse de l'huile par un composé phénolique tel que l'alcool vanillique constitue une voie prometteuse pour l'obtention d'extraits riches en AGPI prêts à l'emploi, combinant une grande stabilité et des propriétés antioxydantes élevées.

Partie.4: Applications of phenolic lipids synthesized to food systems

4.1. Introduction

La dernière partie de ce travail avait pour objectif de préciser le potentiel d'application de l'ester de DHA et d'alcool vanillique (DHA-VE) en l'incorporant dans deux matrices modèles : une émusion eau dans huile de type margarine et une émulsion huile dans eau, la phase huile étant constituée d'huile de saumon. Ces deux systèmes ont été choisis pour les raisons suivantes : la margarine est un modèle commun d'émulsion eau dans huile. C'est aussi un aliment consommé quotidiennement pouvant constituer un bon vecteur de lipides à fort intérêt nutritionnel. L'émulsion huile de saumon dans eau est un milieu intéressant sur le plan nutritionnel en raison de sa teneur élevée en lipides polyinsaturés. De par sa composition, c'est aussi un milieu hautement sensible à l'oxydation. Ce système a été choisi pour évaluer l'efficacité de l'ester DHA-VE en tant qu'agent antioxydant, et notamment sa capacité à interagir avec la phase lipidique. L'approche suivie a consisté à établir les cinétiques d'oxydation des deux systèmes émulsionnés, en présence et en absence d'ester DHA-VE, et à les comparer aux cinétiques obtenues après ajout d'alcool vanillique. Des conditions accélérées d'oxydation ont été appliquées, impliquant différentes températures de stockage. *In fine*, l'objectif était de mettre en évidence l'intérêt d'une association covalente entre l'alcool vanillique et le DHA, comparativement à un simple mélange des deux entités.

Cette étude fera l'objet d'un article soumis dans Journal of Agricultural and Food Chemistry.

4.2. Assessment of antioxidant capacity of DHA phenol ester in food emulsions

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Abstract

Fatty acid esters of phenols appear as interesting derivatives that could enhance the solubility of phenols in lipidic phases, then improving their antioxidant capacity. This work aimed to determine the capacity of DHA vanillyl ester (DHA-VE), to stabilize margarine and salmon oil in water emulsion (O/W) under different storage conditions. Primary oxidation products were evaluated through conjugated dienes (CD) and peroxide value (PV) measurements. Anisidine value (An.v) and thiobarbituric acid assays were used to follow the formation of the secondary oxidation products. Results showed that the addition of DHA-VE or vanillyl alcohol allowed increasing the oxidative stability of the emulsions due to the antioxidant properties of the phenolic entity. DHA-VE was shown to be much more efficient than vanillyl alcohol, especially in the case of margarine. This effect was attributed to a better localization of the antioxidant agent with regard to the lipidic phase. Overall, DHA-VE appears as a promising ingredient that combines the nutritional benefit of DHA while improving the oxidative stability of the media in which it is incorporated.

Keywords

DHA, vanillyl alcohol, lipid oxidation, oxidative stability, margarine, emulsions and fish oil

1. Introduction

Fats and oils play an important role in human nutrition as an important dietary source of energy and supply the body with essential fatty acids. Even though the Western diet is relatively rich in fat, the majority of the population does not consume adequate levels of the important omega-3 long chain polyunsaturated fatty acids (PUFAs), naturally present in marine foods among others. The health benefits of dietary PUFAs include reduced risk of cardiovascular disease (Lorente-Cebrian *et al.*, 2013 ; Nicholson *et al.*, 2013) and improved development of brain and nervous tissue in the infant (Kuratko *et al.*, 2013). They have also been associated with decreased risk of developing Alzheimer's disease and depressions (Cunnane *et al.*, 2013). These benefits have been mainly attributed to eicosapentaenoic acid (EPA) and docosahexaenoic acids (DHA) (Kuratko *et al.*, 2013).

In this view of, the uses of PUFA as functional ingredients in food products have a great interest to improve their nutritional profile. By fortification of common foods the intake of certain basic nutrients such as DHA could be increased and people could then live healthier without changing their dietary habits (Lorente-Cebrian *et al.*, 2013). This is the basic for the growing interest of functional foods (Bortolozo *et al.*, 2013 ; Egert *et al.*, 2012). However, the practical use of omega-3 PUFA is limited due to their high susceptibility to autoxidation which is the major cause of food quality losses. Oxidation could take place during preparation, processing or storage and change appearance in taste, odour and shelf life of food products (Albert B. B. *et al.*, 2013a).

In food products, lipids are often dispersed in an aqueous phase, stabilized by emulsifier that affects their oxidative stability. Oil in water (O/W) emulsion is generally used as a model of food matrices to study the effect of some factors like composition and processing on the lipid oxidation (Berton *et al.*, 2012). Emulsion is a heterogeneous system consisting of one immiscible liquid dispersed in another in the form of small spherical droplets. The two immiscible liquids are typically an oil phase and an aqueous phase that are thermodynamically systems unstable and not formed spontaneously (McClements, 2012). The oxidative stability of these fatty foods depends on several factors such as type of ingredients, fatty acid composition, polarity, concentration of antioxidants, pro-oxidants, increased surface area and interfacial properties.

The use of natural antioxidants is a new trend in both research and industrial applications to reduce lipid oxidation. Several compounds, including herbs extracts (Aladedunye, 2014 ; Gallego *et al.*, 2013) and tocopherol (Azizkhani *et al.*, 2011 ; Azizkhani andZandi, 2010a;2010b) were investigated and were found to be effective than synthetic antioxidant in delaying oxidation. But the use of natural antioxidant is limited due to their poor solubility in hydrophobic media, migration phenomena and phase separation in margarine. Also higher concentrations of these compounds have a pro-oxidant effects that accelerate oxidative damage (Zeb andMurkovic, 2013).

Recently, combination of phenolic compounds with PUFA in one compound is one of the solutions used enhances the hydrophobicity of phenol and protects the PUFA against oxidation. (Mbatia B. *et al.*, 2011) observed that antioxidant activities of rutin and vanillyl PUFA esters showed better activity than α-tocopherol in emulsion systems. Synthesis of lipophilic antioxidants have great interest due to their ability to work in emulsion media (Rudzinska *et al.*, 2014). Antioxidative effect of lipophilized dihydrocaffeic acid (octyl dihydrocaffeate and oleyl dihydrocaffeate) on the oxidation of emulsions was evaluated by (Sørensen *et al.*, 2012a). They observed that lipophilized dihydrocaffeic acid was more efficient than caffeic and dihydrocaffeic acids and that octyl dihydrocaffeate had a significantly higher antioxidative effect than oleyl dihydrocaffeate in emulsions. In another work, (Sørensen *et al.*, 2012b) studied the antioxidative effect of phenolipids in fish oil enriched milk emulsions. The authors confirmed that both dihydrocaffeate esters and rutin laurate showed significantly better antioxidant properties in milk emulsion compared with the original phenolics.

Therefore, the main objective of the present work was to assess the antioxidant properties of DHA-VE in relation to their structure and to investigate their effectiveness in preventing oxidative deterioration of emulsions. Accelerated conditions of oxidation were used in order to evaluate the influence of temperature on the stability.

2. Materials and methods

Vanillyl alcohol (4-hydroxy-3-methoxybenzyl alcohol, VA purity of 98%), α-tocopherol (purity of 98%), Tween 80, thiobarbituric acid (TBA) and 1,1,3,3-tetraethoxypropane (TEP) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Docosahexaenoic acid ethyl ester (DHA-EE) of 95% purity was supplied by KD-Pharma (Bexbach, Germany). DHA vanillyl ester (DHA-VE) 98% purity was prepared by enzymatic acylation method done in our laboratory (LIBio – ENSAIA -

Lorraine University, Nancy, France). Methanol and trichloroacetic acid (TCA) was obtained from Carlos Erba (Paris, France). Butylated hydroxytoluene (BHT) was purchased from Merck (Hohenbrunn, Germany). Margarine samples prima 4 were purchased from local markets in Nancy, France. According to the manufacturer, it contained approximately 60% oil. The oil phase was consisted of rapeseed oil, palm oil and copra oil.

2.1. Oil in water (O/W) emulsions preparation

Oil in water emulsions was prepared from 10% (w/w) salmon oil, 1% (w/w) emulsifier (Tween 80) and 89% ultrapure water. Antioxidants compounds were added to salmon oil to obtain 200 mg/kg final concentration in the emulsion. A coarse emulsion was prepared using a Ultra-Turrax T25 basic (2 min, 11.400 rpm, IKA-Werke, Staufen, Germany) that was sonicated as follow by sonication (pulses 1s on/ 1s off, time 4 min, amplification 40%, *Vibra Cell 75115*, *Bioblock Scientific*, Illkirch, France*)* and circulated through a high pressure valve homogenizer Emulsiflex-C3 (Sodexim S.A, France) four times at 9.000 psi. The freshly prepared emulsions were placed in closed clear bottles and incubated at 4, 20 and 60 ºC.

2.2. Oxidative stability experiments

Lipid samples containing various inhibitors of the oxidation were prepared directly before use. 50 mg/kg and 200 mg/kg of the tested antioxidant compounds were added to margarine and salmon oil in water emulsions, respectively. Accelerated condition was used to study the antioxidative effect of tested antioxidants in margarine and salmon oil emulsion samples. Margarine samples (100 g) containing antioxidant compounds were maintained at 100 ± 2 °C, 20 ± 2°C and 4 ± 1°C and emulsion samples were stored at 37°C ± 2°C and 20± 2°C. Primary oxidation products were determined by peroxide measurements (PV and CD) and secondary oxidation products was measured using the *p*-anisidine value and TBA methods.

2.2.1. Peroxide value (PV)

The peroxide value corresponds to the amount of peroxide present in the sample, expressed in number of milli-equivalents of active oxygen per kilogram of product and oxidizing potassium iodide with liberation of iodine. This reaction is between a solution of potassium iodide and the fat dissolved in chloroform. The

liberated iodine in acidic solution is titrated with sodium thiosulfate. A mixture of acetic acid - chloroform (3-2, v/v, 25 ml) was added to a sample from 1 to 5 g to solubilise the fat. One ml of a saturated solution of KI (5 g/5 ml water) was added and the mixture was stirred for 1 min, then let stand for 5 minutes protected from light. Thirty ml of distilled water were added (to stop the reaction), then 1 ml of starch solution 1% that serve as an indicator. The liberated iodine was titrated with stirring with solution of 0.01 N sodium thiosulfate. A blank test was performed without fat. The results were expressed as follows

$$\text{Peroxide value (meq kg-1)} = \frac{(V_S - V_B) \times N \times 1000}{W}$$

Where V_S is the titre value (ml) of sodium thiosulphate solution for sample, VB is the titre value (ml) of sodium thiosulphate solution for blank; N is the normality of sodium thiosulphate solution and W is the weight of sample in gram.

2.2.2. Conjugated diene value (CD)

The conjugated diene value (CD) was determined as described by (Weber *et al.*, 2008). Briefly, the sample was dissolved in 25 ml *n*-hexane and recorded the optical density (1 cm light path) at 234 nm against *n*-hexane blank. The conjugated diene value was calculated according to the equation:

$$\text{CD value} = [C_{CD} \times (2.5 \times 10^4)] / W$$

$$C_{CD} = A\ 234 / (E \times L)$$

Where CCD is the CD concentration in mmol/ml, A 234 is the absorbance of the lipid solution at 234 nm, E molar absorption coefficient, 26 000 L/mol, L is the path length of the cuvette in cm (1 cm) and 2.5 x 104 is a factor that encompasses the volume of *n*-hexane (25 ml) used to dissolve the oil sample.

2.2.3. Anisidine value (p-An.v)

The *p*-anisidine value (AV) was determined according to Cd 18-90 method suggested by (AOCS, 1995). This method depends on spectrophotometric determination of products formed between aldehyde compounds and *p*-anisidine. Oil samples (0.5-2.0 g) were dissolved in 25 ml iso-octane and absorbance of this fat solution was measured at 350 nm using a spectrophotometer (Shimadzu UV-1605). Five millilitres of the above mixture was mixed with 1 ml 0.25% *p*-anisidine in glacial

acetic acid (w/v) and after 10 min standing, the *p*-anisidine value (AV) was calculated according to the equation:

$$AV = 25 \times (1.2\ A_S - A_B) / m$$

Where A_S is the absorbance of the fat solution after reaction with the *p*-anisidine reagent; A_B is the absorbance of the fat solution and m is the mass of oil sample (g).

Measurements of *p*-anisidine value are commonly used together with peroxide value measurements in describing the total extent of oxidation by the Totox value, which equals the sum of the *p*-anisidine value plus twice the peroxide value. However, the Totox value is an empirical parameter since it corresponds to the addition of two parameters with different units (Fereidoon andUdaya, 2008).

2.2.4. Thoibarbituric acid reactive substances (TBARS) assay

TBARS method was determined according to the method of Tong et al (2000) with some modifications. One hundred microlitres of emulsion was mixed with 0.9 mL of water and 1 mL of TBARS reagent (15% (w/v) TCA and 0.375% (w/v) TBA in 0.25 M HCl), then vortexed. The reaction mixture was heated at 90°C in water bath for 20 min. The samples were cooled to room temperature and centrifuged at 2000 x g for 10 min (MiniSpin Eppendorf). The absorbance of the supernatant was then measured at 532 nm (Shimadzu UV-1605). Concentrations of TBARS were determined from a standard curve prepared using 1, 1, 3, 3-tetraethoxypropane (TEP) in concentrations ranging between 0.6 µM and 40 µM. The results were expressed as µM of TBARS.

2.3. Statistical Analysis

Statistical analyses were conducted using SPSS (Statistical Programme for Social Sciences, SPSS Corporation, Chicago, IL, USA) version 16.0 for Windows. All analyses were performed in triplicate and data reported as means ± standard deviation (SD). Data were subjected to analysis of variance (ANOVA). The confidence limits used in this study were based on 95% ($P < 0.05$).

3. Results and discussion

In general, lipid oxidation goes through different steps or reaction pathways depending on low or high temperature conditions (Fu Xiangjin andDang, 2011 ; Smith *et al.*, 2007). Measurements of oxidative stability are used for lipid shelf life prediction. Methods that measure oxidative stability focus on different pathways in the oxidation process. Several methods are used to give a clearer picture of the stability and state of the lipids. In this study, storage under accelerated conditions of 100 °C and 60 °C and under ambient conditions (20 °C and 4 °C) were used to evaluate the oxidative stability of oils phase in margarine and salmon o/w emulsions.

3.1. Oxidative stability of margarine

In this study, the effect of margarine enrichment with DHA-EE, vanillyl alcohol (VA) and DHA-VE on the oxidative stability was tested. Oxidation of margarine oil phase was monitored by determination of peroxide values, anisidine value and Totox number during storage period under different storage conditions.

3.1.1. Peroxide Value (PV)

The current results showed that PV was increased in all margarine samples at the various storage temperatures during the study period (Fig. 1). Initial rate of PV was slow for margarine enriched with DHA-VE and VA in comparing with control margarine or margarine with DHA-EE at different storage conditions. PV started to increase after 4 h of storage at 100 °C for margarine with DHA-VE and VA and went on increasing further with the increase in storage period. Storage at 20 °C showed that PV was in the range of 0.6 – 58.7 and 0.6 – 145.98 meq/kg for margarine with DHA-VE and VA respectively, whilst this range was 0.6 – 16.4 and 0.6 – 24.6 meq/kg respectively, for the samples stored at 4 °C. The results too showed a significant difference ($P< 0.05$) in PV between the control margarine and margarine containing DHA-VE and VA as antioxidants compound as presented in Fig. (1). Similar trend was observed by (Rudzinska *et al.*, 2014) when they studied the oxidative stability of margarine

enriched in phytosterols/phytostanols. These authors found that storage temperatures at 4 and 20 °C significantly affected PV whereas value was increased to 9.4 and 35.4 meq/kg after 12 weeks of storage at 4 and 20 °C, respectively.

The induction period of margarine samples was presented in Table (1). This period was calculated as the time to reach PV=20 meq/kg at storage temperature of 20 °C and 100 °C and the period of PV reached 5 meq/kg at storage temperature of 4 °C (Economou *et al.*, 1991 ; Pokorny and Trojáková, 2001). In this regard, the highest antioxidative activity at 100, 20 and 4 °C was belonged to DHA-VE with 9h, 10 weeks and 6 months, respectively.

3.1.2. Anisidine Value (AV)

The primary products of lipid oxidation are pretty unstable and readily decompose into various secondary products. The secondary stage of oxidation occurs when the hydroperoxides decompose to form carbonyls and other compounds, in particular aldehydes. Anisidine value (AV) test is a good way to measure secondary oxidation products and should be used together with a primary test like PV to reflect the real extent of oxidation.

In this study, antioxidants compounds were significantly ($p < 0.05$) affected with secondary oxidation and total oxidation in margarine during the storage period. Data presented in Fig (2) showed the oxidative kinetic of margarine oil phase stored at 100, 20 and 4°C. The results demonstrated that storage temperature had a significant effect on AV. It can be clearly seen that the oxidation rate was increased with increasing temperature. Whereas, AV of the samples stored at 4 and 20 °C was increased with long time of storage and at 100°C it need only few hours to increase. Margarine with DHA-VE showed the better stability at all storage conditions than other margarines. Changes of AV in margarine samples stored at 100 °C (Fig. 2A) showed that margarine with DHA-VE and margarine with VA had the highest antioxidative activity among margarine control or with DHA-EE ($p<0.05$). The AV of all samples was increased through storage at 20 °C from 0.5 in week 0 to 75.65, 70.98, 59.48 and 25.60 in week 15 for control margarine, margarine with DHA-EE, margarine with VA and margarine with DHA-VE, respectively (Fig. 2B). In all margarine samples, the trend of AV was comparable to that for PV. Margarine samples stored at 4 °C had significantly lower AV level compared to those at 20°C, and the AV range was 0.5 - 67 for control, 0.5 - 31 for margarine with VA and 0.5 - 14 for margarine with DHA-VE after storage for 10 months (Fig. 2C).

The current results (Fig. 3) illustrate the effects of temperature and time on the Totox numbers of margarine samples. Similar trend with PV and AV was observed for all samples at all storage conditions due to Totox numbers generated from PV and AV. Through storage at 20°C, Totox values were increased from 2 - 476 for control samples, 2 - 351 for margarine with VA and 2 - 143 for margarine containing DHA-VE (Fig. 3B). These values were decreased in case of storage at 4°C (Fig. 3C). As expected, the Totox values of samples stored at 100 °C were higher than those stored at 4 and 25°C in the different groups.

3.2. Oxidative stability of salmon oil in water emulsions

The mechanism of lipid oxidation for oil-in-water emulsions differs from bulk lipids because emulsions have an aqueous phase that contains both prooxidant and antioxidants compounds and oil/water interface that impact on interactions between oil and water components (Waraho *et al.*, 2011 ; Zhou andElias, 2012). The ability of antioxidants to inhibit lipid oxidation in food emulsions depends on factors such as antioxidant concentration; reactivity; partitioning between oil, water and interfacial phases; interactions with other food components; and environmental conditions such as pH, ionic strength and temperature. Antioxidant generally works by scavenging free radicals and/or inactivating pro-oxidant such as transition metals.

Antioxidant activities DHA-VE and vanillyl alcohol (at a concentration of 200 mg/kg) were tested in 10% salmon oil in water emulsions prepared with 1% Tween 80 as emulsifier. The emulsions were left to autoxidized at 37°C and 20°C. Oxidation of oil phase was determined by measurements of conjugated dienes at 234 nm and TBARS at 532 nm. Samples were taken periodically as the main method of monitoring fish lipid oxidation. Formation of CD occurs during the propagation stage through reactions of the lipid with free radicals that were generated at the initiation stage. Conjugated dienes are produced due to the rearrangement of the double bonds in PUFAs, and are used as an indicator of oxidation (Shahidi Fereidoon andZhong, 2005).

According to the results of CD presented in Fig. 4 the emulsions started to get significantly oxidized with induction time in all tested compounds samples and in both temperatures. The kinetics of oil phase autoxidation at 37°C and 20°C in the absence (control sample) and presence of 200 mg/kg concentrations of individual compounds under study (DHA-VE, vanillyl alcohol and tocopherol). It was observed that vanillyl

alcohol and its DHA-VE were able to ensure the higher oxidation stability of lipid substrate and has a better protective effect than control and tocopherol. At 37°C, it was observed that during the first 11 days, no significant differences were observed between tocopherol, vanillyl alcohol and its derivative DHA-VE in suppressing the CD formation than control. After this period, CD was increased for control and the emulsion samples with tocopherol; meanwhile, CD was decreased for the emulsions samples containing vanillyl alcohol and DHA-VE. In the following oxidation stage, the CD decomposes, producing secondary products which then produce volatile compounds. For the emulsions samples stored at 20°C, a little CD was formed and after 11 days it was started to decrease. However, no significant changes were observed for the samples with tocopherol, vanillyl alcohol and DHA-VE.

The development of TBARS in emulsions contained different antioxidants is presented in Fig 5. Emulsions stored at 20°C had a lag phase of around 10 days before these emulsions started to develop the TBARS. The emulsions with DHA-VE and tocopherol were more stables than the others. After 10 days, CD started to disappear while the TBARS started to increase. This trend did not appear for the samples stored at 37°C, no lag phase was observed for emulsions. At this temperature, the development of TBARS was similar for all the formulations that contained the antioxidants, reaching the control in the end of 14 days. The fast oxidation in the emulsion at 37°C could be attributed to prooxidant effect of the tested antioxidant compounds.

In margarine, DHA-VE showed different positive efficiency to inhibit lipid oxidation. While in salmon oil emulsion in water, emulsion DHA-VE showed antioxidant efficiency during the initial days of storage condition, then pro-oxidant activity appeared during the last days of storage. These results confirmed that the interface of the emulsion (the site where oxidation occurred) play an important role in lipid oxidation. Consequently, in margarine, the antioxidant activity was regulated by polarity and ability or rapidity of donating hydrogen atoms of phenolic compounds. Meanwhile, in emulsions, the antioxidant activity of phenolic compounds prefer to the affinity toward emulsifier (tween 80) rather than their polarity.

4. Conclusions

Novel lipophilic vanillyl alcohol derivative was prepared via enzymatic acylation of vanillyl alcohol with DHA-EE. The results showed that modification of vanillyl alcohol didn't affect its antioxidant capacity. Moreover, lipophilic derivatization of vanillyl alcohol effectively increased hydrophobicity and solubility in fats and thus enhanced the efficiency of the esters prepared in lipophilic media. The results also indicated that synthesized vanillyl alcohol derivatives might be useful agents for oil/fat-based food protection against oxidation during storage. Acylation of phenol compounds with fatty acids not only leads to increase the lipophilicity, but also may provide additional advantages by introducing new bioactive functional groups to the antioxidant molecules. These functional groups upon digestive hydrolysis may release the individual compounds that render their own health effects individually or synergistically. The need for novel natural antioxidants is obvious and the industry continues to look for useful natural antioxidants. Furthermore, DHA-VE possesses good antioxidant activity in fatty food type and can be used as a natural antioxidant for margarine products. Hence these antioxidants increase the shelf life of foodstuffs and provide protection to margarine and emulsions, replacing synthetic antioxidants.

Acknowledgements

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Tables and Figures:

Table 1. Induction period (IP) of margarine at different storage conditions with different antioxidant compound. IP was considered as the time of storage needed for PV to reache 20 meq/kg sample.

Samples	Time to reach PV 20meq/kg		Time to reach PV 5 meq/kg at 4°C (months)
	100°C (h)	20°C (Weeks)	
Margarine control	4.0[a]	2.0[a]	3.0[a]
Margarine + DHA -EE	4.0[a]	2.5[a]	3.5[a]
Margarine + VA	6.0[b]	7.0[b]	4.0[b]
Margarine +DHA-VE	9.0[c]	10.0[c]	6.0[c]

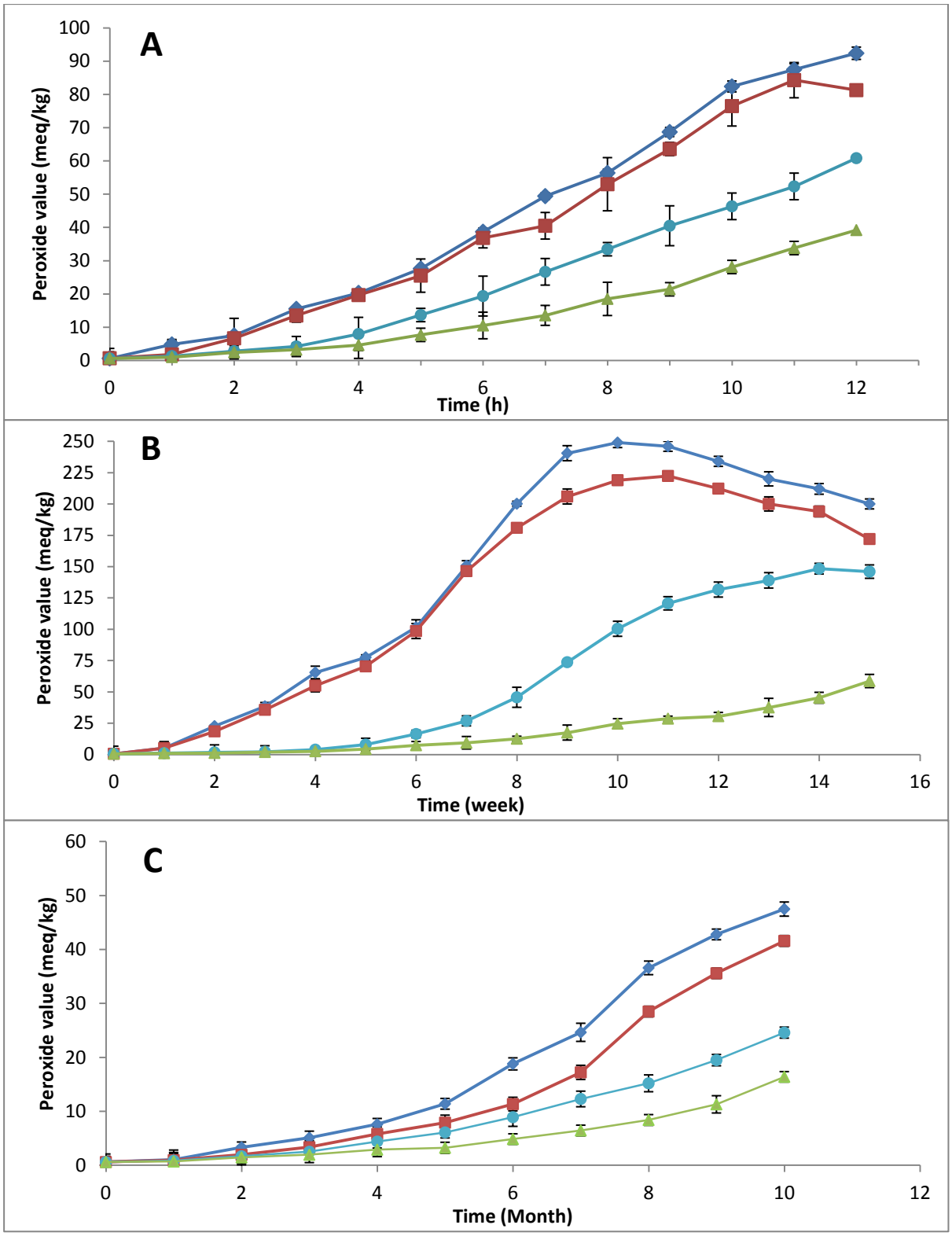

Fig. 1. Peroxide values of margarine oil phase store at different storage conditions, (A) at 100°C, (B) at 20°C and (C) at 4°C. ♦ Margarine control, ■ Margarine + DHA-EE, ● Margarine + vanillyl alcohol and ▲ Margarine + DHA-VE.

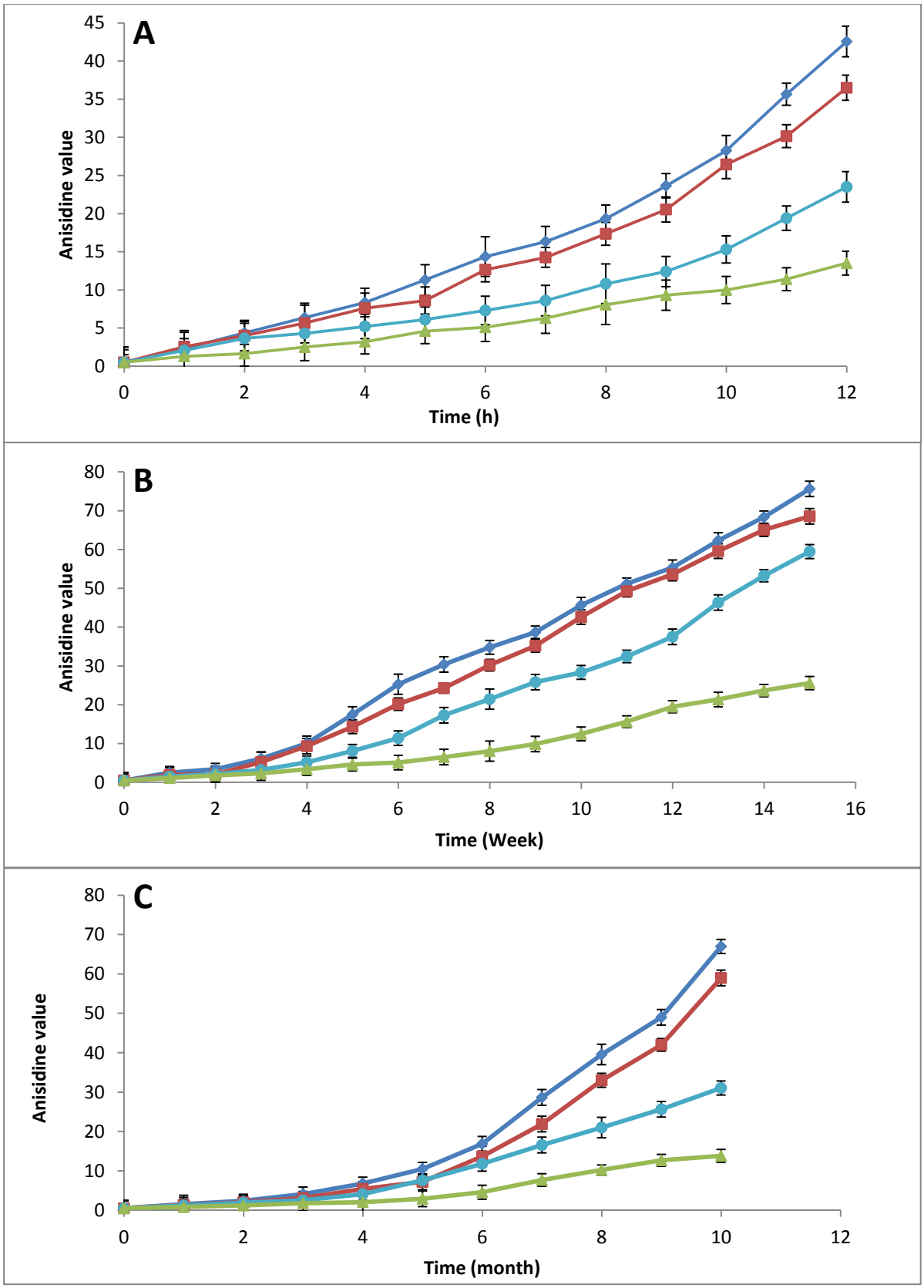

Fig. 2. Anisidine values of margarine oil phase store at different storage conditions, (A) at 100°C, (B) at 20°C and (C) at 4°C. ♦ Margarine control, ■ Margarine + DHA-EE, ● Margarine + vanillyl alcohol and ▲ Margarine + DHA-VE.

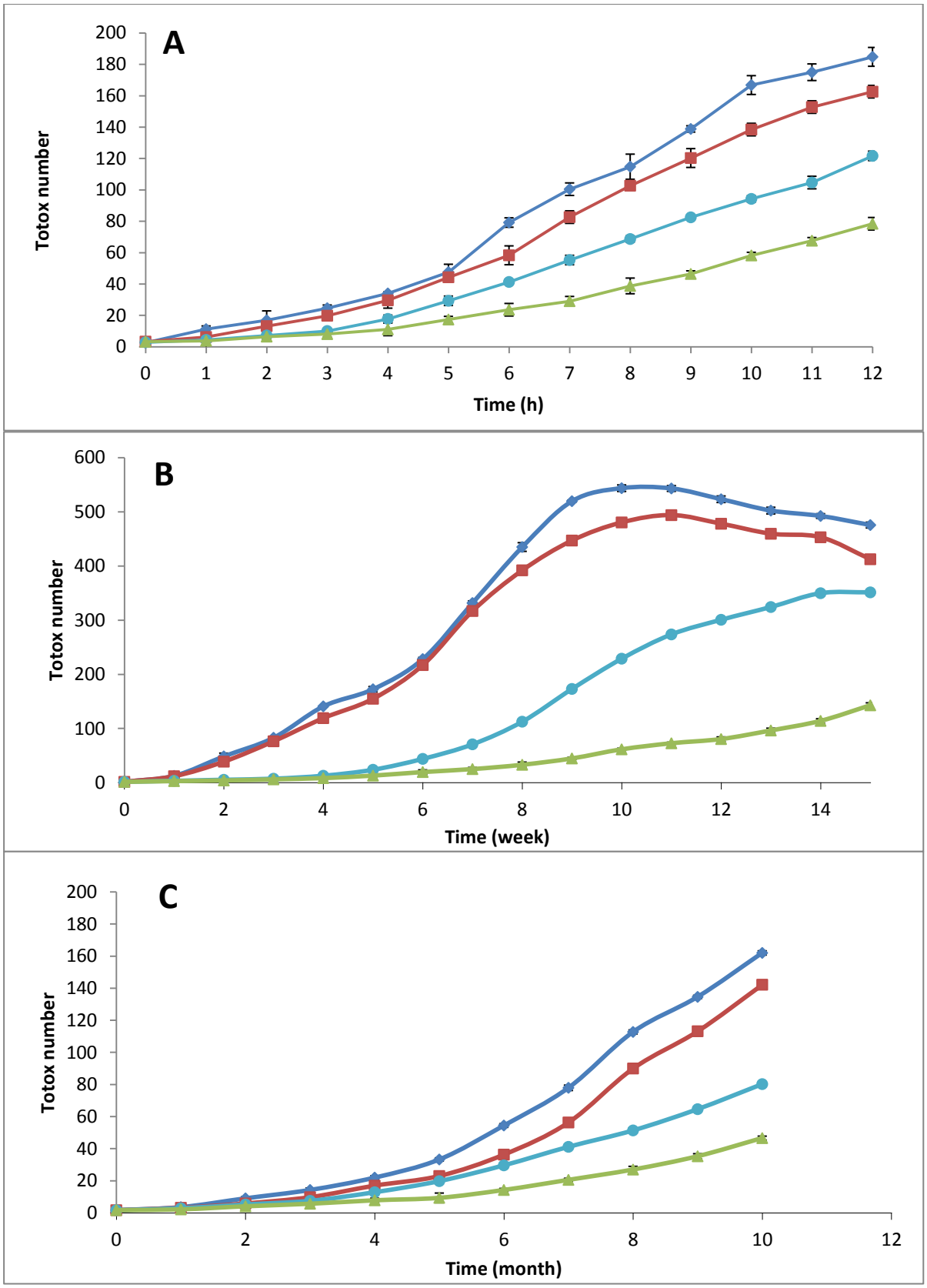

Fig. 3. Totox values of margarine oil phase store at different storage conditions, (A) at 100°C, (B) at 20°C and (C) at 4°C. ♦ Margarine control, ■ Margarine + DHA-EE, ● Margarine + vanillyl alcohol and ▲ Margarine + DHA-VE.

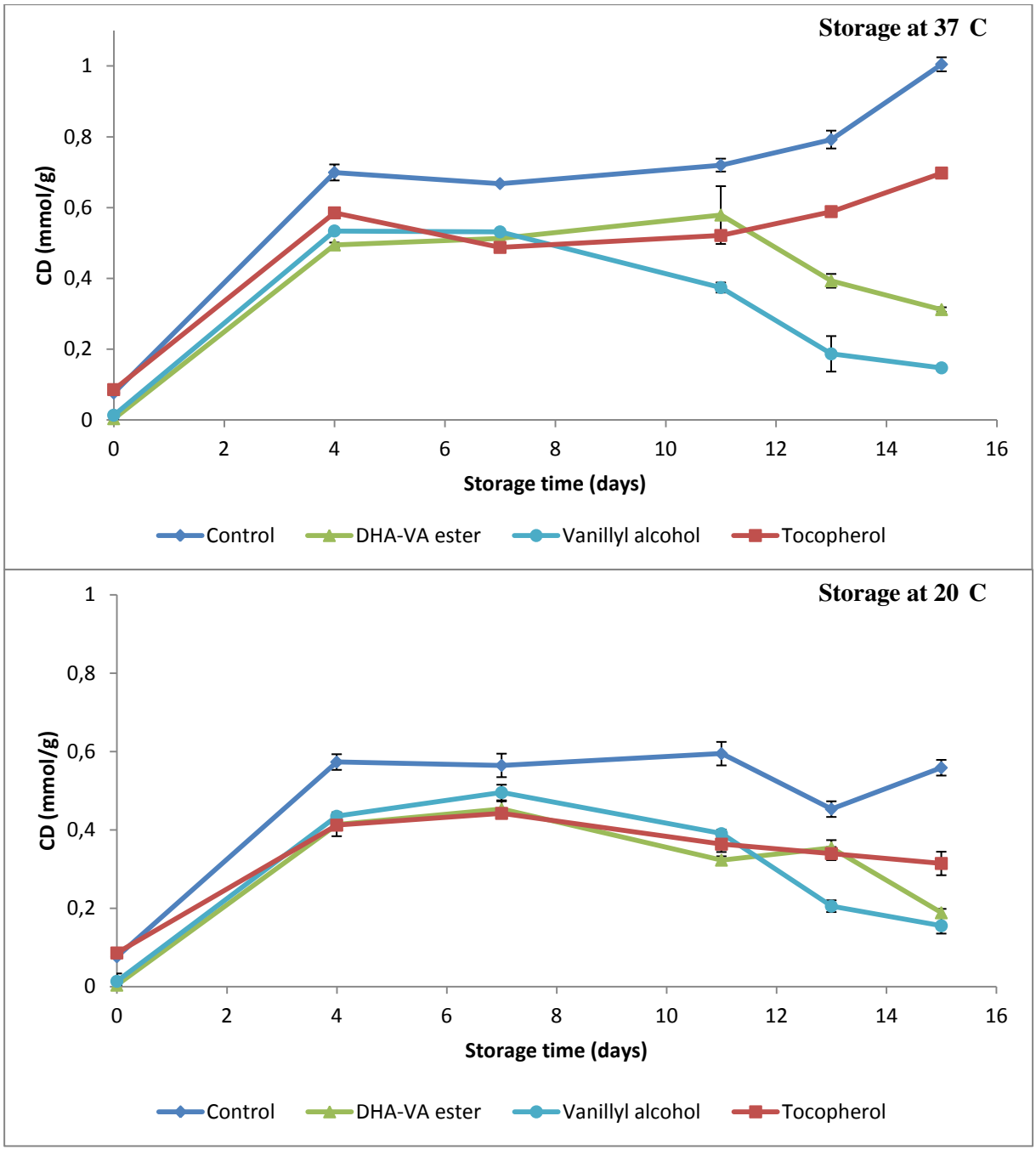

Fig. 4. Oxidation kinetics of salmon oil-in-water emulsion stored at 37°C and 20°C with different antioxidants compounds. Conjugated dienes method (λ = 234 nm). Error bars are confidence intervals (α = 0.05; n = 3).

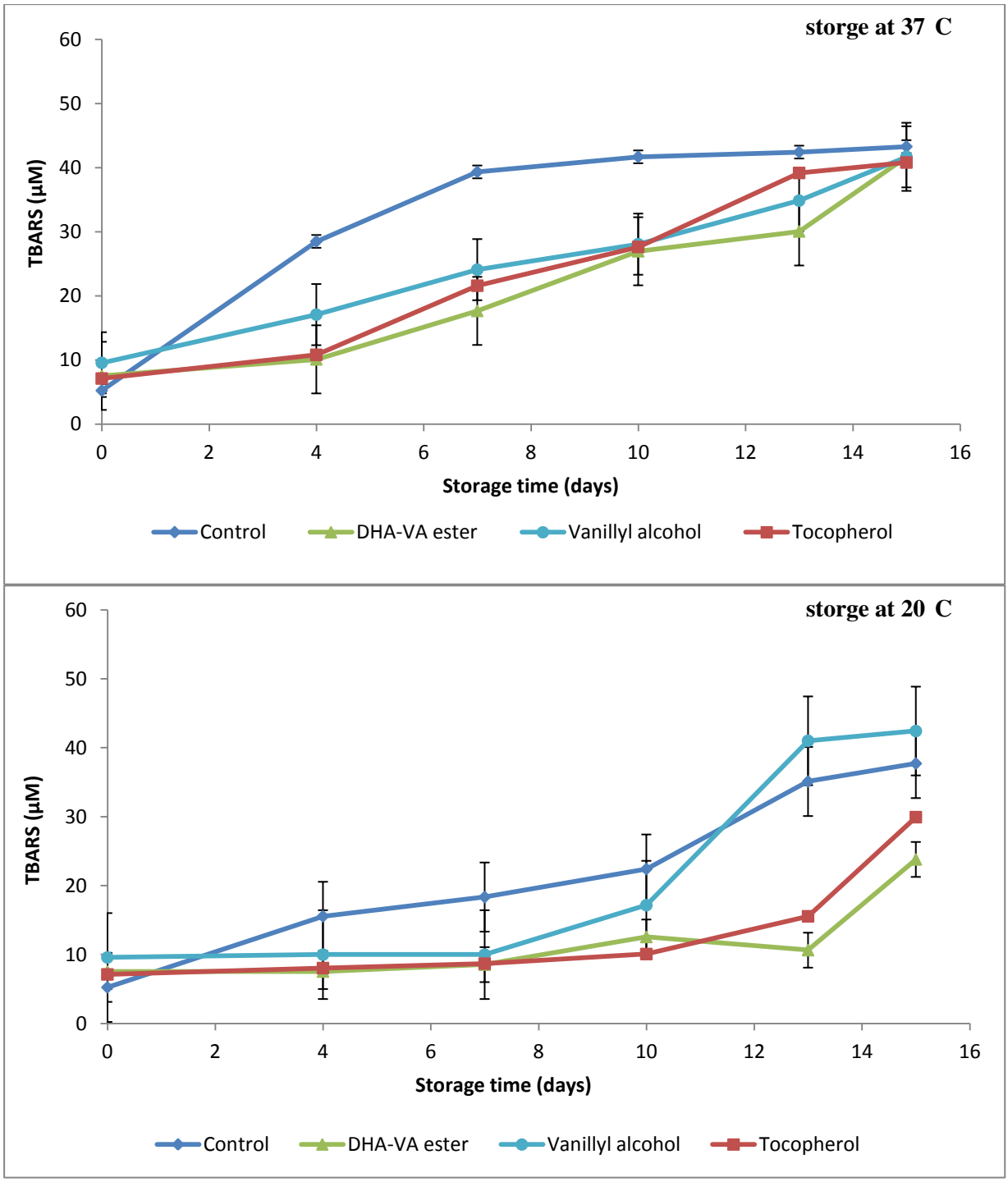

Fig. 5. Oxidation kinetics of salmon oil-in-water emulsion stored at 37°C and 20°C with different antioxidants. TBARS method (λ = 532 nm). Error bars are confidence intervals (α = 0.05; n = 3).

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4.3. Contribution de l'article

La stabilité à l'oxydation d'émulsions enrichies en alcool vanillique ou son ester de DHA (DHA-VE) a été étudiée. Les résultats obtenus montrent que quelles que soient les conditions de stockage, l'ajout de composé phénolique sous sa forme native ou sous la forme d'ester de DHA permet d'améliorer la stabilité des émulsions de manière significative. Par ailleurs, le dérivé DHA-VE se montre plus efficace que l'alcool vanillique natif, notamment dans le cas de la margarine. Ceci s'explique par une meilleure localisation de l'agent antioxydant par rapport à la phase lipidique du système.

Cette étude met en évidence le potentiel de certains esters phénoliques d'acides gras en tant qu'agents antioxydants pour la protection de systèmes émulsionnés sensibles à l'oxydation. Ainsi que d'autres auteurs l'ont déjà démontré précédemment, la lipophilisation des composés phénoliques apparaît comme une bonne solution pour moduler leur solubilité dans diverses matrices d'incorporation et optimiser leur potentiel antioxydant. L'ester DHA-VE est un dérivé un peu particulier, cumulant plusieurs effets positifs : c'est un composé stable, source de DHA et capable de protéger son milieu d'incorporation contre l'oxydation. Les travaux doivent être poursuivis pour évaluer au mieux son potentiel applicatif. Son métabolisme devra notamment être étudié pour recueillir des informations sur son transport et son absorption dans l'organisme. Par ailleurs, l'alcool vanillique présente des propriétés aromatiques intéressantes, susceptibles de masquer l'odeur de poisson liée à la présence du DHA. Une étude devra être menée pour évaluer l'acceptabilité sensorielle de l'ester après incorporation dans des aliments.

Conclusions and Perspectives

Conclusions and perspectives

In this study, an enzymatic process was developed and optimized, using the lipase B from *C. Antarctica*, for the synthesis of bimodular derivatives combining highly oxidizable fatty acids and a phenolic antioxidant. A model derivative was firstly synthesized through an alcoholysis reaction between DHA ethyl ester and vanillyl alcohol.

In that case the synthesis was carried out in organic solvents or in solvent-free medium. Initially, the enzymatic synthesis was carried out in organic solvents such as 2-methyl-2-butanol or acetonitrile, in the presence of a slight excess of DHA (molar ratio vanillyl alcohol: PUFA equal to 1:2). Kinetics obtained showed that these conditions required 8h of reaction to reach the equilibrium and a maximum conversion of vanillyl alcohol of 60%ester. The product was purified; its structure was determined showing that acylation took place on vanillyl alcohol primary hydroxyl group. Although traditional, processes performed in organic solvents are increasingly criticized today because of their cost and the use of media of high environmental impact. Due to growing safety and environmental concerns and with the objective to increase the conversion yield of vanillyl alcohol together with the ester production, a solvent-free process appeared as an attractive alternative. Under these conditions, the acyl donor substrate (DHA ethyl ester) was introduced in large excess comparing to the acyl acceptor substrate (vanillyl alcohol). The main advantages of this approach are the absence of organic solvents (the large excess of one substrate allowed the solubilisation of the other substrate) and the possibility to shift the equilibrium of the reaction in favour of the ester synthesis. This effect can be reinforced by working under reduced pressure, that allows the continuous elimination

of the by-product of the reaction, ethanol. Such conditions were shown to improve the conversion of vanillyl alcohol, and then the production of the ester. The concentration of vanillyl alcohol and the feeding of the reactor with the phenolic substrate were optimized. Finally, a fed-batch process was proposed, leading to high concentration of ester (440 g/L) and avoiding the oxidative degradation of the reaction medium. Alternatively, a high amount of vanillyl alcohol can be introduced at one time (saturation conditions).

Given all these results, different elements must be taken into account to choose the most appropriate process for the production of phenolic fatty acid esters as the efficiency of the process, the complexity and the cost of synthesis and purification steps, the stability of the reaction medium towards oxidation.

The biological activities of the ester were investigated, leading to promising conclusions. In fact, the ester exhibits interesting potential for food industry and nutrition: (i) improved organoleptic qualities of DHA-VE-supplemented diet; (ii) elevated antioxidant activity that should stabilize DHA as well as various food matrices such as oils, fats and emulsions; (iii) increased bioavailability of DHA leading to higher DHA levels in erythrocytes and neurons; (iv) combined beneficial effects of phenols and ω3 PUFAs; (v) increased neuroprotection against amyloid stress; (vi) no visible toxicity.

Moreover, its oxidative stability was monitored by using different spectroscopic methods. Conjugated dienes (CD) determination is a widespread and inexpensive technique providing information about the first stage of oxidation that leads to primary oxidation products. In the present work, CD determination was particularly effective to study the thermal and temporal stability of DHA-based compounds. Furthermore, FTIR is now well-known for its high efficiency to follow structural changes in complex

evolving systems. The different regions of FTIR spectra provide useful information about functional groups and their chemical environment. On a practical point of view, this rapid and non-destructive method does not require any sample or chemical preparation, and then allows significant time- and cost-savings in comparison with classical analyses. In this work, FTIR was shown to be an efficient tool to follow lipid oxidation thanks to significant changes in the frequency and the intensity of characteristic bands. More specifically, the intensity of the band related to =C-H bond stretching vibration of cis-double bonds at 3013 cm-1 depended on the degree of unsaturation of the samples, and then was used as a marker for DHA oxidation. Another sensitive indicator was the ratio between the absorbance at 3013 cm-1 and the absorbance at 2853 cm-1 that corresponds to the vibration of saturated C-H bonds.

Unsurprisingly, the oxidative stability of the compounds was negatively affected by increasing temperature and storage time. All results indicated a higher stability of DHA-VE in comparison with DHA-EE, showing the interest of combining this highly oxidizable lipid with vanillyl alcohol in a single structure. According to FTIR data, oxidation was delayed till 8 weeks in the case of pure DHA-VE stored at 20°C against 2 weeks in the case of pure DHA-EE. A midway stability was determined for the crude reaction medium made of 45% DHA-VE. Main advantages of such a medium are, firstly, a high stability despite a significant content in DHA, secondly, easy preparation and use that do not require any purification step. Phenolic esters of DHA undoubtedly appear as promising derivatives that could make easier the use of polyunsaturated lipids.

Lipase-catalyzed alcoholysis of salmon oil with vanillyl alcohol was performed through an efficient solvent-free bioprocess, leading to a mixture of SFA, MUFA and

PUFA vanillyl esters (FA-VE). Structural analyses allowed identifying the major products. Vanillyl alcohol was almost totally converted after 24 h of reaction, starting from an initial concentration of 50g/L. The crude reaction medium recovered from salmon oil alcoholysis exhibited a high stability during storage compared to salmon oil.

The overall experimental results obtained through the present study could lay the ground for the use of vanillyl fatty acid esters as nutraceuticals and/or functional ingredients for food products. Future work should focus on bioavailability studies in order to get a better understanding of the transport and the absorption of these molecules in the human body.

To develop and complete this work, some ideas seem promising:

- Biological studies must be undertaken in order to get information about the metabolism the esters produced.
- The organoleptic properties of the synthesized products must be evaluated through sensory analyses.
- The process could be even more improved by studying the effect of specific parameters like water activity, enzyme concentration, reaction temperature and agitation speed.
- Biological studies about the neuroprotective effect of the esters must be pursued and intensified. The mechanism of action of the esters appears as a key issue of this project.
- The alcoholysis process could be applied to other types of oils aiming to extend it to other application areas like cosmetic ingredients.

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Abstract

An efficient solvent-free bioprocess was developed for the synthesis of DHA phenolic ester, using the lipase B from *Candida antarctica*. The protocol developed here led to high-level production (440 g/L) of DHA vanillyl ester (DHA-VE) that exhibits interesting application potential as food ingredient. DHA-VE was characterized by a high stability and a high radical scavenging activity towards DPPH, ABTS and hydroxyl radicals. Neuroprotective properties of DHA-VE were also demonstrated in rat primary neurons exposed to amyloid-β oligomers. Enzymatic esterification of DHA with vanillyl alcohol (VA) led to increased DHA levels in erythrocytes and brain tissues of mice fed DHA-VE-supplemented diet comparing with DHA. No visible toxicity of the ester was found. Enrichment of emulsions with DHA-VE improved significantly their oxidative stability whatever the conditions of storage, showing the potential of DHA-VE to enrich various food matrices with DHA while protecting them against oxidation. The enzymatic process was applied to salmon oil as a source of omega-3 polyunsaturated fatty acids (PUFA). The total conversion of VA (50 g/L) was achieved after 24 h of reaction, leading to the production of a wide variety of esters that mirror the initial composition of the oil. The crude reaction medium recovered from salmon oil alcoholysis exhibited a high stability together with high antioxidant properties in comparison with native salmon oil. In conclusion, the approach that consists in bringing phenolic compounds and PUFA-rich lipids together within a single structure is expected to provide stable bioactive ingredients that should broaden the scope of application of omega-3 PUFAs whose health benefits are increasingly sought.

Key words: Omega-3, DHA, phenolic compounds, vanillyl alcohol, enzymatic esterification, oxidative stability, neuroprotection, and fish oil.

Résumé

Un procédé enzymatique sans solvant a été développé permettant la synthèse d'un ester phénolique de DHA. L'optimisation des paramètres réactionnels a permis d'atteindre des rendements élevés (440 g/L) d'ester de DHA et d'alcool vanillique (DHA-VE), dont les activités biologiques et le potentiel applicatif ont été évalués. L'activité inhibitrice du DHA-VE vis-à-vis des radicaux ABTS, DPPH et hydroxyle a été démontrée. Un effet neuroprotecteur de l'ester a également été mis en évidence sur des neurones primaires de rat, exposés aux oligomères du peptide β-amyloïde. Une étude *in vivo* a permis de montrer que le greffage d'alcool vanillique conduit à une augmentation du taux de DHA au niveau des globules rouges et des neurones, indiquant une biodisponibilité accrue du DHA lorsque celui-ci est couplé au composé phénolique. Aucune toxicité visible de l'ester n'a été constatée. Par ailleurs, l'incorporation de DHA-VE dans divers systèmes émulsionnés a permis d'accroître leur stabilité à l'oxydation, quelles que soient les conditions de stockage. Ceci montre le potentiel de cet ester pour enrichir diverses matrices alimentaires en DHA, tout en améliorant leur stabilité à l'oxydation. Le procédé enzymatique développé a été appliqué à de l'huile de saumon, utilisée comme source d'acides gras polyinsaturés de la série oméga-3. L'incorporation totale de l'alcool vanillique (50 g/L) a été obtenue après 24 h de réaction, conduisant à la production d'une grande variété d'esters, représentatifs de la composition initiale de l'huile en acides gras. Le milieu réactionnel brut issu de l'alcoolyse de l'huile présente une grande stabilité et des propriétés antioxydantes importantes par rapport à l'huile de saumon native. En conclusion, l'approche consistant à assembler des composés phénoliques et des lipides polyinsaturés au sein d'une même structure semble prometteuse pour renforcer le potentiel applicatif de ces deux familles de biomolécules et produire de nouveaux ingrédients bioactifs stables.

Mots clés : Omega-3, DHA, composés phénoliques, alcool vanillique, estérification enzymatique, stabilité à l'oxydation, neuroprotection et l'huile de poisson.