Université de Liège
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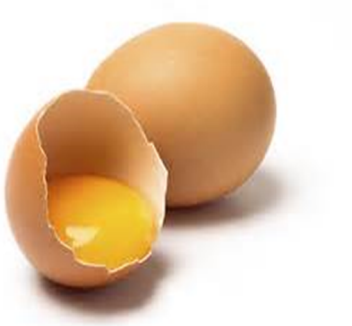
DEVELOPMENT OF ANALYTICAL TOOLS FOR THE DETERMINATION OF MARKERS OF LIPID ALTERATION IN FOOD

DEVELOPPEMENT D'OUTILS ANALYTIQUES POUR LA DETERMINATION DE MARQUEURS DE L'ALTERATION DES LIPIDES ALIMENTAIRES



Caroline DOUNY

Thèse présentée en vue de l'obtention du grade de Docteur en Sciences vétérinaires
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ABSTRACT

It is now well-known that n-3 polyunsaturated fatty acids (omega-3 PUFA) have beneficial effects on health but are also rapidly oxidized in potentially toxic compounds, which means that healthy food products, rich in n-3 PUFA, can become toxic for the consumer if not stored or processed appropriately. The main objective of this work was to develop new analytical tools to study markers of lipids alteration in food, in order to allow an adequate monitoring of possible toxic compounds in n-3 PUFA rich food products.

The **first chapter** of this PhD thesis is a literature review about fatty acids, their oxidation and the different oxidation products generated. The different analytical techniques used to study the oxidative stability of polyunsaturated fatty acids and their degradation products in food are also described.

Chapter 2 describes the development of a gas chromatography coupled to mass spectrometry (GC-MS) method to analyze the fatty acids in food. The method was applied to eggs and pork (both standard and n-3 enriched) submitted to various cooking processes and storage conditions. This study confirmed that the fat of standard eggs or pork from the Belgian market cannot be considered as a source ofn-3 PUFA.. Indeed, in this work, we measured no n-3 PUFA in meat or only around 2 % for eggs, while the fat of both enriched eggs and pork contained about 10 % of n-3 fatty acids. After storage or cooking without culinary fat no change of the fatty acid profile was observed, in standard eggs or pork. We made the same observation for n-3 enriched products, except for some cooking processes (pan-frying and oven cooking), which induced a slight but significant loss of n-3 fatty acids in n-3 enriched eggs or pork. The fairly stable behavior of the fatty acids after various cooking and storage experiments indicates that the fatty acid profile is not a good marker of lipid alteration. As a consequence, this work was oriented to the development of analytical tools to evaluate the presence of toxic aldehydes in food and feed as secondary oxidation products coming from polyunsaturated fatty acids.

Chapter 3 is dedicated to the study of the oxidative stability of linseed oil, an oil rich in n-3 fatty acids.

Section 3.1 describes the development of a liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) method to evaluate the aldehyde content in linseed oil. After optimisation of the extraction, derivation, detection and quantification steps, the method was validated according to guidelines from the European legislation as well as the recommendations of the AFNOR protocol NF V 03-110 (AFNOR, 2010). The

evaluated parameters included specificity/selectivity, recovery, precision, accuracy, uncertainty, limits of detection and quantification, determined using the concept of accuracy profiles. When the method was applied to first pressure linseed oil stored for several days at 60 °C according to the Schaal oven test, it was shown that 4-hydroxy-2E-hexenal (4-HHE) was the most produced aldehyde.

In **Section 3.2**, the different patterns of oxidation of linseed oil in real-time and accelerated aging assays were investigated, in order to verify the hypothesis commonly accepted for vegetal oil that one day aging at 60 °C is equivalent to one month real time aging at room temperature. Oxidation was evaluated with the peroxide value and *para*-anisidine value, as well as the content in conjugated dienes and aldehydes. All four indicators of oxidation showed very different kinetic behaviours at 20 and 60 °C, showing that the hypothesis (1 day at 60 °C = 1 month at 20 °C) is not valid for linseed oil.

Chapter 4 describes the adaptation and the validation of a LC-MS/MS method to determine aldehydes in animal feed samples. Indeed, as animal feed is often enriched with linseed to increase the human intake of n-3 fatty acids through the consumption of food from animal origin, the study of the oxidative stability of n-3 enriched animal feed is also of interest. To achieve that goal, the LC-MS/MS method previously developed for linseed oil was adapted to include more aldehydes and was validated using the same guidelines as in section 3.1.

Finally, **chapter 5** presents the discussion and conclusions about the developed methods and the results obtained as well as their comparison with literature. Future prospects to improve the evaluation of and the exposure to the markers of lipid alteration in food are proposed.

RÉSUMÉ

Il est actuellement bien connu que les acides gras polyinsaturés n-3 (oméga-3) ont des effets bénéfiques sur la santé mais peuvent être également rapidement oxydés en composés potentiellement toxiques. C'est pourquoi l'objectif principal de ce travail a consisté en l'étude de marqueurs d'altération des lipides dans les aliments.

Le **premier chapitre** de cette thèse brosse un aperçu général concernant les acides gras, leur oxydation et les différents produits générés suite à leur oxydation. De plus, les différentes techniques d'analyse utilisées pour étudier la stabilité à l'oxydation des acides gras polyinsaturés et leurs produits de dégradation dans les denrées alimentaires sont également décrites.

Dans le **chapitre 2,** la méthode par chromatographie en phase gazeuse couplée à la spectrométrie de masse (GC-MS) mise au point pour analyser les acides gras dans l'alimentation est décrite. La méthode a ensuite été appliquée aux échantillons d'œufs et de viande de porc (tous deux standards et enrichis en acides gras n-3) soumis à divers processus de cuisson et durées de stockage. Cette étude a confirmé que la graisse des œufs ou du porc standards vendus sur le marché Belge ne peut pas être considérée comme une source d'acides gras n-3 car, dans ce travail, aucun acide gras n-3 n',a été détecté dans le porc ou seulement 2 % dans les œufs, alors que la graisse des œufs et de la viande de porc enrichis en acides gras n-3 contiennent environ 10 % d'acides gras n-3. Suite au stockage ou à la cuisson sans matière grasse, aucun changement dans le profil en acides gras n'a été observé dans les œufs ou la viande de porc standards. De manière surprenante, la même observation a pu être faite pour les produits enrichis en acides gras n-3, sauf pour certains procédés de cuisson (à la poêle ou au four), qui ont induit une légère perte significative des acides gras n-3 dans les œufs et le porc enrichis en acides gras n-3, mais aucune diminution dramatique n'a été observée. Étant donné que les acides gras ont été trouvés relativement stables après diverses expériences de cuisson et de stockage, ce travail a été orienté vers le développement d'outils analytiques pour évaluer la présence d'aldéhydes dans l'alimentation humaine et animale comme produits d'oxydation secondaire provenant des acides gras polyinsaturés.

Le **chapitre 3** est consacré à l'étude de la stabilité à l'oxydation de l'huile de lin, une huile riche en acides gras n-3.

La **section 3.1** décrit le développement d'une méthode de chromatographie en phase liquide couplée à la spectrométrie de masse en tandem (LC-MS/MS) pour évaluer le

contenu en aldéhydes dans l'huile de lin. Après optimisation des étapes d'extraction, de dérivation, de détection et de quantification, la méthode a été validée selon les recommandations formulées par la législation européenne et des exigences du protocole issu de la norme AFNOR NF V 03-110 (AFNOR, 2010). Les paramètres évalués comprennent la spécificité/sélectivité, récupération, précision, exactitude, incertitude, limites de détection et de quantification, en utilisant le concept des profils d'exactitude. Lorsque la méthode a été appliquée à de l'huile de lin première pression stockée pendant plusieurs jours à 60 °C selon le « Schaal oven test », le 4-hydroxy-2E-hexenal (4-HHE) était l'aldéhyde le plus produit.

Dans la **section 3.2**, les différents comportements de l'huile de lin face à l'oxydation dans un essai de vieillissement accéléré et en temps réel ont aussi été examinés de manière à vérifier l'hypothèse communément acceptée pour les huiles végétales selon laquelle un vieillissement de un jour à 60 °C est équivalent à un vieillissement de un mois à température ambiante. L'oxydation a été évaluée par l'indice de peroxyde et la valeur de *para*-anisidine, ainsi que par le contenu en diènes conjugués et aldéhydes. Ces quatre indicateurs de l'oxydation ont montré des comportements cinétiques très différents à 20 et 60 °C, montrant que l'hypothèse de départ (1 jour à 60 °C = 1 mois à 20 °C) n'est pas valide pour l'huile de lin.

Le **chapitre 4** décrit l'adaptation et la validation de la méthode LC-MS/MS pour doser les aldéhydes présents dans des échantillons d'aliments pour animaux. En effet, les aliments pour animaux étant souvent enrichis en graines de lin de manière à augmenter notre apport alimentaire en acides gras n-3 par le biais de la consommation d'aliments d'origine animale, une étude de la stabilité à l'oxydation de ces aliments riches en acides gras n-3 est intéressante à envisager. Pour atteindre cet objectif, la méthode LC-MS/MS mise au point pour l'huile de lin a été adaptée de manière à doser plus d'aldéhydes et a été validée à l'aide des mêmes référentiels que dans la section 3.1.

Enfin, le **chapitre 5** présente la discussion, les conclusions concernant les méthodes mises au point ainsi que les résultats obtenus et leur comparaison avec la littérature. En outre, quelques perspectives d'avenir afin d'améliorer l'évaluation et l'exposition aux marqueurs d'altération des lipides dans les aliments sont proposées.

LIST OF ABBREVIATIONS

1PLO	First pressure linseed oil
2,4-Deca	2,4-Decadienal
2,4-Nona	2,4-Nonadienal
4-HHE	4-Hydroxy-2E-hexenal
4-HNE	4-Hydroxy-2E-nonenal
ALA/LNA	*Alpha*-linolenic acid (C18:3 n-3)
ARA	Arachidonic acid (C20:4 n-6)
ASE	Accelerated solvent extraction
AU	Absorbance unit
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BNZ	Benzaldehyde
CRM	Certified reference material
CRT	Crotonaldehyde
DHA	Docosahexaenoic acid (C22:6 n-3)
DNPH	2,4-Dinitrophenylhydrazine
DPA	Docosapentaenoic acid (C22:5 n-3)
EI	Electronic Ionization
EPA	Eicosapentaenoic acid (C20:5 n-3)
FA	Fatty acid
FAME	Fatty acid methyl ester
FFA	Free fatty acid
GC-MS	Gas chromatography coupled to mass spectrometry
HPLC	High performance liquid chromatography
HXL	Hexanal
IS	Internal standard
LA	Linoleic acid (C18:2 n-6)
LC-PUFA	Long chain polyunsaturated fatty acid
LC-MS/MS	Liquid chromatography coupled to tandem mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
LSM	Least square mean
MDA	Malondialdehyde
MeMDA	Methylmalondialdehyde
MRM	Multiple reaction monitoring
MUFA	Monounsaturated fatty acid
PA	*para*-anisidine
PUFA	Polyunsaturated fatty acid
PV	Peroxide value
QC	Quality control
QIT	Quadrupole Ion Trap
RLO	Refined linseed oil
SD	Standard deviation
SE	Standard error
SFA	Saturated fatty acid
SIM	Selected ion monitoring
SRM	Selected reaction monitoring
TBARS	Thiobarbituric acid reactive species
TEP	1,1,3,3-Tetraethoxypropan

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PREFACE AND OBJECTIVES OF THE THESIS

For decades, many researchers were interested in different nutritional habits of various populations in the world and in the impact of these diet habits on the human health. One of the findings of these studies was that populations of certain countries including Greenland and Canada had ten times less cardiovascular problems than Western populations for the same total fat contribution in the diet (Bjerregaard *et al.,* 1988).
Northern people having a higher consumption of n-3 fatty acids compared to other people in the world, it seems that the nature of the consumed fat is of great importance for a good health. What is more, a low n-6 to n-3 ratio has been associated with a low incidence of cardiovascular disease and other chronic diseases (Simopoulos, 2008).

For food industries, the fight against the dietary imbalance is not only an ethical duty. Indeed, it is also a vast economic issue and a source of innovation of food products with high added value. A certain part of the food industry has thus taken that opportunity to develop various food products containing high amounts of n-3 fatty acids (eggs, meat, margarine…) which are nowadays presented in the shelves of the supermarkets. These products possess nutritional properties that have a positive impact on the health and/or welfare of consumers. Following that, a huge enthusiasm for n-3 fatty acids was observed. This trend fulfils the consumer's interest for a healthier diet and, more specifically here, for the numerous nutritional and health recommendations promoting the consumption of products rich in n-3 fatty acids. Since then, many commercials advertise the health benefits of this type of fatty acids through their products. But, in a context of advertising escalation where the worst meets the best, the development of so called « functional » food has to be based on nutrition and health claims that must be scientifically validated (European Parliament and Council Regulation No 1924/2006, 2006).

The presence of at least one double bond makes the polyunsaturated fatty acids very sensitive to oxidation (Belitz *et al*., 2004). This oxidation can occur during storage and/or processing of food products in the presence of light, air, heat etc. What is worse, some of the degradation products generated from the oxidation of polyunsaturated fatty acids, mainly aldehydes, can be considered as cytotoxic and genotoxic for human or animal health by reacting with proteins and nucleic acids (Esterbauer *et al*., 1991; Vander Jagt *et al*., 1997; Reed, 2011).

Considering both the health interest for n-3 polyunsaturated fatty acids and the potential generation of toxic degradation products in the food matrices, there is a need to provide analytical techniques to evaluate the markers of lipid alteration in food containing

these polyunsaturated fatty acids. Degradation products are usually detected by fast and simple enzymatic or colorimetric methods (Papastergiadis *et al.*, 2012; Ulu, 2004; Pikul *et al.*, 1989). However, these methods lack specificity. As a result, more recently, chromatographic techniques have been developed.

The main objective of this work being the study of markers of lipids alteration in food, this thesis focuses on the oxidative stability of polyunsaturated fatty acids in food products naturally rich in n-3 fatty acids (linseed oil) or coming from animals with a diet supplemented with n-3 fatty acids (eggs or pork). The *first objective* of the thesis was to develop a method using gas chromatography coupled to mass spectrometry (GC-MS) to determine the fatty acid profile of different foodstuffs and then evaluate their content in n-3 fatty acids. The development of this analytical method and the results obtained concerning the effect of storage and cooking on fatty acids profile of omega-3 enriched eggs and pork marketed in Belgium are presented in **chapter 2**.

The *second objective* of this work was to develop and validate an analytical technique to measure toxic or potentially toxic aldehydes as secondary oxidation products of fatty acids in food (linseed oil) and feed. The development of the analytical method and its application to the study of the oxidative stability of linseed oil are presented in **chapter 3** while the method developed for feed is described in **chapter 4**.

CHAPTER 1.
INTRODUCTION

1.1. Fatty acids

1.1.1. Fatty acids nomenclature, health effects and sources

Fatty acids (FA) belong to the lipid family. Food lipids may be classified in three main categories: sterols, phospholipids and triglycerides. Fatty acids present in food are mainly present as triglycerides, the latter being constituted of a glycerol molecule bound by esterification to three fatty acid molecules. These fatty acids are organic molecules composed of an aliphatic and hydrophobic chain of variable length terminated by a carboxylic group (-COOH).

A. Fatty acid classification and sources

- **Saturated fatty acids**

Fatty acids are called saturated (SFA) when their aliphatic chain doesn't contain any carbon-carbon double bond but only simple bonds between carbon atoms leading to a rigid and straight structure. SFA have the general formula: $CH_3(CH_2)_nCOOH$.
The major contribution of SFA in the diet occurs through palmitic (C16:0) and stearic (C18:0) acids, mainly present in food as products of animal origin such as bacon, fatty cheese, butter or plant origin such as palm or coconut oils…

- **Unsaturated fatty acids**

When the carbon chain contains at least one double bond, fatty acids are called unsaturated: either monounsaturated (MUFA, one single double bond), or polyunsaturated (PUFA, at least two double bonds).
Unsaturated fatty acids can be distinguished by the length of their carbon chain, the number of double bonds and the position of the double bond(s) on the carbon chain but also the spatial structure of the double bond(s). Indeed, a double bond can be qualified of *cis* (or Z) if the two lateral chains or atoms bound to the two carbon atoms of the double bond are located on the same side of the double bond. The *trans* (or E) isomer has the two lateral chains or atoms bound to the two carbon atoms of the double bond located on opposite sides of the double bond (Nawar, 1996; Cuvelier *et al*., 2004).

Monounsaturated fatty acids have the general formula: $CH_3(CH_2)_xCH=CH(CH_2)_yCOOH$. They are mainly present in food as n-9 fatty acids, the most

abundant being oleic acid (C18:1 n-9). Some vegetable oils contain high amounts of MUFA such as hazelnut, olive, rapeseed or peanut oils with 78.0, 72.8, 60.5 and 56.2 g MUFA/100 g oil, respectively (Nubel, 2009). Besides, oleic acid can also be synthetized *in vivo* from the enzymatic dehydrogenation of stearic acid, a saturated fatty acid.

Typical chemical structures of mono- and poly-unsaturated fatty acids are presented in Figure 1, with stearic acid as SFA (C18:0), oleic acid (C18:1 n-9) as MUFA and linoleic (LA, C18:2 n-6) and *alpha*-linolenic acid (ALA or LNA, C18:3 n-3) as n-6 and n-3 PUFA, respectively.

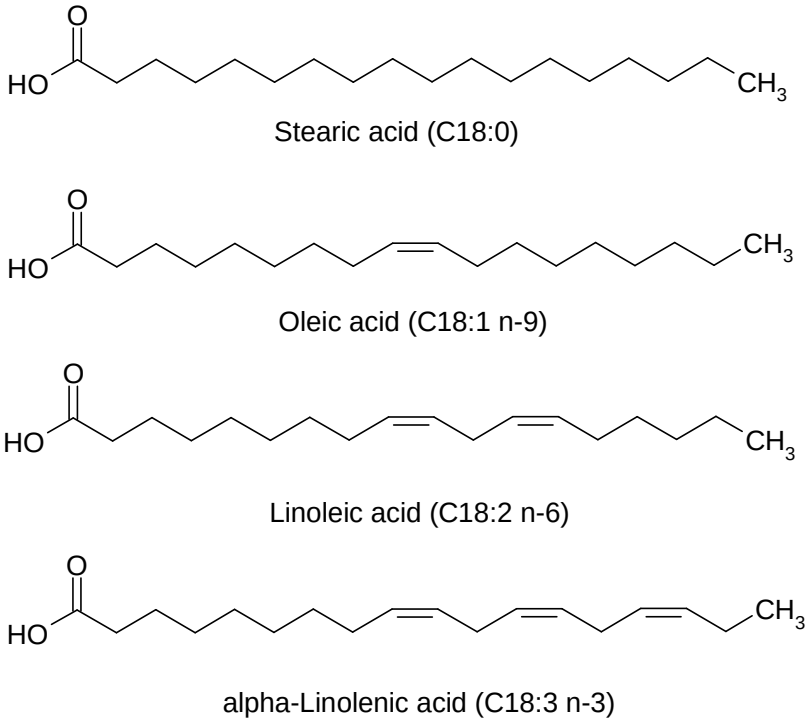

Figure 1: Chemical structures of SFA, MUFA, n-3 and n-6 PUFA.

Different types of **polyunsaturated** fatty acids can be found; they are discriminated by the position of the first double carbon-carbon bond starting from the terminal methyl group. Thus, PUFA from the n-3 (or omega-3) family and n-6 (or omega-6) are polyunsaturated fatty acids that have their first double bond on the third and the sixth carbon from the terminal methyl group, respectively. The most important n-3 and n-6 fatty acids from a nutritional point of view are the *alpha*-linolenic (ALA, C18:3 n-3) and linoleic acids (LA, C18:2 n-6). These PUFA are called “essential fatty acids” because the human body cannot synthetize them, so they must be brought by the diet. Table 1 presents the main dietary sources of the different families of fatty acids (adapted from Martin, 2001; AFSSA, 2003).

Table 1: Main food sources of the fatty acids (adapted from Martin, 2001; AFSSA, 2003).

Fatty acids	**Main food sources**
SFA	Bacon, cheese, butter
MUFA	Vegetable oils : olive, rapeseed, hazelnut, peanut
PUFA	
n-3	
ALA	Vegetable oils : linseed, walnut, rapeseed and soy
EPA, DHA	Marine animal products (fish oil), other animal products
n-6	
LA	Vegetable oils : sunflower, corn
ARA	Terrestrial animal products (meat, eggs, milk)
Trans fatty acids	Partially hydrogenated oils, margarine, dairy

SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; n-3=n-3 fatty acids; n-6= n-6 fatty acids; ALA= *alpha*-linolenic acid; EPA= eicosapentaenoic acid; DHA= docosahexaenoic acid; LA= linoleic acid; ARA= arachidonic acid.

The most frequent n-3 fatty acid found in food is ALA. It can be found in various vegetable oils, mainly in linseed, walnut, rapeseed and soy oils (AFSSA, 2003). Other n-3 PUFA that can also be found in high amounts in fish tissues and oil are eicosapentaenoic (EPA, C20:5 n-3) and docosahexaenoic acids (DHA, C22:6 n-3). These two compounds belong to the n-3 long chain polyunsaturated fatty acids (LC-PUFA) and can be synthetized from ALA in the human body, mainly in the liver, through different elongation and desaturation reactions. However, the conversion of ALA in n-3 long chain fatty acids is limited and depends on the n-6 fatty acid content. Consequently, EPA and DHA are referred to as "semi-essential", because a sufficient contribution from the diet remains nevertheless necessary to cover nutritional requirements. The consumption of fatty fish such as salmon, herring, mackerel and sardines, rich in EPA and DHA, is therefore recommended (Alexander, 1998; Sioen *et al*., 2008). EPA is also known to be a precursor of DHA and of group 3 eicosanoids (prostaglandins, thromboxanes and leukotrienes).

For the consumer, the intake of ALA and LC n-3 FA will depend on his level of consumption of the food sources mentioned above. In Belgium, according to the Superior Health Council, an adult is supposed to ingest each day 2.8 g (men) or 2.2 g (women) of ALA

and between 0.25 and 0.5 g of EPA+DHA (Belgian Superior Health Council, 2016). In a survey study in the Belgian population published in 2013, Sioen and co-workers showed that the intake of n-3 PUFA is lower than the recommended amount (Sioen *et al.*, 2013).

The most common n-6 fatty acid found in the food is LA. It is present in many plant-derived oils with a particularly high content in sunflower and corn oils (AFSSA, 2003). This fatty acid can also be found in food of plant origin such as borage, evening primrose and black currant seed oils. The elongase and desaturase enzymes in animals are only able to insert new double bonds between an existing double bond and the carboxyl group. LA, therefore, serves as the precursor of a family of fatty acids that is formed by desaturation and chain elongation, in which the terminal (n-6) structure is retained (Figure 2). Enzymatic action on LA leads then to the formation of *gamma*-linolenic acid (GLA, C18:3 n-6), which is the precursor of the n-6 dihomo-γ-linolenic acid (DGLA, C20:3 n-6). This one is the precursor of group 1 eicosanoids and of arachidonic acid (ARA, C20:4 n-6), the precursor of group 2 eicosanoids.

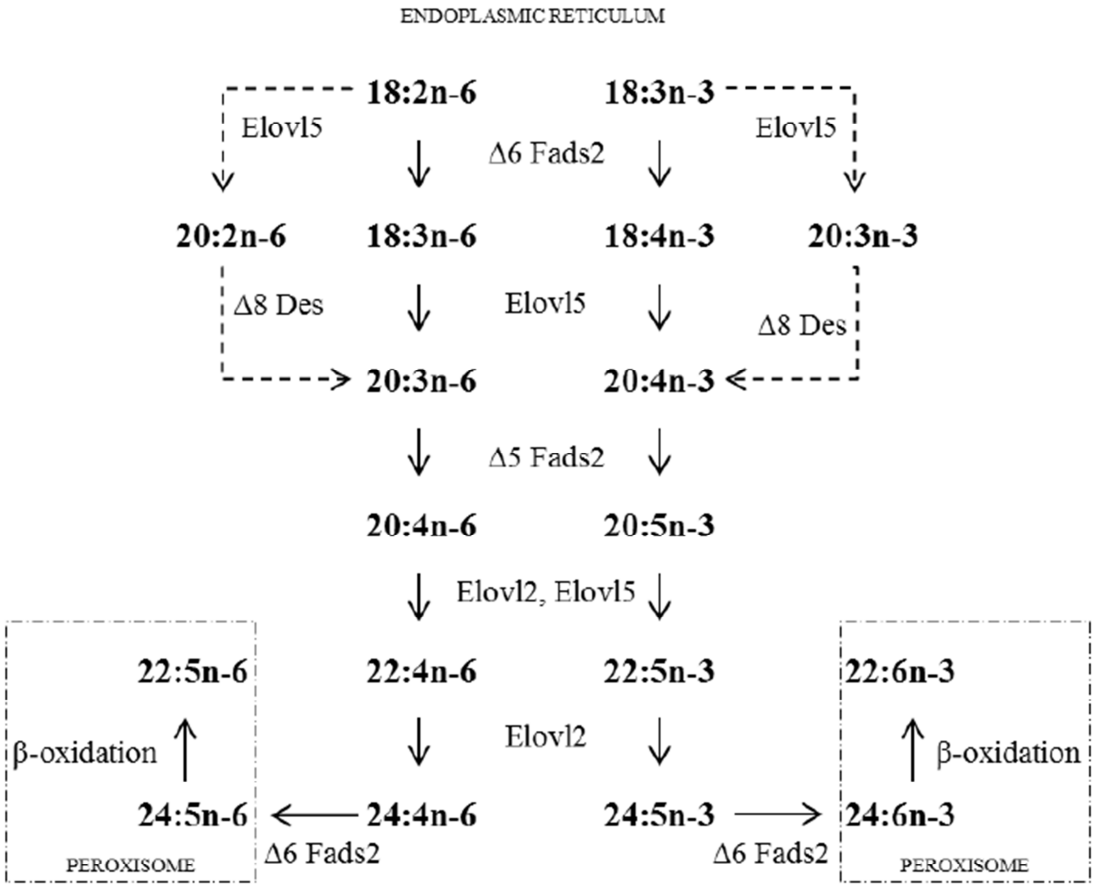

Figure 2: Biosynthesis of long-chain polyunsaturated fatty acids by chain-elongation and desaturation of linoleic and linolenic acids (Mellery, 2016).

B. Health effects of n-3 and n-6 fatty acids

There are numerous physiological properties related to the **n-3 fatty acids**. Various studies (Alexander, 1998; AFSSA, 2003; Sioen *et al*., 2009) have proved their role in protecting against cardiovascular diseases by the decreasing of blood pressure (hypotensor effect), LDL-cholesterol and blood triglycerides. What is more, they have anti-inflammatory and antiplatelet effects. They are involved in the regulation of the expression of many genes via the modulation of nuclear receptors and transcription factors.

In addition, n-3 PUFA contribute to the development of the retinal and cerebral nervous systems during foetal development and infants' breastfeeding, as well as during the growth of children. For the elderly, they act also as protection against dementia, Alzheimer's disease and psychiatric illnesses such as depression and schizophrenia.

As n-3 fatty acids can also be used as substrates for cyclooxygenase and lipoxygenase, they are involved in the formation of eicosanoids, leading to the reduction of inflammation (Alexander, 1998; Kohli *et al*., 2009), platelet aggregation, fibrinogen production and also the risk of thrombosis (Radack *et al*., 1989; Din *et al*., 2008). Oxygenated metabolites derived from EPA and DHA, the resolvins, (neuro)protectins and maresins, have potent anti-inflammatory and immunoregulatory actions (Duvall *et al.*, 2016; Weylandt, 2016).

According to WHO, n-3 PUFA play also a role in the immunological response and the protection of diseases such as type 2 diabetes, dyslipidemia and the metabolic syndrome (WHO, 2003; Poudyal *et al*., 2011). Finally, these fatty acids would contribute to the protection against some types of cancer (breast, colon and maybe prostate) (Rose *et al*., 1999). However, according to other studies, no direct link could be established between n-3 fatty acids and the incidence of human cancer (Kris-Etherton *et al*., 2003; Din *et al*., 2004; Deckelbaum *et al.*, 2006; Thissen, 2006; Ruxton *et al*., 2007).

Concerning **n-6 polyunsaturated fatty acids**, they are known to participate in the development and functioning of the nervous and immune systems, to modulate allergic and inflammatory reactions, to regulate the cardiovascular system and to modulate the body composition (particularly the lipoprotein lipase level). They could also have inhibitory effects on carcinogenesis *in vitro* and *in vivo* (Pariza *et al*., 2001; Bhattacharya *et al*., 2006).

C. The n-6 to n-3 ratio

Experts in nutrition recommend more and more to prefer, for their beneficial effects on health, a balanced and sufficient consumption of n-3 and n-6 polyunsaturated fatty acids

and to avoid saturated fatty acids. It is advised not to exceed a certain n-6 to n-3 ratio in order to limit the competition for key enzymes in the metabolism of polyunsaturated fatty acids, such as those leading to the synthesis of EPA and DHA from their ALA precursor. Thus, in 2001, the French Agency for Food, Environmental and Occupational Health & Safety (ANSES), formerly known as AFSSA, advised nutritional intakes in which the ideal n-6 to n-3 ratio was of 5 to 1 (AFSSA, 2003). In 2003, this recommendation was adapted by the WHO who proposed an ideal n-6 to n-3 ratio of 3 to 1 (WHO, 2003). At the moment, the n-6 to n-3 ratio is far too high in most of the European and North American populations. The same observation can be made in Belgium where the n-6 to n-3 ratio was estimated to be of 20 in 2009 (Belgian Superior Health Council, 2009). In 2016, Belgian Superior Health Council published new nutritional recommendations suggesting minimum intakes for n-6 and n-3 PUFA. For adults, the recommended minimum intake for PUFA per day should be of 5 to 10 % of the total energy needs, with the intake of n-6 fatty acids of 4 to 8% of the total energy needs, the intake of n-3 fatty of 1 to 2 % of the total energy needs and the intake of EPA and DHA of 250-500 mg (Belgian Superior Health Council, 2016).

Excessive amounts of n-6 PUFA and a very high n-6/n-3 ratio promote the pathogenesis of many diseases, including cardiovascular disease, cancer, inflammatory and autoimmune diseases, whereas increased levels of n-3 PUFA (a lower n-6/n-3 ratio), exert suppressive effects (Simopoulos, 2008; WHO, 2003).

1.1.2. n-3 fatty acid rich food

Increasing the amount of n-3 fatty acids in animal feed is a way to increase human intake of those compounds through the consumption of food from animal origin other than fatty fish, the major natural dietary source of long chain n-3 fatty acids.

Examples from literature have shown the possibility to obtain n-3 fatty acids enriched meat (Raes *et al*., 2003a, 2004a & 2004b; Wood *et al*., 2004), meat products (D'Arrigo *et al*., 2004), eggs (Cherian *et al.,* 2007a,b; Jiang *et al*., 1992) or dairy products (Oeffner *et al.,* 2013). In Belgium, some Belgian companies produce food enriched with n-3 fatty acids such as: meat, milk and dairy products, eggs… For example, Belovo (www.belovo.com) developed a patented animal feed (Columbus™ feed) containing 5 % of linseed oil (Remacle *et al*., 2001) suitable for hens that produce Columbus™ eggs with a ratio of n-6 and n-3 fatty acids of 1:1. The same strategy was used more recently to produce Columbus™ pork, which was commercialised by Marcel Biron et Fils SA or "Mieux pour tous" pork, which was commercialised by Delhaize supermarkets

Concerning animal feed, Dumoulin (www.dumoulin.eu) is providing an animal feed called Nutex that is containing more than 50 % of extruded linseed, bringing then a considerable quantity of n-3 fatty acids (Dang Van *et al*., 2008, 2011a & 2011b). Linseed can also be found on the market as linseed oil. Indeed, Vandeputte (www.vandeputte.com) is providing among other things raw, first pressure and refined linseed oils.

Dietary sources of n-3 PUFA is a combination between concentration in the food and food consumption. Indeed, some vegetable oils contain interesting amounts of n-3 PUFA but their consumption is so low that the daily intake is insufficient. These n-3 PUFA can then be brought in a more efficient way through food like eggs and meat.

Due to their high level of n-3 fatty acids, all those food or feed products are of great nutritional interest but are also of concern because of their very high sensitivity to oxidation. That is why they will all be further investigated in this work.

1.1.3. Fatty acids oxidation

Oxidation is a chemical reaction composed of the transfer of one or more electrons from a reducing compound (generally a metal) to an oxidant compound (generally a non-metal) and leading to the formation of free radicals (Arnaud, 1998).

The fatty acid oxidation processes are complex dynamic phenomena leading to the formation of a variety of primary and secondary oxidation products, also known as degradation products (Labuza *et al.,* 1969 & 1971; Frankel, 1980; Afaf *et al.,* 2005; Guichardant *et al*., 2006b; Juita *et al*., 2011 & 2012a).

The processes of lipid alteration can be classified in three categories according to the initiating agent:

- auto-oxidation catalysed by the temperature, metallic ions and free radicals
- photo-oxidation, initiated by light in the presence of photo-sensibilisators such as ketones, chlorophyll…
- enzymatic oxidation initiated by oxidation enzymes (lipoxygenases).

In food, most of the fatty acids are not free but are located in triglycerides. However, the level of oxidation could be influenced by the position of fatty acids on glycerol. Indeed, unsaturated acyl bound in position n-1 and n-3 would be more susceptible to oxidation than those in position n-2 (Belitz *et al*., 2004). In addition, oxidative degradation reactions are major phenomena for polyunsaturated fatty acids since the level of oxidation increases with the number of unsaturated bonds as well as with the position of the double bonds (Visioli *et al.*, 1998). Consequently, n-3 fatty acids, because of their structure, are more

susceptible to oxidation than n-6 fatty acids. Concerning saturated fatty acids, they are fairly stable and therefore undergo this phenomenon very lightly. They are oxidized at temperatures above 60 °C while the polyunsaturated fatty acids can be oxidized at temperatures below 0 °C (Wood, 1984).

Auto-oxidation, also called lipid peroxidation, is the main process found in dietary lipids. It is a radical reaction initiated by the loss of a hydrogen atom, usually on a carbon nearby a double bond (Belitz *et al*., 2004) giving a lipid hydroperoxide, which will lead to chain reactions. This set of reactions follows a growing exponential kinetics, which is decomposable in three stages: initiation, propagation and termination. Figure 3 shows the main steps of lipid peroxidation.

The initiation step is very slow and results in the formation of organic free radicals (R•), following the departure of some hydrogen radicals (H•) from the lipid chains near the double bonds of unsaturated fatty acids. This step requires the presence of reactive oxygen species (ROS) or the presence of initiator substrates such as metal cations (Fe^{2+}, Cu^{+} can catalyze decomposition of hydrogen peroxide via the Fenton reaction which yields hydroxyl radicals (Valko *et al*., 2016)), pro-oxidant components (haemoglobin, myoglobin, chlorophyll…) and extrinsic factors (light, energy input, temperature, irradiation...). The presence of pre-existing hydroperoxides also promotes oxidation reactions. Natural antioxidants, important constituents of fruits and vegetables, such as tocopherols, carotenoids, ascorbic acid, as well as synthetic antioxidants absorb free radicals and contribute to the decrease in lipid oxidation (Lee *et al*., 2006).

During the **propagation** step, the generated reactive species, very unstable, react rapidly with molecular oxygen to form peroxide free radicals (ROO•). These can react with the hydrogen atom present on lipid chains of PUFA (RH) to be transformed into various primary oxidation products such as organic hydroperoxides (ROOH) and other lipid radicals. Also very reactive and unstable, these compounds can be quickly degraded, especially in the presence of metal ions, and will form many complexes due to various chain reactions. Among the formed primary oxidation products, hydroperoxides are considered as the more present. The cleavage of their chain easily leads to the formation of by-products or secondary oxidation products, such as pentanal, hexanal, malondialdehyde and 4-hydroxy-2E-nonenal (4-HNE) (Raharjo *et al*., 1993).

Initiation :

$$RH + initiator \rightarrow R\bullet + H\bullet$$

Propagation :

$$R\bullet + O2 \rightarrow ROO\bullet$$
$$ROO\bullet + RH \rightarrow ROOH + R\bullet$$
$$RO\bullet + RH \rightarrow ROH + R\bullet$$
$$ROOH \rightarrow RO\bullet + \bullet OH$$
$$ROOH + ROOH \rightarrow ROO\bullet + RO\bullet + H2O$$

Termination :

$$ROO\bullet + ROO\bullet \rightarrow \text{stable products}$$
$$ROO\bullet + R\bullet \rightarrow \text{stable products}$$
$$R\bullet + R\bullet \rightarrow \text{stable products}$$

Figure 3: Unsaturated fatty acid (RH) auto-oxidation process (adapted from Belitz *et al.*, 2004). Radical species (•), organic free radicals (R•), hydrogen radical (H•), organic hydroperoxides (ROOH), alkoxyl radical (RO•).

In the **termination** step, reactive species finally combine to form non-reactive secondary oxidation products, volatile or not. Among these products, high molecular weight molecules are obtained by (co)polymerization of the free radical degradation products, while molecules of lower molecular mass (such as alcohols, ketones, aldehydes, hydrocarbons, furans...) are obtained by cleavage of the hydroperoxide chains. Some of these low molecular weight compounds, mainly volatile aldehydes, are responsible for food rancidity. In addition, some of these aldehydes, volatile or not, were identified as highly reactive compounds because they accumulate more than one functional group on the molecule (Figure 4). This is the case for alkanals (hexanal), hydroxyl alkenals (mainly 4-hydroxy-2E-nonenal), malondialdehyde (MDA) and acrolein (Salvayre *et al.*, 2002). Indeed, MDA, 4-hydroxyalkenals and acrolein are highly reactive with thiols and amines (Esterbauer *et al.*, 1991) and may induce derivatization of various proteins (Uchida, 1999).

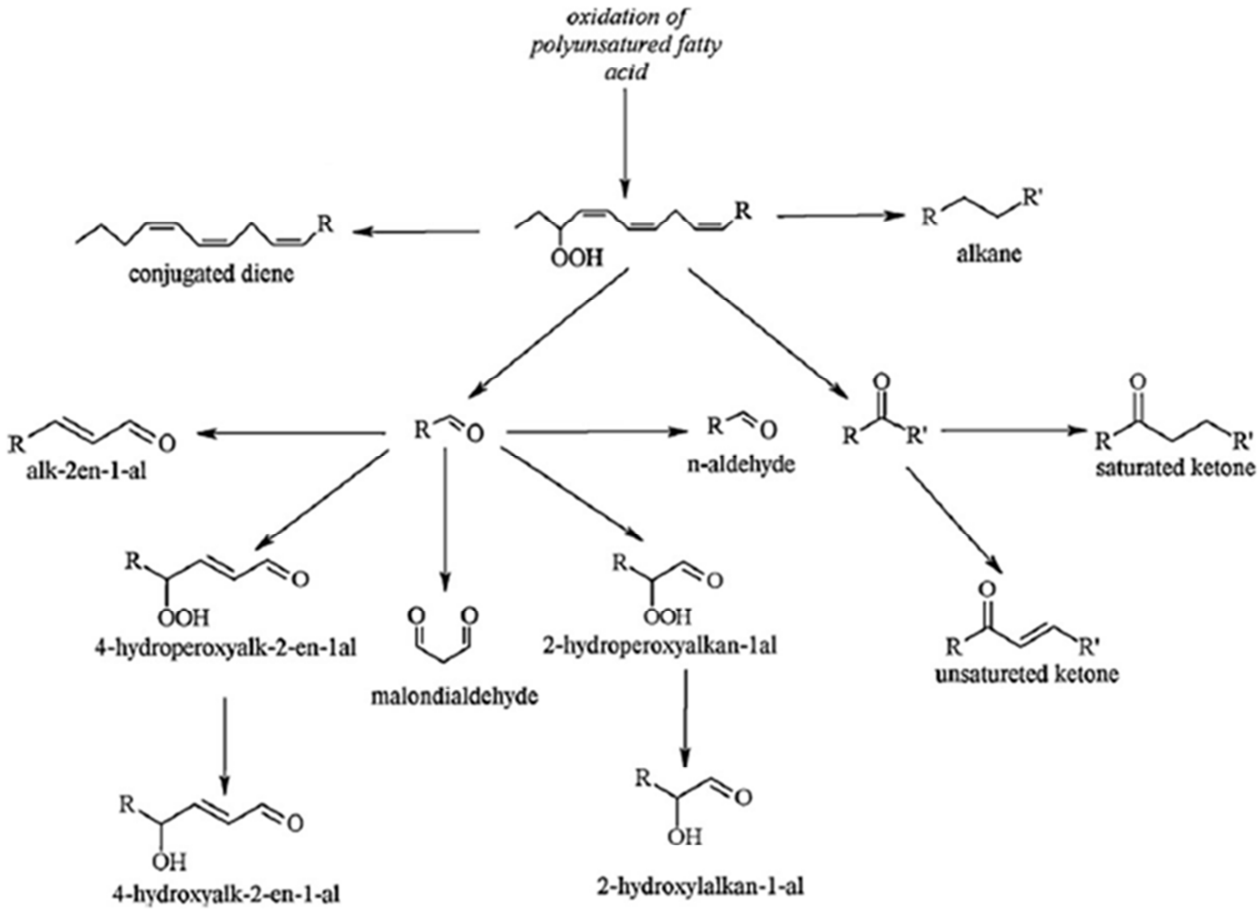

Figure 4: Formation of degradation products from polyunsaturated fatty acid oxidation (Adapted from Syslová *et al*., 2009).

Lipolysis, the hydrolysis of triglycerides into glycerol and free fatty acids, can occur in food following enzymatic action. In meat and meat products, muscle lipases and phospholipases appear to be responsible for lipolysis (Motilva *et al.,* 1993; Andres *et al*., 2005), while lipases from various sources, particularly the milk and cheese microflora, appear to be the main contributors of lipolysis in cheeses (Hickey *et al.,* 2007; McSweeney 2007). The lipoxygenase enzyme, also called LOX (linoleate:oxygen oxidoreductase), belongs to a large family of non-iron containing fatty acid dioxygenases) that occur widely in plants (cucumber, soybean, potato, sunflower) and animals (mammals) (Gunstone, 1996; Juita *et al*., 2012b). These enzymes are present naturally in linseeds and, in particular, the lipoxygenase enzyme catalyses the oxidation reaction of polyunsaturated fatty acids with cis,cis-1,4-dienes structure to form hydroperoxides (Gunstone, 1996).

Some authors claim that an increase in free fatty acid content following lipolysis leads to a higher susceptibility to lipid oxidation (Ansorena *et al*., 1998; Aubourg, 2001 & 2005).,

1.1.4. Fatty acid oxidation in omega-3 rich food

Fatty fish are typical and well known natural dietary source of n-3 fatty acids. These fatty fish, such as salmon, sardines, anchovies etc., containing long chain n-3 fatty acids, are generally cooked before consumption. It is thus interesting to look in the literature if some data are available about the stability of these LC n-3 PUFA. Pan-frying experiments of salmon showed that, when using culinary fats such as oils, margarines or butter, EPA and DHA diffuse from salmon meat into the culinary fat, resulting in decreased levels of these fatty acids in the cooked salmon (Gladyshev *et al*., 2006; Kitson *et al*., 2009; Sioen *et al*., 2006). About LC n-3 FA stability, Gladyshev and co-workers (2006) concluded that heat treatment in general did not decrease content of EPA and DHA in salmon, and proposed that this protection could be due to natural antioxidant present in salmon flesh.

1.2. Secondary oxidation products

Volatile or non-volatile compounds such as alcohols, ketones and aldehydes appear during the final step of lipoperoxidation as **secondary oxidation products**. Secondary oxidation products are indeed responsible for the degradation of the organoleptic properties of food due to rancidity, which is accompanied by alterations of the taste, development of unpleasant odours and flavours, changes of the aspect, the colour (browning of meat, yellowing of fats) or the texture of food (Belitz, 2004; Velasco *et al*., 2010). Losses in the nutritional quality (deterioration of essential fatty acids) plus alterations in the biochemical properties and reactions of chemical cross-linking with the proteins and DNA can also be observed with possible consequences of cytotoxic, mutagenic and carcinogenic effects (Belitz, 2004). It is therefore essential to monitor the storage (presence of oxygen, light, antioxidants...) and processing (cooking, frying, grilling...) conditions of food products given their significant influence on the level of lipid oxidation and the significant negative impact that this oxidation creates on the nutritional value of food as well as on food safety.

Because of their toxicity, aldehydes particularly caught the attention of many researchers. Aldehydes are compounds belonging to the carbonyl compounds family. They are characterized by the terminal position of the double bond C=O of the function (-CHO). The terminal position of this carbonyl function in aldehydes enables to distinguish them from ketones where this function is not terminal. Figure 5 shows the chemical structures of an aldehyde and a ketone.

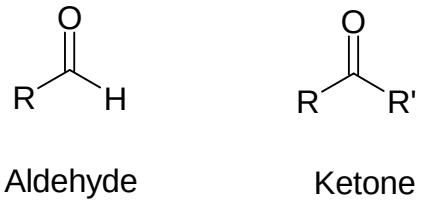

Figure 5: Chemical structures of an aldehyde and a ketone.

Among the bioactive and potentially toxic aldehydes generated by the oxidation of PUFA, four compounds were presented by the Belgian Superior Health Council as probably dangerous for human health. These aldehydes are: malondialdehyde, acrolein, crotonaldehyde and 4-hydroxy-2E-nonenal (4-HNE) (Belgian Superior Health Council, 2011). What is more, a study of the scientific literature was conducted during this work to determine the aldehydes predominantly formed from MUFA and PUFA contained in food. The results of this study are summarized in Table 2. Depending on their toxicological properties, their presence in water, food and/or environment or their relevance as fatty acid oxidation products according to the literature (Table 2), some other aldehydes like acetaldehyde and formaldehyde would be interesting to be followed. However, due to their high volatility at room temperature and their natural presence in the environment of the laboratory, it was decided to exclude them from this work because of their lack of specificity.

1.2.1. Chemical and toxicological properties of studied oxidation products

Even if the formation in food of malondialdehyde and other aldehydes due to lipid peroxidation is well documented, the role and importance of these aldehydes when absorbed through the diet is not extensively investigated to date apart from a few studies (Papastergiadis *et al*., 2014b). Some authors studied the bio-accessibility of unsaturated aldehydes *in vitro* and demonstrated that a certain proportion of these compounds remained unaltered after digestion (Goicoechea *et al.,* 2008). Thus it was suggested that they are bio-accessible in the gastrointestinal tract and so could reach the systemic circulation. In another investigation, it was demonstrated that trans-2-alkenal compounds known to be produced from the thermally induced autoxidation of PUFA are readily absorbed from the gut into the systemic circulation in vivo, metabolized and excreted in the urine as C-3 mercapturate conjugates in rats (Grootveld *et al.,* 1998). The chemical and toxicological properties of eight of the aldehydes found in Table 2 and that were

investigated further during the present work are detailed below. Their structure is presented in Figure 6.

Table 2: Synthesis of the main secondary oxidation products of monounsaturated and polyunsaturated fatty acids referenced in the literature.

FATTY ACIDS	SECONDARY OXIDATION PRODUCTS : ALDEHYDES AND KETONES	REFERENCES
Oleic acid (18:1 n-9)	- alkanals (methanal, **hexanal**, heptanal, octanal, nonanal, decanal) - alkenals (nonenal, 2-decenal, 2-undecenal) - alkadienals (2,4-heptadienal, **2,4-decadienal**)	Frankel, 1980 & 1985; Miyashita *et al.*, 1991; Belgian Superior Health Council, 2011.
Linoleic acid (LA, 18:2 n-6)	- alkanals (ethanal, methanal, propanal, pentanal**, hexanal**, heptanal, octanal) - 2-alkenals (2-propenal, **2-butenal**, 2-hexenal, 2-heptenal, 2-octenal, 2-nonenal, 4,5-epoxy-2-decenal) - alkadienals (2,4-heptadienal, **2,4-nonadienal, 2,4-decadienal**) - 2-hydroxyalkanals (2-hydroxyheptanal) - 4-hydroxy-2-alkenals (**4-hydroxy-2E-nonenal**, 4-hydroxy-2,6-nonadienal) - 5-oxodecanal, 4-oxo-2-nonenal - dialdehydes (ethanedial, **propanedial**, 2-butene-1,4-dial) - heterocyclic aldehyde (furfural)	Frankel, 1980 & 1985; Miyashita *et al.*, 1991; Tamura *et al.*, 1991; Miyake *et al.*, 1996; Pan *et al.*, 2002; Spiteller *et al.*, 2001; Kawai *et al.*, 2007; Long *et al.*, 2010; Juita *et al.*, 2012; Onyango, 2012.
Arachidonic acid (ARA, 20:4 n-6)	- alkanals (methanal, pentanal, **hexanal**) - 2-alkenals (2-heptenal, 2-octenal, 2-propenal, 4,5-epoxy-2-decenal) - 2,4-dialkadienals (4-hydroxy-2,6-nonadienal, **2,4-decadienal**) - 2-hydroxyalkanals (2-hydroxyheptanal, 2-hydroxy-4-decenal) - 4-hydroxy-2-alkenals (**4-hydroxy-2E-nonenal**, 4-hydroxy-2,6-dodecadienal) - 4-oxo-2-nonenal - dialdehydes (ethanedial, **propanedial**)	Frankel, 1980 & 1985; Esterbauer *et al.*, 1990; Tamura *et al.*, 1991; Mlakar *et al.*, 1996; Pan *et al.*, 2002; Kawai *et al.*, 2007; Long and Picklo, 2010; Onyango, 2012.
α-linolenic acid (ALA, 18:3 n-3)	- alkanals (methanal, ethanal, butanal, propanal, pentanal, **hexanal**, octanal) - 2-alkenals (2-propenal, **2-butenal**, 2-pentenal, 2-hexenal, 2-heptenal, 4-oxo-2-pentenal, 4-oxo-2-hexenal) - alkadienals (2,4-hexadienal, 2,4-heptadienal, **2,4-decadienal**) - 4-hydroxy-2-alkenals (4-hydroxy-2-butenal, 4-hydroxy-2-pentenal, **4-hydroxy-2E-hexenal**, 4-hydroxy-2,6-nonadienal) - dialdehydes (ethanedial, **propanedial**) - heterocyclic aldehyde - 2-hydroxyethanal	Frankel, 1980 & 1985; Miyashita *et al.*, 1991; Miyake *et al.*, 1996; Pan *et al.*, 2002; Kawai *et al.*, 2007; Juita *et al.*, 2012; Onyango, 2012.
Docosahexaenoic acid (DHA, 22:6 n-3)	- alkanals - 2-alkenals (2-pentenal, 2-octenal, 2-propenal, **2-butenal**) - 2,4-alkadienals (2,4-heptadienal) - 2-hydroxyalkanals (2-hydroxybutanal) - 4-hydroxy-2-alkenals (**4-hydroxy-2E-hexenal**, 4-hydroxy-2,6-hexadienal, 4-hydroxy-2,6-nonadienal, 4-hydroxy-2,6,9-dodecatrienal) - 4-oxo-2-hexenal - dialdehydes (ethanedial, **propanedial**)	Frankel, 1980; Miyake *et al.*, 1996; Kawai *et al.*, 2007; Long *et al.*, 2010; Onyango, 2012.
Eicosapentaenoic acid (EPA, 22:5 n-3)	- 2-alkenals (2-propenal, **2-butenal**, 2-pentanal) - 4-hydroxy-2-alkenals (4-hydroxy-2-butenal, **4-hydroxy-2E-hexenal**, 4-hydroxy-2,6-hexadienal) - dialdehydes (**propanedial**)	Miyake *et al.*, 1996; Pan *et al.*, 2002; Kawai *et al.*, 2007; Long *et al.*, 2010; Onyango, 2012.

Compounds indicated in bold are those studied in this thesis. Malondialdehyde (MDA) is also known as malonaldehyde or propanedial and crotonaldehyde as 2-butenal. 4-hydroxy-2E-nonenal is also known as 4-HNE and 4-hydroxy-2E-hexenal as 4-HHE.

A. Malondialdehyde ($C_3H_4O_2$, CAS 542-78-9, MW 72.063)

Malondialdehyde (MDA), also known as malonaldehyde or propanedial, is an organic compound with the chemical formula $CH_2(CHO)_2$. MDA is formed by β-scission of PUFA and exists under two mesomeric forms: an enolic major form HOHC=CH–CHO and a dialdehyde form OCH–CH_2–CHO (IARC, 1999). Malondialdehyde is produced and used in small quantities, principally for research purposes but it is also found in many foodstuffs (vegetable oils, fish, milk, fat...) and at high levels in rancid foods (United States National Library of Medicine, 1997). It is present as a lipid metabolite in human and animal tissues. In tissues, an excess of MDA can lead to the formation of adducts with protein amino groups (mainly lysine) altering their biological properties and turning them into potentially immunogenic compounds (Michel *et al*., 2008). MDA also reacts with DNA to produce adducts (Fang *et al*., 1996), including deoxyguanosine (M1G), deoxyadenosine (M1A), deoxycytidine (M1C) which are mutagenic (Marnett, 1999 & 2012). Malondialdehyde has been classified by the IARC as *Group 3*, meaning that the compound is not classifiable as to its carcinogenicity to humans due to a lack of data (IARC, 1999).

B. Crotonaldehyde (C_4H_6O, CAS 4170-30-3, MW 70.09)

Crotonaldehyde or 2-butenal is an α,β-unsaturated organic compound with the chemical formula CH_3CH=CHCHO. The compound can be present in 2 isomeric conformations: more than 95 % under *trans* (E) conformation and less than 5 % under *cis* (Z) conformation, the 2 different conformations differing according to the relative spatial position of the methyl (-CH_3) and formyl (-CH=O) groups on the double bound. Its production comes mainly from acetaldehyde aldol condensation, followed by dehydration in acidic medium as an acetanilide (IARC, 1995). Its main industrial uses are the manufacture of sorbic acid, a preservative, and trimethylhydroquinone, a vitamin E precursor. It is also used as slow release fertilizer or as alcohol denaturant. In addition, it is produced during the combustion of paper, cotton and plastics as well as in cigarette smoke (IARC, 1995). It is a powerful eye, respiratory and cutaneous irritant, listed as substance extremely dangerous, able to interfere with many immune functions (Chung *et al*., 1986; Belgian Superior Health Council, 2011). In addition, like acrolein, crotonaldehyde would be

able to form adducts with DNA that can cause mutations and loss of stability of the genome by interfering with its repair mechanisms (Stein *et al.*, 2006; Chung *et al.*, 1984). Crotonaldehyde would also be potentially carcinogenic. However, it has been classified by the IARC as *Group 3*, meaning that the compound is not classifiable as to its carcinogenicity to humans (IARC, 1995).

C. 4-Hydroxy-2E-Nonenal (4-HNE, $C_9H_{16}O_2$, CAS 29343-52-0, MW 156.22) and 4-Hydroxy-2E-Hexenal (4-HHE, $C_6H_{10}O_2$, CAS 160708-91-8, MW 114.1)

4-hydroxy-2E-nonenal (4-HNE) and 4-hydroxy-2E-hexenal (4-HHE) are α,β-unsaturated aldehydes mainly formed during n-6 (mainly ARA) and n-3 (mainly DHA) polyunsaturated fatty acid oxidation, respectively (Van Kuijk *et al.,* 1990; Surh *et al*., 2010). They have three reactive functions: an aldehyde function on carbon 1, a double bond between the 2nd and 3rd carbon and a hydroxylated function on carbon 4. These functions can explain their highly reactive properties, including with thiol and amino functions, giving it a very toxic activity. Studies have shown that they are able to react with proteins, lipids, DNA and other nucleophiles (Nair *et al*., 2007; Long *et al.,* 2010). Only few toxicological information are available for 4-HHE while it is known that 4-HNE would be involved in the development of many inflammatory and degenerative diseases affecting both digestive and central nervous systems (such as Alzheimer's and Parkinson's diseases) (McCracken *et al.*, 2000; Siegel *et al.,* 2007), but also cardiovascular (Mali *et al*., 2013), respiratory and urinary systems (Leonarduzzi, 2005). However, there is a small group of enzymes (such as glutathione S-transferase, aldose reductase, aldehyde dehydrogenase, cytochrome P450...) that are able to detoxify and eliminate 4-HNE from cells. But, these enzymes have a critical action threshold beyond which they cannot provide this function properly and then apoptosis is inevitable (Spickett, 2013). 4-HNE is also frequently used as a marker of oxidative stress (Zarkovic, 2003).

The LD50 of 4-HNE for mice has been reported as 0.44 mmol kg^{-1} body weight if given intra-peritoneally and of 4-HHE 0.98 mmol kg^{-1} of body weight (Esterbauer *et al.*, 1991). Interestingly, mice which orally received a 4-HNE-rich fraction of oxidized linoleic acid showed severe lymphocyte necrosis in the thymus 24 h later (Oarada *et al.*, 1988).

D. Benzaldehyde (C_7H_6O, CAS 100-52-7, MW 106.12)

Benzaldehyde is an organic compound consisting of a benzene ring with a formyl substituent. It is the simplest aromatic aldehyde and one of the most industrially useful. Benzaldehyde is used as a flavoring and fragrance in food, cosmetics, pharmaceuticals, and soap and is "generally regarded as safe" (GRAS) by the US Food and Drug Administration (Brühne *et al.,* 2011). This colorless liquid has a characteristic almond-like odor. Benzaldehyde is the primary component of bitter almond oil and can be extracted from a number of other natural sources. Synthetic benzaldehyde is the flavoring agent in imitation almond extract, which is used to flavor cakes and other baked goods.
Encephalopathy in rats and nephropathy in rats and mice were observed after subchronic oral exposure to benzaldehyde (Kluwe *et al*., 1983). Benzaldehyde has been classified as a hazardous substance by the United States Environmental Protection Agency (U.S. Environmental Protection Agency, 1988).

E. 2,4-nonadienal ($C_9H_{14}O$, CAS 5910-87-2, MW 138.1) and 2,4-decadienal ($C_{10}H_{16}O$, CAS 25152-84-5, MW 152.23)

2,4-decadienal and 2,4-nonadienal are aldehydes derived from polyunsaturated fatty acids oxidation. 2,4-decadienal is a substance also found in butter, cooked beef, fish, potato chips, roasted peanut, buckwheat and wheat bread crumb (Janeš *et al*., 2009; Vermeulen *et al.,* 2007). A study conducted by Chang *et al.* (2005) suggests a potential new role of 2,4-decadienal as a tumor promoter in human lung epithelial cells and another study conducted by Cabré and coworkers suggests that 2,4-decadinal may be the molecular cause of lipid oxidation cytotoxicity to human vascular smooth muscle cells (Cabré *et al*., 2003).

F. Hexanal ($C_6H_{12}O$, CAS 66-25-1, MW 100.16)

Hexanal is an aldehyde and a major component of air pollutants compounds (Svedberg *et al*., 2004). According to Cho and coworkers, it has adverse effects on human health and could be linked to diverse disease categories such as cancer, respiratory tract disease, and immune system disease (Cho *et al*., 2015 & 2016). However, a toxicological

literature survey showed that the available scientific information on hexanal is insufficient to determine the potential risks to health.

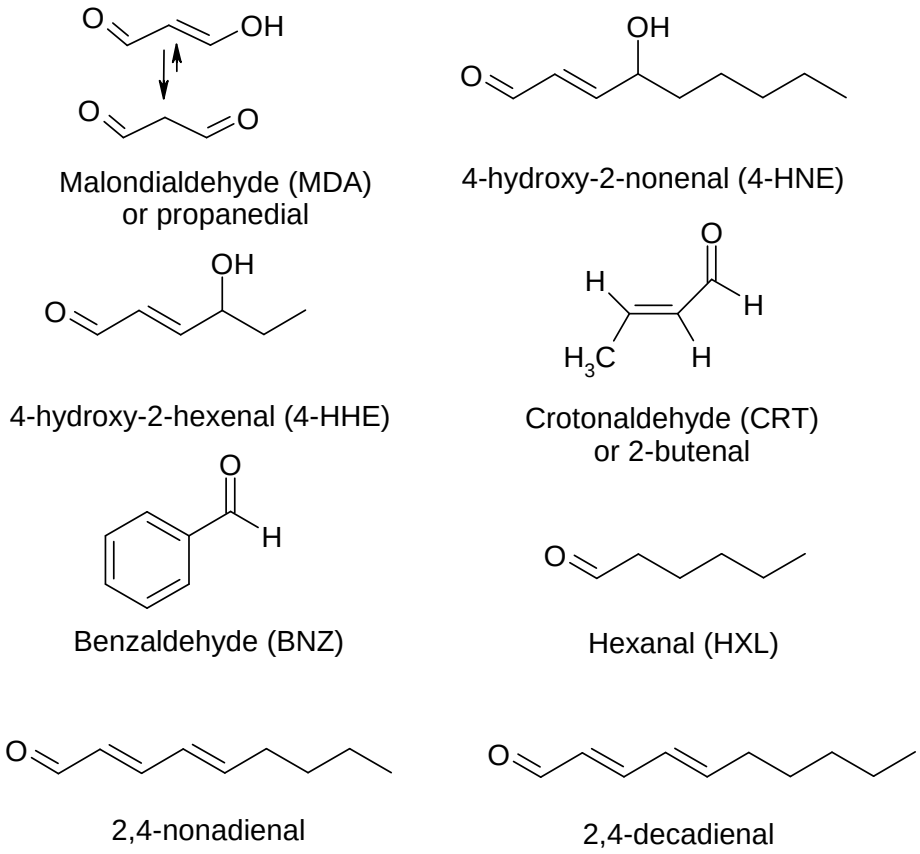

Figure 6: Structures of the 8 aldehydes analyzed: malondialdehyde (or propanedial), 4-hydroxy-2E-nonenal (4-HNE), 4-hydroxy-2E-hexenal (4-HHE), crotonaldehyde (or 2-butenal), benzaldehyde, hexanal, 2,4-nonadienal and 2,4-decadienal.

1.2.2. Further decomposition of aldehydes

Saturated aldehydes can easily oxidize to form the corresponding acids, and they can also participate in dimerization and condensation reactions. Unsaturated aldehydes can undergo classic autoxidation with oxygen attack at α-methylenic positions giving rise to short-chain hydrocarbons, aldehydes and dialdehydes like malondialdehyde (Nawar, 1996; Frankel, 2005). For oxidation of aldehydes with conjugated double bonds like 2,4-nonadienal and 2,4-decadienal, the proposed mechanism involves formation of epoxides by oxygen attack at the olefinic centers leading to a 2,3- or 4,5-epoxy derivative as intermediate. In the case of 2,4-decadienal, the decomposition of the 2,3-epoxide can lead to the formation of 2-octenal, 2-octene, glyoxal and acetaldehyde while the decomposition of the 4,5-epoxide leads to the formation of hexanal, 2-butenal, hexane and 2-butene-1,4-dial (Matthews *et al*., 1971).

Recently, the formation of non-substituted furan has been advanced from the oxidation of lipids. According to Locas and coworkers, furan comes from toxic 4-hydroxy-2-unsaturated aldehydes, themselves coming from polyunsaturated fatty acid oxidation (Locas *et al*., 2004).

1.2.3. Analysis of oxidation products

The evolution of the oxidation level of edible oil or fat can be followed with classic, titrimetric, colorimetric or spectrometric analytical methods. These methods give a global vision of the oxidation reactions of a chemical function and/or a family of molecules and are relatively simple, reliable, reproducible and quite sensitive. Among those techniques, there are the thiobarbituric acid reactive species (TBARs) method, the conjugated dienes dosage, the measure of the *para*-anisidine and the peroxide values. However, while quick and easy, these methods have the disadvantage of having a reduced scope that does not allow a complete and detailed characterization of the fatty acids oxidation.

Recently, advances in technology allowed the development of analytical methods to identify specific products of oxidation, especially secondary oxidation products. They include liquid or gas phase chromatography (HPLC or GC) coupled to mass spectrometry but also the electron spin resonance (ESR), infrared (IR) and nuclear magnetic resonance (NMR) spectroscopies (Shahidi *et al*., 2008).

In this section, a brief description of some of these techniques has been written.

A. Peroxide value

Primary oxidation products such as hydroperoxides can be evaluated by measuring the peroxide value (PV). The measurement of the peroxide value is well known and is generally performed using a titration method (AOAC, 1995). Briefly, a saturated potassium iodide solution is added to a solution of oil or fat diluted in acetic acid/chloroform, the titration is then realized with a $Na_2S_2O_3$ solution with starch solution as indicator. However, the peroxide value can also be estimated with a colorimetric method adapted from the ISO 3976 method used for anhydrous milk fat. That method consists in the oxidation of iron (II) in iron (III) by the peroxides followed by the spectrophotometric determination of the red iron (III) complex formed (International Organization for Standardization, 2006). The determination of the peroxide value is systematically

performed as a quality control to evaluate the quality of oils. This measure provides an overall estimation of the primary stage of their oxidation. This method can be applied to all PUFA, regardless of the number of unsaturation, but does not provide information on the nature of the hydroperoxides or their origin. The determination of the peroxide value is very easy in the case of oils but is more complicated in more complex matrices because it is necessary to previously extract lipids selectively and quantitatively while avoiding any oxidation (Villière *et al.,* 2006). The limit of this method lies in the fact that primary oxidation products being highly reactive and unstable, their quantification is difficult. Indeed, the rate of generated peroxides is the result of the formation and degradation of the hydroperoxides. However, the speed of formation and degradation does not change similarly depending on the temperature. Indeed, the formation of hydroperoxides is more important than their degradation up to 60-70 °C and then their degradation is more important than their formation beyond this temperature range (Frankel, 2005). That is why evaluation of secondary oxidation products, which are more stable, is preferred.

B. Conjugated dienes and trienes

Primary oxidation products containing conjugated double bonds can be measured by Ultra-Violet (UV) spectrometry (Corongiu *et al*., 1994). Indeed, following the displacement of double bonds by formation of the hydroperoxides, conjugated dienes and trienes are formed. Conjugated dienes absorb at 232-234 nm while conjugated trienes absorb at 268 nm (Dieffenbacher, 1992). These oxidation products can then be followed by measuring the absorbance of fat diluted in hexane at these wavelengths. The dosage of the conjugated dienes and trienes in oxidized oil is fast but requires the lipid extraction of the sample and their solubilization in an organic solvent. This measure is, moreover, poorly specific and can lead to interferences with antioxidants. However, the increase in the amount of conjugated dienes and trienes is proportional to the oxygen consumption and to the hydroperoxides formation (Casimir *et al*., 2008).

C. para-anisidine value

The *para*-anisidine value can also be measured to evaluate the rancidity of a fatty product by measuring the amount of aldehydes (mainly 2-alkenal and 2, 4-dienal) obtained from the degradation of the hydroperoxides in oxidized oil or fat. This value is defined in the AOCS method Cd 18-90 as the absorbance of a solution obtained when mixing 1 g oil or

fat in 100 mL isooctane with *para*-methoxyaniline (*para*-anisidine) (0.25 % in glacial acetic acid), leading to a yellow reaction product absorbing at 350 nm (American Oil Chemists' Society, 2011). The *para*-anisidine value is a reliable indicator of oxidative rancidity of oil and is well correlated with the amount of total volatile products (Van der Merwe *et al.,* 2003). In addition, a strong correlation has been observed between this value and the peroxide value (List *et al.,* 1977). However, this method gives a higher value with unsaturated aldehydes because the colored (yellow) complex formed with *para*-anisidine absorbs more strongly at 350 nm than with saturated aldehydes. What is more, this value is only comparable for the same type of oil as the initial value varies according to the sources, an oil rich in PUFA presenting a high value of *para*-anisidine value even if the oil is fresh (Guillén *et al*., 2002).

D. Thiobarbituric acid reactive species value

The TBARS method (thiobarbituric acid reactive species) is measuring the total aldehyde content of a sample (expressed as milliequivalents of malondialdehyde/kg of sample) (Janero, 1990; Fenaille *et al*., 2001). Two molecules of thiobarbituric acid (TBA) react with one molecule of malondialdehyde, produced during fatty acid oxidation, to form a pink TBA-MDA complex absorbing at 532-535 nm. The TBARS test has the disadvantage to overestimate the concentration of malondialdehyde because MDA, but also all aldehydes present in the sample, react with thiobarbituric acid and are evaluated simultaneously (Korchazhkina *et al*., 2003). Indeed, aldehydes such as 4-hydroxy-alkenal, 2,4-alkadienal and 2-alkenal react also with TBA to form a pink complex. The TBARS test remains the most used technique to measure lipid oxidation in matrices such as meat and oils (soybean, cotton, corn and sunflower) (Papastergiadis *et al*. 2012; Ulu, 2004; Pikul *et al.,* 1989). However, this technique has been criticized for its lack of speed, sensitivity and specificity (Guillén-Sans *et al.,* 1998; Casimir *et al.,* 2008). An example of the non-specificity of the TBARS assay was published by Ohkawa and coworkers where they observed that linoleic acid hydroperoxide obtained by a lipoxygenase system reacts with TBA (Ohkawa *et al*., 1978). Moreover, according to Papastergiadis and coworkers, in products such as dry nuts, pork sausages, cooked fish and Gouda cheese, an overestimation of MDA was observed, indicating that the TBARS test was unsuitable for accurate determination of MDA (Papastergiadis *et al.*, 2012).

Several technologies for the determination of free and total MDA, which are more sensitive and selective compared to the classic TBARS assay, have been developed during the last decade (Giera *et al*., 2012). Although the superior performance of all these methods has been proven, the TBARS assay is still widely applied, even though its unspecific nature is known today. The most likely reason for this might be its convenient utilization in particular for the analysis of large sample numbers.

E. Total oxidation value

The total oxidation value, also known as TOTOX value, can be useful because it reflects the total oxidation of oil by combining data from primary oxidation products (peroxide value) and data from secondary oxidation products (*para*-anisidine value). It is often calculated as 2 x peroxide value + the *para*-anisidine value. According to Wanasundara and coworkers, it can also be defined as 2 x PV + TBARs value when the *para*-anisidine value cannot be calculated (Wanasundara *et al.,* 1995). Nevertheless, the TOTOX value has no rigorous scientific basis because it combines two values with different units.

F. Chromatographic techniques

Chromatographic techniques of separation such as gas or liquid chromatography coupled to simple UV-based methodologies or mass spectrometry can be used to separate, identify unambiguously and quantify individually the aldehydes present in a sample (Deighton *et al*., 1997; Kölliker *et al*., 1998 & 2001; Cighetti *et al.,* 1999; Zwiener *et al.,* 2002; Giera *et al*., 2012; Papastergiadis *et al*., 2014a).

Concerning the analysis of aldehydes as degradation products of polyunsaturated fatty acids, a liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) method was developed in this work. The next section gives a short description of the analytical techniques developed and used in this work.

1.3. Chromatographic techniques coupled to mass spectrometry

1.3.1. Chromatographic techniques of separation

A. Gas chromatography

The gas chromatography (GC) is an analytical technique used to separate, in time, the different molecules present in a sample. A determined volume (generally 1 to 2 µL) of preextracted and purified sample is injected on the chromatographic column present in an oven. Then, when a temperature gradient is applied, the different components present are desorbed from the column more or less rapidly depending on their boiling point and their affinity for the stationary phase and are pushed with the carrier gas to the detector. The time needed to reach the detector (here, the mass spectrometer) is called the retention time.

B. Liquid chromatography

Liquid chromatography (LC) or high performance liquid chromatography (HPLC) is also an analytical technique used to separate the different components present in a mixture, using a liquid mobile phase. These components are first dissolved in a solvent, and then forced to flow through a chromatographic column under a high pressure. In the column, the mixture is resolved into its components. The interaction of the solute with the mobile and stationary phases can be manipulated through the choices of both the solvent and the stationary phase. As a result, HPLC acquires a high degree of versatility not found in other chromatographic systems and it has the ability to easily separate a wide variety of chemical mixtures. The time needed for each component of the mixture is eluted and goes from the column to the detector (here, the mass spectrometer) is also called the retention time and is determined by the molecular structure of the components.

There are two elution types: isocratic and gradient. In the first type, constant eluent composition is pumped through the column during the whole analysis. In gradient elution, eluent composition is progressively changed during the run.

In both separation techniques, the quality of the separation is mainly based on the nature and the dimensions (length, particle size and internal diameter) of the chromatographic column. Indeed, a long column will extend the analytical run time but will improve the separation of the components due to the number of theoretical plates.

Those separation techniques coupled to various detectors can be used to analyze fatty acids (Raes *et al*., 2001 & 2003b; Sioen *et al.,* 2006; Lee *et al.*, 2006) as well as aldehydes (Tsaknis, 1998; Cighetti *et al.,* 1999; Fullane *et al*., 2004; Wei *et al*., 2011; Papastergiadis *et al*., 2014a) or other degradation products like hydroperoxides (Yang *et al*., 1991; Yang, 1992; Hartvigsen *et al*., 2000) in food.

1.3.2. Mass spectrometry detection

Mass spectrometry (MS) is used for the determination of the structure of a compound by analysing its fragments as well as for the quantification of compounds within a sample (Hoffmann *et al*., 2001).

The mass spectrometer is composed of:

- **a source**, used for the production of positive or negative ions of the molecules coming from the chromatographic device.
- **an analyzer**, used for the separation of produced ions based on their mass-to-charge ratio (*m/z*). A plot of the relative intensity of each ion related to its *m/z* is obtained and called a mass spectrum.
- **a detector**, used for the counting of the ions.
- **a data recording system**, who usually is a computer with a specific software to associate the respective *m/z* and the retention time of the ion and to convert them into a chromatogram called Total Ion Current (TIC).

A. The source

Different types of MS sources exist, each type having specific characteristics. Atmospheric Pressure Chemical Ionization (APCI) and ElectroSpray Ionization (ESI) are used to produce ions when liquid chromatography is coupled to MS. Matrix Assisted Laser Desorption Ionization (MALDI) is used to analyze biomolecules (DNA, proteins, peptides and sugars) and large organic molecules (polymers…), which tend to be fragile and fragment when ionized by more conventional ionization methods. In GC-MS, usually, Electronic Ionization (EI) is used for ions production.

The EI source, formerly known as Electron Impact ionization, was created in 1918 by Dempster and improved by Bleakney and Nier (Nier *et al*., 2016). In this type of source, the electrons are produced by heating a wire filament and they are accelerated to 70 eV in the source block. The electrons collide with neutral molecules, transfer their kinetic energy and excite the neutrals above the ionization threshold. The excess of electronic energy is transformed into vibrational energy dissipated by fragmentation. The parent ion itself may be absent in the mass spectrum, if its fragmentation is high (Scholl, 2013).

In LC-MS, in this work, the change of ions from liquid phase to gas phase was done by the electrospray (ESI) source of the mass spectrometer. The transfer of ionic species

from solution into the gas phase by ESI involves three steps: (1) dispersal of a fine spray of charged droplets, followed by (2) solvent evaporation and (3) ion ejection from the highly charged droplets. Within an ESI source, a continuous stream of sample solution is passed through a stainless steel or quartz silica capillary tube, which is maintained at a high voltage (e.g. 2.5 - 6.0 kV). A mist of highly charged droplets (aerosol) with the same polarity as the capillary voltage is generated (Figure 7).

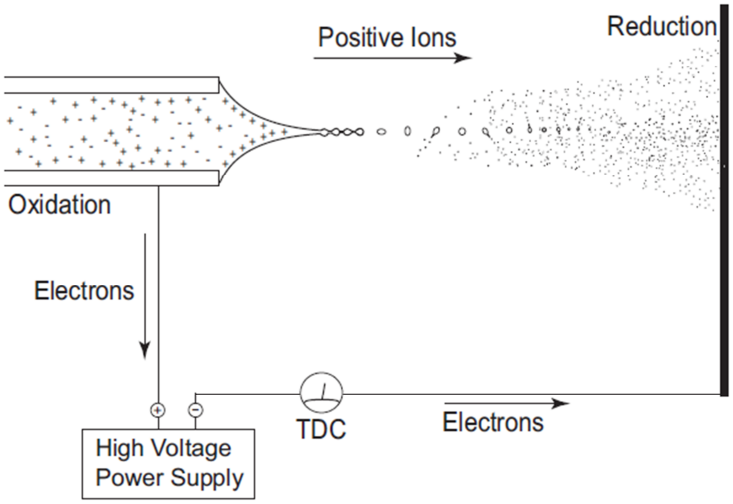

Figure 7: Illustration of major processes in the atmospheric pressure region of an ESI ion source run in the positive ion mode (Kebarle *et al.*, 2009).

The charged droplets, generated at the exit of the electrospray tip, pass down a pressure gradient and potential gradient toward the analyzer region of the mass spectrometer. With the aid of an elevated ESI-source temperature and/or another stream of nitrogen drying gas, the charged droplets are continuously reduced in size by evaporation of the solvent, leading to an increase of surface charge density and a decrease of the droplet radius. Finally, the electric field strength within the charged droplet reaches a critical point at which it is kinetically and energetically possible for ions at the surface of the droplets to be ejected into the gaseous phase. The emitted ions are sampled by a sampling skimmer cone and are then accelerated into the mass analyzer for subsequent analysis of molecular mass and measurement of ion intensity (Ho *et al.*, 2003; Kebarle *et al.*, 2009).

Because the ion formation involves extensive solvent evaporation (also called desolvation), the typical solvents for electrospray ionization are prepared by mixing water with volatile organic compounds (e.g. methanol or acetonitrile).

B. The analyzer

Like for sources, different types of analyzer exist: Quadrupole Ion Trap, Simple Quadrupole, Triple Quadrupole, Time of Flight (ToF), Sector. In this work, the detection part of the analytical methods developed for FAME or aldehydes was performed in both cases on ion trap mass spectrometers (ThermoFinnigan, Austin, Texas, USA). The ion trap mass spectrometer used for FAME analysis was a PolarisQ system while the one used for aldehydes analysis was a LCQ™Deca, both from Thermo Fisher Scientific (Waltham, Massachusetts, USA).

The Quadrupole Ion Trap (QIT), or Ion Trap, is constituted of three electrodes: one ring electrode, surrounded by two end-cap electrodes (Figure 8). A radio frequency voltage is applied on the ring electrode to trap ions produced by the source on a stable trajectory. "Stable" ions are ejected from the trap by an axial modulation mode consisting of applying an alternative tension at a specific frequency to the end-cap electrodes. Ions come into resonance and this is reflected by a rapid increase of the amplitude of their movements. Ions are resonantly ejected (process called Resonant ejection). The ejected ions hit a conversion dynode to be counted at the detector (Scholl, 2013).

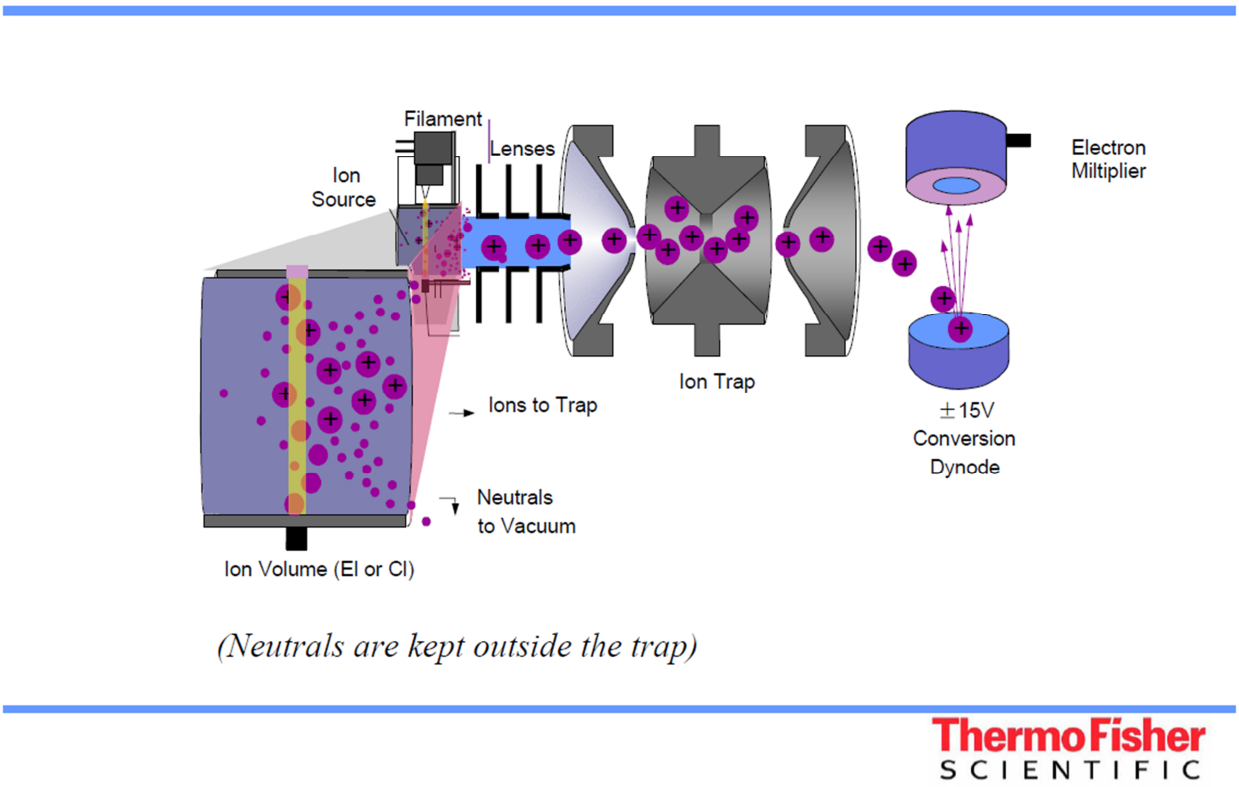

Figure 8: Internal structure of the PolarisQ Ion Trap mass spectrometer (ThermoFisher Scientific).

Whatever the ionization technique used, there are three different acquisition modes: the full scan, the selected ion monitoring (SIM) and the selected reaction monitoring (SRM). In the full scan mode, mass spectra are recorded repeatedly in a specified range of mass during a specified time. All ions in the specified range of mass reaching the detector in the specified range of time will be counted.

If the goal of the analysis is to detect a specific molecule with known spectral characteristics and with a maximal sensibility, the selected ion monitoring could be used. Thus, if specific fragments of the molecule are selected, the analyzer will jump from one mass to the other rather than scanning a range of mass. Therefore, no acquisition time is wasted by recording non relevant signals. It results in a better signal to noise for target compounds.

A third acquisition mode, the selected reaction monitoring (SRM), can be used to obtain higher sensibility and selectivity compared to the SIM mode. That mode is used in tandem mass spectrometry to follow fragments with a selected mass obtained from ions with a selected mass.

By configuring correctly the trap parameters, only ions of a selected mass can be kept in the trap. Over time, these ions (called parent ions) will be fragmented by collisions with helium in the trap, which will be containing all the fragments produced (called daughter ions). This technique is called Collision Induced Dissociation (CID). Then, the fragments can be selectively ejected and a spectrum of the fragment ions of a molecule with selected mass is generated. This is called tandem mass spectrometry or MS/MS in time instead of in space in coupled analyzers. This process can be repeated many times by selecting successive parent ions (MSn).

C. The detector

The detectors commonly used in mass spectrometry are photomultiplicator and electron multiplier. On the PolarisQ and LCQ™Deca systems, continuous electron multipliers were used.

The detection system includes a 15-kV conversion dynode and a channel electron multiplier. The conversion dynode is a concave metal surface that is located at a right angle to the ion beam. When an ion strikes the surface of the conversion dynode, one or more secondary particles are produced. These secondary particles can include positive ions,

negative ions, electrons and neutrals. They are focused by the curved surface of the conversion dynode and are accelerated by a voltage gradient into the electron multiplier. As seen on Figure 9, secondary particles from the conversion dynode strike the inner walls of the electron multiplier cathode with sufficient energy to eject electrons. The ejected electrons are accelerated farther into the cathode, drawn by the increasingly positive potential gradient. Due to the funnel shape of the cathode, the ejected electrons strike again the inner surface of the cathode, thereby causing the emission of more electrons. Thus, a cascade of electrons is created that finally results in a measurable current at the end of the cathode where the electrons are collected by the anode. The current that leaves the electron multiplier via the anode is converted to a voltage by the electrometer circuit and is recorded by the data system (ThermoQuest , 1999).

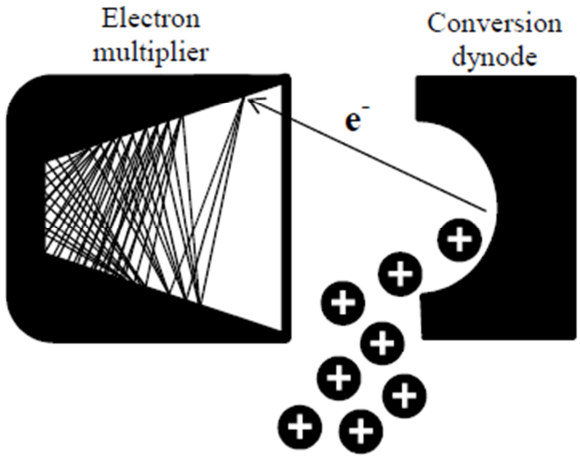

Figure 9: Scheme of an electron multiplier (Scholl, 2013).

D. Data recording system

The signal obtained from the detector is recorded by the data recording system who usually is a computer. Thanks to specific softwares installed on the computer, the signal intensity can be associated with its respective m/z and the GC retention time and this association can be converted into a chromatogram called Total Ion Current (TIC) (Scholl, 2013).

The LC-MS system used the Xcalibur 2.0.7 software while the GC-MS system used the Xcalibur 3.0 software.

In the literature, mass spectrometry was used as detector to measure 4-HNE and 4-HHE in food (Papastergiadis *et al*., 2014a), aldehydes in various foodstuffs (Wang *et al*.,

2014), MDA in human blood, human plasma and rat liver (Cighetti *et al*., 1999) or carbonyl compounds in ambient air (Brombacher *et al*., 2002).

1.3.3. Quantification

The method used for the quantification of the analyzed compounds is the **internal standard method**. It is based on the comparison of the intensities of the signal corresponding to the compound to be quantified with that of a reference called internal standard, introduced at an early stage of the analytical procedure. This method allows to get rid of different sources of errors other than the minimum intrinsic error due to statistical reasons. Indeed, if a molecule with chemical and physical properties as close as possible to the properties of the molecule to be dosed is chosen as internal standard, the molecule to be assayed and the internal standard will undergo the same loss during the extraction and derivatization steps or the same mistakes during the introduction of the sample in the mass spectrometer. As the two molecules undergo the same losses and mistakes, their ratio remains unchanged throughout the analytical procedure. As a consequence, standard recovery correction is not any more necessary.

First, the method involves performing measurements on synthetic samples containing a known and constant amount of internal standard and increasing amounts of the compound to be assayed. Using this result, a calibration curve is created. It allows obtaining a mathematical relationship between the ratio of the signals of the analyte and internal standard and the quantity of the substance present in the sample. In a second step, the measurements are performed on unknown samples to which a constant and known amount of internal standard is added before processing them according to the established experimental protocol. Based on the established calibration curve, the amount of substance to be assayed in each unknown sample can be calculated. The internal standard must have physicochemical properties the closest to the properties of the molecule to be assayed. It must be pure, must not be naturally present in the sample and must obviously be inert to the substances composing the sample.

When available, the best internal standard for the quantification is a labeled analogue of the target analyte. Labeling is done by substituting at least one of the analyte atoms by one of its stable uncommon isotope such as Deuterium (^{2}H or D) or ^{13}Carbon. The use of this stable isotope as internal standard is obviously limited to mass spectrometry

detection. It is a rather expensive approach but it definitely gives the best accuracy on results.

1.4. Method validation

When the development of an analytical method is over, most of the time a validation is performed to demonstrate the performances of the developed method.

As there is no permitted limit or specific validation criteria defined in the legislation for fatty acids or aldehydes in food matrices, the validations realized in the present work were based primarily on the performance criteria and validation protocols described in the Decision of the European Commission 2002/657/EC (European Parliament and Council Directive, 2002), a guideline used for the analysis of residues of veterinary drugs. That document presents requirements concerning chromatographic techniques and mass spectrometry that can be applied here and recommends the evaluation of the following criteria: specificity/selectivity, recovery, precision (intermediate precision/repeatability), accuracy and stability (Commission of the European Communities, 2002). Other parameters were also assessed according to the recommendations of the AFNOR protocol NF V 03-110 (AFNOR, 2010): the linearity of the results, the limit of detection (LOD), the limit of quantification (LOQ) and the accuracy profile.

For fatty acids, the treatment of the validation data has been made using Microsoft Office Excel 2007 while, for aldehydes, it has been made using the e-Noval 3.0a PROD software from Arlenda (Liège, Belgium) and Microsoft Office Excel 2007.

- **Performance criteria definitions**

All definitions are coming from the Decision of the European Commission 2002/657/EC (European Parliament and Council Directive, 2002), except when another reference is mentioned.

- **Specificity** means the ability of a method to distinguish between the analyte being measured and other substances. This characteristic is predominantly a function of the measuring technique described, but can vary according to class of compound or matrix.

- **Recovery** means the percentage of the true concentration of a substance recovered during the analytical procedure. It is determined during validation, if no certified reference material is available.

- **Precision** means the closeness of agreement between independent test results obtained under stipulated (predetermined) conditions. The measure of precision usually is expressed in terms of imprecision and computed as standard deviation of the test result. Less precision is determined by a larger standard deviation.
- **Intermediate precision** (also called within-laboratory reproducibility) means precision obtained in the same laboratory under stipulated (predetermined) conditions (concerning e.g. method, test materials, operators, environment) over justified long time intervals.
- **Repeatability** means precision under conditions where independent test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment.
- **Accuracy** means the closeness of agreement between a test result and the accepted reference value. It is determined by determining trueness and precision.
- **Trueness** means the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value. Trueness is usually expressed as bias.
- The **linearity** of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample (ICH, 2005).
- The **limit of detection (LOD)** of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value (ICH, 2005).
- The lower or upper **limit of quantification (LOQ)** of an individual analytical procedure is the lowest or highest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy (ICH, 2005).

CHAPTER 2. EFFECT OF STORAGE AND COOKING ON FATTY ACIDS PROFILE OF OMEGA-3 ENRICHED EGGS AND PORK MEAT MARKETED IN BELGIUM

Most of the time, fatty acids are analyzed with the use of gas chromatography coupled to flame ionisation detector (FID), electron-capture detector (ECD) or mass spectrometry (MS). Liquid chromatography coupled to UV detector, differential refractometer, evaporative light-scattering detector (ELSD) or mass spectrometry can also be used as analytical techniques (Christie, 2003). In this work, to determine the fatty acid profile of fat extracted from food with the required specificity and sensitivity, a gas chromatography coupled to mass spectrometry (GC-MS) method was developed as separation and detection technique.

Fatty acids had then to be derivated to be separated with gas chromatography. That is why a saponification/methylation step was realized on the fat extracted from food to free fatty acids from triglycerides and turn them into fatty acid methyl esters (FAME) (Figure 10). The saponification/methylation protocol that was optimized is described in Douny *et al*. (2015a).

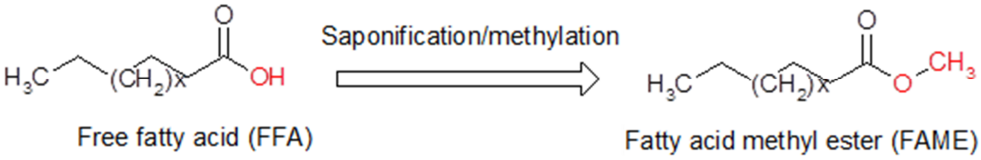

Figure 10: General saponification/methylation scheme of free fatty acids.

Figure 11 shows a chromatogram of 26 fatty acid methyl esters analyzed with the developed GC/MS method.

The developed method was then applied on samples coming from different studies concerning the storage and/or cooking of food containing n-3 fatty acids to determine their FA profile. The studies realized were concerning linseed oil, eggs and pork and were initiated during the Walnut-20 research project. For eggs and meat rich in n-3 fatty acids, the samples were systematically compared to standard eggs and pork. The results observed were published in Food Science & Nutrition (Douny *et al*., 2015a).

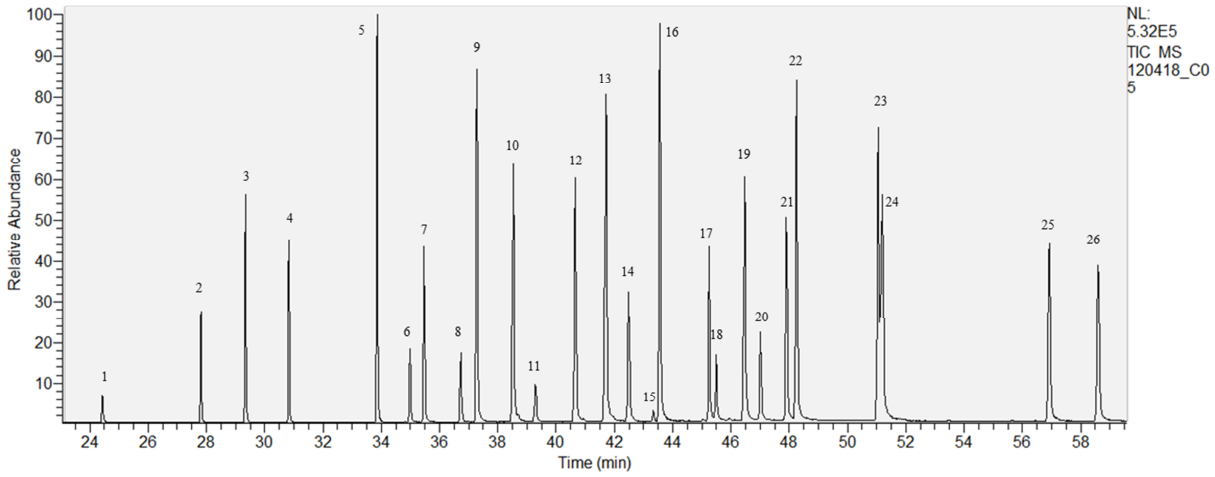

Figure 11: Chromatogram of fatty acid methyl esters obtained by GC/MS. Peaks of fatty acid methyl esters: 1, C10:0; 2, C12:0; 3, C13:0; 4, C14:0; 5, C16:0; 6, C16:1n-7; 7, C17:0; 8, C17:1n-7; 9, C18:0; 10, C18:1n-9; 11, C19:0 (Internal std); 12, C18:2n-6; 13, C20:0; 14, C13:3n-6; 15, C20:1n-9 (Injection std); 16, C18:3n-3; 17, C18:4n-3; 18, C20:2n-6; 19, C22:0; 20, C20:3n-6; 21, C20:3n-3; 22, C20:4n-6; 23, C24:0; 24, C20:5n-3; 25, C22:5n-3; 26, C22:6n-3.

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ORIGINAL RESEARCH

Effect of storage and cooking on the fatty acid profile of omega-3 enriched eggs and pork meat marketed in Belgium

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Abstract

The fatty acids (FA) profile was determined in n-3 enriched (Columbus™) Belgian eggs and pork in order to evaluate to what extent the n-3 fatty acids, which are very sensitive to oxidation, are resistant to storage or cooking. In standard eggs or pork, no change of the fatty acid profile was observed after storage or cooking without culinary fat, as well as in Columbus™ eggs and pork after storage. Some cooking processes (eggs in custard and meat in oven) induced a slight significant loss of n-3 fatty acids in Columbus™ eggs or pork (11.1% in fat from eggs cooked in custard vs. 15.3% in raw Columbus™ eggs and 11.0% in fat from oven cooked meat vs. 11.6% in raw Columbus™ meat). As expected, when Columbus™ pork is cooked with culinary fat, its fatty acid profile is modified according to the nature of the fat used.

Introduction

The essential C18 polyunsatuared fatty acids (PUFA) n-6 linoleic (LA) and n-3 α-linolenic (LNA) acids are precursors of long chain C20 and C22 highly unsaturated fatty acids, and compete, in this process of elongation, for the same Δ^6 desaturase enzymes. So, an excessive intake of n-6 compared to n-3 fatty acids could lead to a deficiency in n-3 eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are said semi-essential fatty acids. EPA and DHA are well known for their beneficial effects, in particular, to prevent different pathologies, mainly cardiovascular diseases (Delgado-Lista et al. 2012; Lovegrove and Griffin 2013). Nowadays, it is considered that the general western diet contains an excess of n-6, with a ratio between n-6 and n-3 FA around 10–15 (Simopoulos 2008). This imbalance is thought to contribute to the appearance of the "modern day" metabolic syndrome, including health problems such as cardiovascular diseases, type 2 diabetes, obesity, allergies, inflammations, cancers, stress etc. For several years, it was considered that for humans, the ideal ratio between fatty acids n-6 and n-3 in food was 1:1. A recent study realized for the American Heart Association (Harris et al. 2009) showed that n-6 fatty acids have no pro-inflammatory effects in humans. Furthermore, this study confirms the hypo-cholesterolemia potency of n-6 fatty acids and recommends abandoning the idea that n-3 and n-6 fatty acids display

opposite effects and to not use anymore the ratio n-6/n-3 to qualify the intake of these fatty acids. Moreover, this study recommends to increase the n-3 and to maintain the n-6 fatty acids dietary intake.

Increasing the amount of n-3 fatty acids in animal feed is a way to increase human intake of those compounds through the consumption of food from animal origin other than fatty fish, the major natural dietary source of long chain n-3 fatty acids. Examples from literature have shown the possibility to obtain n-3 fatty acids enriched meat (Raes et al. 2004; Wood et al. 2004), eggs (Jiang and Sim 1992; Cherian et al. 2007a,b) or dairy products (Oeffner et al. 2013). Subsequently, many products enriched with n-3 fatty acids can be found in the market: meat, milk and dairy products, eggs... For example, in Belgium, a Walloon company developed a patented animal feed (Columbus™ feed) containing 5% of linseed oil (Remacle et al. 2001) suitable for hens that produce Columbus™ eggs with a ratio of omega-6 and omega-3 fatty acids of 1:1. The same strategy was used more recently to produce Columbus™ pork.

Polyunsaturated fatty acids oxidation in food is generally favored by thermal processing as well as storage (Lopez-Bote et al. 1998; Nurnberg et al. 1999; Hayat et al. 2010). Eggs and pork are categories of foods that are eaten cooked, that is, after thermal processing. Consequently, due to the possible oxidation, the quantity of PUFA remaining in the food when it is eaten, that is, after storage and/or cooking, could be lower than the initial content in the raw product.

Our study investigated the impact of storage and cooking on the polyunsaturated fatty acids content of two products rich in omega-3 fatty acids marketed in Belgium: Columbus™ eggs and Columbus™ pork.

Materials and Methods

Chemical reagents and cooking ingredients

Free and methylated fatty acids standards were purchased from Sigma-Aldrich (St. Louis, MO). Hexane and toluene were of Picograde quality and provided by Promochem (Wesel, Germany). Methanol and water were of Chromanorm quality and provided by VWR International (West Chester, PA). Hydrochloric acid, 37%, was from Merck (Darmstadt, Germany).

Individual stock solutions of each fatty acid standard in hexane were used to prepare a pool of 23 FA standards, for the external calibration. Nonadecanoic acid (C19:0) was used as internal standard and gadoleic acid methyl ester (C20:1-ME) was used as injection standard.

The certified reference material (CRM) BCR-162R (made of soya-maize oil blend) was purchased at the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium).

Skimmed milk, sugar, flour, corn starch, palm oil, butter, margarine, sesame, and peanut oils were purchased in a local supermarket.

Fat extraction

Samples were weighed, homogenized (for raw whole eggs) or minced (meat and cooked eggs), and lyophilized for 48 h (Benchtop, Virtis; SP Industries, Warminster, PA). The dry matter was weighed and the water content was then calculated. Then, extraction of the total lipids was done using hexane at 125°C for 20 min in an accelered solvent extraction (ASE) system (ASE 200; Dionex, Sunnyvale, CA) and the fat residue was weighed.

Preparation of fatty acid methyl esters

Fifty milligrams of fat extracted with ASE were mixed with 5 mL hexane and 10 μL were used for the saponification/methylation of the fatty acids. Internal standard nonadecanoic acid (C19:0) was then added and hexane was evaporated to dryness under a stream of nitrogen. One milliliter toluene and 2 mL sulfuric acid 2% (v/v, in methanol) were added to the fat and the capped tube was heated in a water bath at 100 °C for 1 h, with vigorous agitation thanks to a magnetic stirrer. Then, 3 mL NaCl 5% were added and the methyl esters were extracted with two times 2 mL hexane. The extract was washed with 4 mL K_2CO_3 2% (w/v) and Na_2SO_4 was added to a part of the extract. The extract was then evaporated to dryness in a Savant™ Universal SpeedVac™ Vacuum System (Thermo Fisher Scientific, Waltham, MA) in order to eliminate the toluene. Three hundred and fifty-five microliters hexane was added and the tube was vortexed. Finally, 80 μL was transferred into an injection vial and 20 μL gadoleic acid methyl ester (C20:1-ME) was added to be used as the injection standard.

For the calibration curve, the same protocol was applied to hexane solutions containing a pool of 23 fatty acids, at six different concentration levels (from 0.06 to 16.68 ng μL^{-1}).

GC–MS separation, detection, and quantification of fatty acids

The method used to analyze fatty acid methyl esters (FAME) was adapted from Aldai et al. (2006). FAME were separated on a Focus GC gas chromatographer (Thermo Fisher Scientific) using a CP-Sil88 column for FAME (100 m × 0.25 mm, 0.2 μm) (Varian; Agilent Technologies, Santa Clara, CA) and analyzed with an ion trap PolarisQ mass spectrometer (Thermo Fisher

Scientific). The GC conditions were: inlet: 250°C; splitless injection; helium as the carrier gas at 1.5 mL min^{-1}; temperature program: 55°C for 1 min, followed by an increase of 5°C min^{-1} to 180°C, then 10°C min^{-1} to 200°C for 15 min, then an increase of 10°C min^{-1} to 225°C for 14 min; total run time was 59.50 min. Injection volume was 1 μL. The peaks were identified by comparing their mass spectrum and retention times with those of the corresponding standards. The MS conditions were: transfer line: 250°C; ion source: 220°C; collision energy: 35 eV; positive ionization mode. The FAME were detected using selected ion monitoring (SIM) mode in five segment windows. In each chromatographic run, different ions were monitored for each fatty acid analyzed, which allowed to perform detection and quantitative analysis: m/z 101 + 143 for saturated, 79 + 91 for mono and polyunsaturated fatty acids.

The 23 FAME, the internal standard, and the injection standard were separated in a run time of 1 h using the optimized GC–MS parameters described in Meat samples section.

For quantification, a 6-point calibration curve containing standard solutions and the internal standard was performed for each of the 23 fatty acids methyl esters determined. The response (ratio between fatty acids methyl esters and the internal standard peak areas) was plotted against standard concentrations. A linear regression was used and no "fit weighting" was applied.

Eggs

Columbus™ ("omega-3 rich") and standard eggs were bought in a local supermarket and were from barn raised hens (purchase date corresponding to 1 week after laying). For both types, all eggs were of medium size (53–63 g). A total of 252 eggs coming from four different batches were used for two studies: one batch for the study of the impact of the storage conditions and three batches for the study of the impact of cooking.

Egg cooking experiment

Eggs were cooked in a water bath (hard-boiled 4, 10, and 15 min), in a pan (scrambled and "au plat") and in an oven (in Savoy cake and in custard). For each cooking experiment, eggs were kept at room temperature 30 min before cooking. Temperatures in water bath, oven, and eggs were monitored during cooking procedures with temperature probes from Testo (Lenzkirch, Germany), with a measurement uncertainty of 0.5°C.

- Water-bath cooking: Water temperature was set at 100 °C. Hard-boiled eggs (4, 10, and 15 min) were cooled 5 min in ice to stop the cooking.
- Pan cooking: eggs were cooked (during 3 min in each case), scrambled (two eggs), and "au plat" (one egg) without oil, with the use of a polytetrafluoroethylene (PTFE) cooking foil.
- Custard (oven cooking): 250 mL skimmed milk was boiled and mixed with 50 g sugar. Then, two eggs were beaten and added to the milk. The mixture was cooked in the oven set at 180°C for 45 min in a water bath.
- Savoy cake: Three egg yolks were mixed with 50 g sugar then 40 g flour, and 30 g corn starch were added. Three egg whites were beaten firmly and 20 g sugar were added. The beaten whites were then folded into the egg yolk mixture; then the mixture was poured into a cake pan and baked for 30 min in the oven set at 160°C.

All eggs have been weighed before and after cooking. Each cooking experiment used 15 eggs of each type: one raw, three hard-boiled (1 for 4 min, 1 for 10 min, and 1 for 15 min), two pan cooked "au plat" (1 without fat and 1 with fat), four pan cooked scrambled (2 without fat and 2 with fat), two in custard and three in Savoy cake. Each experiment was repeated three times with eggs from different batches, leading to a total of 45 eggs of each type.

Egg storage experiment

Raw and hard-boiled (10 and 15 min) Columbus™ and standard eggs were stored 6 weeks in the dark at +4°C and +20°C. Each week, until the sixth week of storage, two eggs were sampled for the fatty acid profile determination. Each condition was in triplicate for day zero and in duplicate for the other storage times. A total of 81 eggs of each type were used for the storage experiment. All eggs from one type were from a single batch.

Egg control samples

For each type of eggs, a total of six raw eggs were analyzed to be used as control for both experiments: three raw eggs coming from the cooking experiment (three different batches) and three raw eggs coming from the storage experiment.

Meat samples

The meat was coming from the shoulder of one Columbus™ pig and one standard pig (both Piétrain), provided by the company Marcel Biron & Fils (Bouffioulx, Belgium). Samples of ground meat of ~70 g each, coming from two different batches, were used for cooking and storage experiment: one batch for the study of the impact

of the storage conditions and one batch for the study of the impact of cooking.

Pork cooking experiment

Samples of ground meat (~70 g) coming from one Columbus™ pig and one standard pig were used for each cooking procedures. A total of 18 samples of each type of meat were used for the cooking experiment.

Oven cooking experiment: the meat (four samples of each category of pig) was cooked in a glass beaker in the oven set at 180°C without culinary fat until the core temperature reached 80°C (between 20 and 22 min). During the oven cooking process, the core temperature was monitored continuously with thermic probes (TC type T; Testo).

Pan-frying experiment: the meat (two samples) was cooked in the pan without fat or with four different culinary fats: butter, margarine, sesame, and peanut oils. The amount of culinary fat used was around 5% of the weight of the raw meat. Each condition was realized in duplicate and pan frying took 20 min, during which the meat was cooked 15 min on one side and 5 min on the other side.

Raw pork storage experiment

Different conditions of storage were investigated for raw ground meat samples: vacuum-packed, plastic-bag-packed, and polypropylene tray with plastic wrap. Samples stored in plastic-bag and polypropylene tray with plastic wrap were kept at +4°C and at −20°C, while samples stored under vacuum were kept at +4°C. All samples were stored in the dark and analyzed in duplicate after a determined number of weeks, depending on the package type and temperature: 1 and 2 weeks for plastic bag and plastic tray at +4°C; 2, 4, 6, 8 weeks for plastic bag and plastic tray at −20°C and 3, 4, 6 weeks for under vacuum at +4°C. A total of 33 samples of each type of meat were used for the storage experiment.

Meat control samples

For each category of pigs (Columbus™ or standard), a total of seven meat samples were analyzed to be used as control for both experiments: four samples coming from the cooking experiment and three samples coming from the storage experiment.

Statistical analysis

Statistical Analysis System (SAS Institute, Cary, NC) was used for statistical analysis. Significant differences between treatments were tested using the analysis of the variance (two-way ANOVA) and generalized linear models (GLM) procedure of the SAS software. Post hoc analyses were used to compare conditions and levels of significant effects were compared using least-square means and associated standard error (significant for $P < 0.05$). The least-square mean corresponds to the mean corrected for all other effects in the model.

Results and Discussion

Choice of the extraction technique

To extract lipids from the samples, ASE was used, with hexane at high pressure and at a temperature above its boiling point. To our knowledge, no data were found in the literature about the use of ASE using hexane as extraction solvent. Indeed, most of the methods developed with this technique use either chloroform/methanol (2:1, v/v) or isopropanol/hexane (2:3, v/v) to extract lipids from biological matrices such as food (Ruiz-Rodriguez et al. 2010). Nevertheless, many authors propose to extract lipids from food using another technique called microwave Soxhlet extraction, with hexane as extraction solvent (Priego-Capote et al. 2004, 2007; Virot et al. 2007, 2008). The results obtained in this study with ASE using hexane were in good agreement with those obtained with microwave Soxhlet extraction using hexane for major fatty acids identification in eggs or pork.

Performances of the GC–MS analytical method

The developed method to measure fatty acids fulfills the criteria indicated in the Commission Decision 2002/657/EC (European Parliament and Council Directive No 2002/657/EC 2002/657/EC 2002/657/EC, 2002) which provides guidelines to evaluate the performance of the screening and confirmatory methods used for organic residues and contaminants analysis: retention time and ions ratios, selectivity, specificity, repeatability, reproducibility (data not shown).

A good separation was achieved for the peaks of all compounds except for the methyl esters of C24:0 and C20:5n-3 (EPA). Nevertheless, C24:0 being a saturated fatty acid and C20:5n-3 being a polyunsaturated fatty acid, the problem was solved by quantifying them with different m/z ratios such as 101 + 143 for C24:0 and 79 + 91 for C20:5n-3 (EPA). For calibration curves, the linear regression provided a good curve fitting, that is, with low residue values and correlation coefficients R^2 associated with those curves higher than 0.98 for the 23 fatty acids, and demonstrated the linearity of the

dose–response curve within the working range (data not shown).

The limit of quantification (LOQ) was fixed as the content of fatty acids corresponding to the first point of the calibration curve (after checking that the signal to noise ratio was higher than 10 at that level), and corresponds to about 0.1–0.5% of total fatty acids, depending on the standard. The limit of detection (LOD) was set at LOQ/2, after checking that the signal to noise ratio was higher than 3 at that level.

A CRM was used to assess the performance of the developed method. The CRM BCR-162R (soya-maize oil blend) contains assigned percentage of 10.74% of palmitic acid, 2.82% of stearic acid, 25.4% of oleic acid, 54.13% of linoleic acid, and 3.35% of α-linolenic acid. The measured values (n = 39) were, respectively, 105.9%, 104.1%, 107.9%, 99.9%, and 103.0% of the certified content (data not shown).

Eggs

Characterization of raw eggs

The average water content and fat contents were, respectively, 75.7 ± 1.5% and 8.8 ± 1.1% for Columbus™ eggs and 76.6 ± 0.9% and 7.5 ± 0.6% for standard eggs (n = 6), expressed on fresh weight basis. In both standard and Columbus™ whole raw eggs, the three major fatty acids are, in decreasing order, oleic acid (45.8 vs. 40.7%), palmitic acid (23.7 vs. 19.1%), and linoleic acid (16.3 vs. 14.3%) (Table 1). As expected, Columbus™ eggs contained a much higher proportion of n-3 LNA fatty acid (12.7%) than standard eggs (1.1%), and consequently, more PUFA (30.2% in Columbus™ eggs vs. 20.2% in standard eggs) and less SFA and MUFA (26.3% and 43.5%, respectively, in Columbus™ eggs vs. 31.1% and 20.2%, respectively, in standard eggs). Interestingly, the DHA (C22:6, n-3) content (1.4% in Columbus™ eggs vs. 0.7% in standard eggs) is also increased in eggs from hens fed with linseed oil containing diet, while the ARA (C20:4, n-6) content is decreased (0.6% in Columbus™ eggs vs. of 1.4% in standard eggs). The n-6/n-3 ratio was found equal to 7.5 for standard eggs and to 1.0 for Columbus™ eggs, which is a value that meets nutritional recommendations of reducing this value below 5 (AFSSA - Agence Française de Sécurité Sanitaire des Aliments 2003).

The proportion of 12.7% of α-linolenic (LNA) acid in the Columbus™ eggs appeared to be higher than the values (between 3.4% and 10.7%) reported in the literature for eggs from hens fed with diet containing linseed products (Meynier et al. 2014; Galobart et al., 2001; Baucells et al. 2000; Halle and Schöne 2013; Ferrier et al. 1995; Ren et al. 2013; Botsoglou et al. 2012a,b,c). The same authors reported for the same eggs a DHA content varying from 1.5% to 2.3% versus 1.4% in Columbus eggs, while a proportion of 3.2% or 5.8% of DHA can be reached when hens are fed with fish oil containing feed (reported in, respectively, Baucells et al. 2000 and Botsoglou et al. 2012a). Another author reported 4.5% of LNA and 2.1% of DHA in commercial omega-3 eggs in Australia (Samman et al. 2009).

Columbus™ eggs can be considered as "high omega-3 fatty acids" products, according to the Regulation (EC) no. 1924/2006 (European Parliament and Council Directive No 1924/200620062006, 2006), as they contain

Table 1. Fatty acid composition (g/100 g) of standard and n-3 enriched (Columbus™) raw eggs.

	Fatty acid content	
	Columbus™ egg (n = 6)	Standard egg (n = 6)
10:0	ND	ND
12:0	ND	ND
13:0	ND	ND
14:0	0.5 ± 0.1	0.5 ± 0.1
16:0	**19.1 ± 0.4**	**24.2 ± 1.3**
17:0	ND	ND
18:0	6.9 ± 0.2	6.6 ± 0.5
20:0	ND	ND
22:0	ND	ND
24:0	ND	ND
16:1 (n-7)	2.7 ± 0.3	2.9 ± 0.7
17:1 (n-7)	ND	ND
18:1 (n-9)	**40.7 ± 1.8**	**43.9 ± 4.1**
18:2 (n-6) (LA)	14.2 ± 0.8	17.9 ± 4.7
20:2 (n-6)	ND	ND
18:3 (n-3) (LNA)	**12.7 ± 2.0**	**1.2 ± 0.4**
18:3 (n-6)	ND	ND
20:3 (n-3)	ND	ND
18:4 (n-3)	ND	ND
20:4 (n-6) (ARA)	**0.6 ± 0.1**	**1.4 ± 0.1**
20:5 (n-3) (EPA)	**0.6 ± 0.1**	**0.3 ± 0.04**
22:5 (n-3) (DPA)	**0.6 ± 0.1**	**0.3 ± 0.1**
22:6 (n-3) (DHA)	**1.4 ± 0.1**	**0.8 ± 0.2**
Σ SFA	**26.5 ± 0.7**	**31.3 ± 1.4**
Σ MUFA	**43.4 ± 1.7**	**46.8 ± 4.5**
Σ PUFA	**30.1 ± 2.0**	**21.9 ± 5.3**
n-6 fatty acids	14.8 ± 0.8	19.3 ± 4.8
n-3 fatty acids	**15.3 ± 2.0**	**2.6 ± 0.6**
n-6/n-3	**1.0 ± 0.2**	**7.5 ± 0.8**

Mean ± standard deviation (SD) of six eggs from four different batches: three eggs coming from the storage experiment (one single batch) and three eggs coming from the cooking experiment (three different batches). Significant differences comparing Columbus™ and standard eggs are indicated in bold ($P < 0.05$). SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; ND, not detected.

more than 0.6 g LNA per 100 g (based on 7.5% fat containing 12% LNA, they contain about 0.9 g of LNA per 100 g).

Egg cooking experiment

Evolution of the core temperature during egg cooking

The evolution of the core temperatures during each cooking experiment was monitored with Testo probes. Temperature values are expressed as mean ± standard deviation of three independent cooking experiments. In the different cooking procedures where the temperature was monitored, the maximum core temperatures recorded at the end of the cooking time in the standard eggs were 118.3 ± 4.3°C, 102.6 ± 15.6°C, 49.3 ± 2.0°C, 71.9 ± 5.2°C, and 80.5 ± 7.0°C for Savoy cake, custard, and hard-boiled eggs cooked 4, 10, and 15 min, respectively. In Columbus™ eggs, the maximum core temperatures recorded were 97.4 ± 4.3°C, 88.7 ± 2.1°C, 37.4 ± 4.5°C, 65.5 ± 5.8°C, and 77.4 ± 4.9°C for Savoy cake, custard, and hard-boiled eggs cooked 4, 10, and 15 min, respectively. Surprisingly, the core temperatures recorded for Columbus™ eggs were significantly lower than for standard eggs in the Savoy cake during the last 10 min of cooking.

Fatty acid profile of eggs before and after cooking

Table 2 shows the results obtained for the sum of SFA, MUFA, PUFA, n-3, and n-6 fatty acids, and the LNA (C18:3 n-3), DHA (C22:6 n-3), and ARA (C20:4 n-6) proportion, in raw and cooked eggs. The n-6/n-3 ratio has been calculated as well.

Results are expressed in percent of total identified fatty acids, as least-square means and standard errors of six eggs from four different batches for raw and hard-boiled eggs and three eggs from three different batches for each other cooking condition. The statistical analysis applied to the data corrected the heterogeneity observed between batches.

The fatty acids composition of the standard eggs was not significantly influenced by any cooking procedure. Indeed, the SFA, MUFA, and PUFA, as well as the n-3 and n-6 fatty acids contents showed no significant difference between raw and cooked eggs. The LNA, DHA, and ARA contents were neither influenced (Table 2). Murcia et al. (1999) studied the effect of cooking on the fatty acid profile of standard eggs (containing 36.5% of C18:1, 29.2% of C16:0, and 26.2% of C18:2). Concerning the PUFA, they reported a decrease of C18:2 (LA), C18:3 (LNA), and C20:4 (ARA) in scrambled standard eggs (omelette) and microwaved eggs compared to raw eggs. For boiled standard eggs, they observed an increase of LA and ARA after boiling 3 min

Table 2. Effect of cooking on the fatty acid composition (g/100 g) of standard and Columbus™ eggs.

		Σ SFA		Σ MUFA		Σ PUFA		n-3		n-6		C18:3(n-3) LNA		C22:6 (n-3) DHA		C20:4 (n-6) ARA		n-6/n-3	
Egg type	Condition	LSM	SE	LSM	SE	LSM	SE	LSM	SE	LSM	SE	LSM	SE	LSM	SE	LSM	SE	LSM	SE
Standard	Raw	31.3	0.8	46.8	2.2	21.9	2.9	2.6	0.3	19.3	2.6	1.2	0.2	0.8	0.1	1.4	0.1	7.5	0.8
	HB 4 min	31.1	0.8	47.4	2.2	21.5	2.9	2.4	0.3	19.1	2.6	1.2	0.2	0.7	0.1	1.3	0.1	8.2	0.8
	HB 10 min	32.6	0.8	44.5	2.2	22.8	2.9	2.6	0.3	20.3	2.6	1.2	0.2	0.8	0.1	1.6	0.1	7.9	0.8
	HB 15 min	31.8	0.8	44.9	2.2	23.4	2.9	2.6	0.3	20.8	2.6	1.2	0.2	0.8	0.1	1.5	0.1	8.2	0.8
	"Au plat"	31.7	0.9	46.8	2.5	21.5	3.3	2.4	0.4	19.1	3.0	1.1	0.2	0.7	0.1	1.5	0.1	8.2	0.9
	Scrambled	32.2	0.9	45.4	2.5	22.3	3.3	2.4	0.4	19.9	3.0	1.2	0.2	0.8	0.1	1.5	0.1	8.3	0.9
	Custard	32.6	1.1	46.0	3.1	21.4	4.1	2.9	0.5	18.5	3.7	1.3	0.2	0.6	0.2	1.1	0.1	6.3	1.1
	Savoy cake	32.1	0.9	45.3	2.5	22.6	3.3	2.5	0.4	20.1	3.0	1.3	0.2	0.7	0.1	1.3	0.1	8.2	0.9
Columbus™	Raw	26.5	1.0	43.4	1.2	30.1	1.8	15.3	1.5	14.8	0.5	12.7	1.6	1.4	0.1	0.6	0.1	1.0	0.1
	HB 4 min	25.5	1.0	42.2	1.2	32.4	1.8	16.9	1.5	15.5	0.5	14.3	1.6	1.5	0.1	0.7	0.1	0.9	0.1
	HB 10 min	27.2	1.0	43.3	1.2	29.5	1.8	14.8	1.5	14.7	0.5	12.4	1.6	1.3	0.1	0.6	0.1	1.0	0.1
	HB 15 min	28.2	1.0	43.1	1.2	28.7	1.8	13.7	1.5	15.0	0.5	11.2	1.6	1.4	0.1	0.7	0.1	1.1	0.1
	"Au plat"	27.0	1.1	41.4	1.4	31.7	2.0	16.2	1.7	15.5	0.5	13.6	1.9	1.4	0.1	0.6	0.1	1.0	0.1
	Scrambled	28.0	1.1	42.2	1.4	29.8	2.0	14.6	1.7	15.3	0.5	12.0	1.9	1.5	0.1	0.6	0.1	1.1	0.1
	Custard	**31.7***	1.1	42.6	1.4	**25.8***	2.0	**11.1***	1.7	14.6	0.5	**8.8***	1.9	**0.9***	0.1	0.5	0.1	**1.4***	0.1
	Savoy cake	**28.9***	1.1	42.4	1.4	28.7	2.0	13.6	1.7	15.1	0.5	11.3	1.9	1.3	0.1	0.5	0.1	1.1	0.1

Least-square means (LSM) and Standard errors (SE) of six eggs from four different batches for raw and hard-boiled eggs, two eggs from two different batches for standard custard, and three eggs from three different batches for each other cooking condition. Significant differences comparing to the raw SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; n-3, n-3 fatty acids; n-6, n-6 fatty acids; LNA, α-linolenic acid; DHA, docosahexaenoic acid; ARA, arachidonic acid; HB, hard-boiled eggs condition are indicated by asterisks (*) and in bold ($P < 0.05$).

and a decrease of LA and ARA after boiling 10 min, while LNA was constant in both boiling conditions. No information concerning the statistical significance of the observed results was provided.

For Columbus™ eggs, hard-boiling, scrambling, or "au plat" cooking of had no significant effect on PUFA and n-3 fatty acids contents, compared to raw eggs, while significant effects of the cooking were observed in custard and Savoy cake (Table 2). In custard, the percentage of LNA and DHA in the total fatty acids decreased to 8.8% and 0.9%, respectively (it was 12.7% and 1.4%, respectively, in raw eggs), with a subsequent decrease of the percentage of PUFA (25.8%) and an increase of the percentage of SFA (31.7%) (in raw eggs, PUFA and SFA were 30.1% and 26.5%, respectively). These changes resulted in an increase of 40% of the n-6/n-3 ratio (1.4 instead of 1.0 in raw eggs). In Savoy cake, the only significant difference, compared to raw eggs, was recorded for the SFA content, but to a lesser extent than in custard (28.9% and 31.7% in Savoy cake and custard, respectively, vs. 26.5% in raw eggs). This increase of SFA in Savoy cake probably comes from the slight, apparently not significant, decrease of n-3 content (15.3% in raw eggs vs. 13.6% in Savoy cake). The decrease in PUFA observed in Savoy cake and custard prepared with ColumbusTM eggs can be explained by the temperatures reached in the core of the egg preparation (97.4 ± 4.3°C and 88.7 ± 2.1°C, respectively), which are the highest temperatures recorded among the different cooking experiments (see Evolution of the core temperature during egg cooking). The fact that such a decrease is not observed in standard eggs can be explained by the 10 times higher content of ColumbusTM eggs in LNA, a polyunsaturated fatty acid displaying three double bonds, thus much more sensitive to oxidation that the two double bonds LA contributing the most to the PUFA content of standard eggs.

Other studies reported that boiling or scrambling omega-3 enriched eggs, slightly decreased the C18:3 (LNA) and C22:6 (DHA) content (LNA: 7.41% and 7.28% in boiled and scrambled eggs, respectively, vs. 7.84% in raw eggs; DHA: 1.10% and 1.02% in boiled and scrambled eggs, respectively, vs. 1.62% in raw eggs) (Botsoglou et al. 2012c), while Cortinas et al. (2003) showed a significant decrease for DHA (and not LNA) in eggs coming from hens fed with fish oil containing feed, after scrambling only (and not boiling). Van Elswyk et al. (1992) reported that cooking (boiling or scrambling) did not alter the fatty acid composition of omega-3 enriched eggs.

Egg storage experiment

Eggs were stored raw, hard-boiled 10 and 15 min, at +4°C and +20°C, during 6 weeks. As no significant difference of the LNA, ARA, and DHA content was observed between the +4°C and +20°C storage conditions, the results from the two different storage temperatures were pooled leading to four repetitions for storage conditions from 1 to 6 weeks. The LNA, ARA, and DHA content in Columbus™ and standard eggs after storage from 0 to 6 weeks are shown in Figure 1. After 6 weeks storage, the LNA, ARA, and DHA content measured in Columbus eggs as well as standards eggs showed no significant tendency for a decrease in cooked (hard-boiled) or raw eggs.

Our results are corroborated by Meluzzi et al. (2000) who reported that n-3 rich eggs stored for 28 days at room temperature showed a fatty acids composition similar to that observed in fresh eggs or by Yang et al. (2004) who reported that conjugated linoleic acid (CLA) was stable in eggs during storage for a period of 6 months at 0–4°C. Similarly, Ahn et al. (1999) did not observe the effect of storage on the fatty acid composition when fresh eggs were stored for 49 days at 4°C. Marshall et al. (1994) noticed that storage stability of shell eggs from hens fed 1.5% dietary menhaden oil, a commercial fish oil containing approximately 30% of n-3 fatty acids, is comparable to that from hens fed a no-added fat diet.

On the contrary, a reduction in total n-3 fatty acids of eggs from hens fed with fish oil or olive leaves after 60 days of storage at +4°C was reported (Cherian et al. 2007a,b; Botsoglou et al. 2012c).

As suggested in different studies, the global stability of n-3 fatty acids observed in our experiments of storage and cooking could be explained by the protective effect of α- tocopherol. Indeed, α-tocopherol is naturally present in raw eggs (Murcia et al. 1999) and is also brought by specific hens feed. According to the inventors of the Columbus feed, the α-tocopherol content is around 100 mg kg^{-1} egg in Columbus™ eggs and around 10 mg kg^{-1} egg in standard eggs (Remacle et al. 2001).

Pork

Characterization of raw meat from standard and Columbus ™ pork

The average water content and fat contents were, respectively, 65.8 ± 0.6% and 15.7 ± 0.8% for Columbus™ pork and 67.2 ± 2.7% and 13.9 ± 3.6% for standard pork (n = 7), expressed on fresh weight basis. The fatty acid profile of standard and Columbus™ raw meat used as reference in the cooking experiment are shown in Table 3. The major difference in the fatty acids composition was coming from the content in n-3 fatty acids, which was of nearly 12% of the total fatty acids in Columbus™ pork, while they were not detectable in stan-

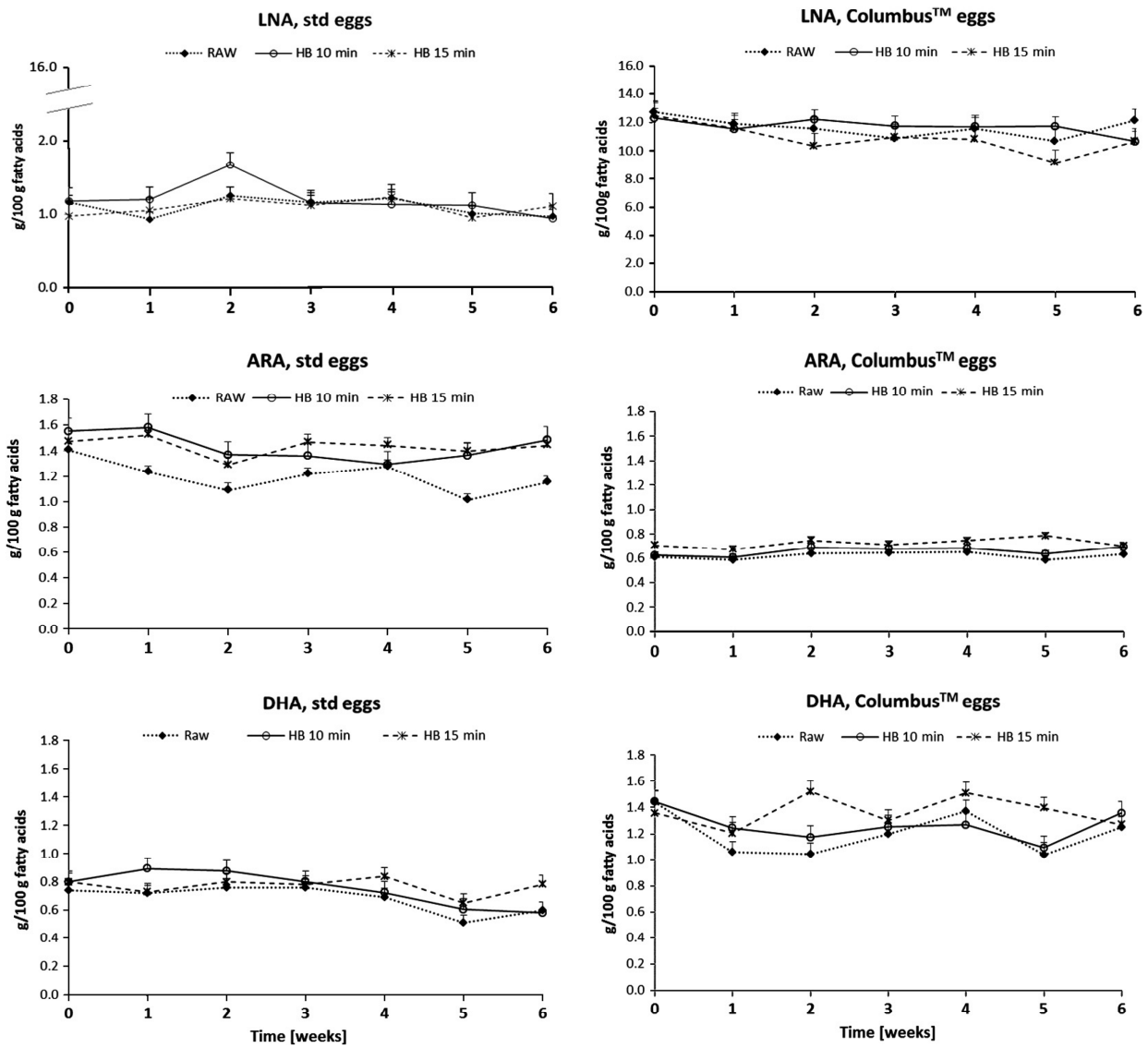

Figure 1. LNA, ARA, and DHA fatty acids content in Columbus™ and standard (std) eggs after storage from 0 to 6 weeks. Eggs were stored raw, hard-boiled 10 min (HB10) and hard-boiled 15 min (HB15). Least Square Means (LSM) ± standard error (SE) of 6 eggs from 4 different batches (day 0) or 4 eggs from one single batch (week 1 to 6) for each storage condition.

dard meat. These omega-3 fatty acids were α-linolenic acid (10.4 ± 0.2% LNA of the total fatty acids) and 11,14,17-eicosatrienoic acid (C20:3 n-3) (1.2 ± 0.1% of the total fatty acids), and the n-6/n-3 ratio was close to 1. Longer chain PUFA were not detected neither in Columbus™ meat, neither in standard pork. The nutrition claim "high omega-3 fatty acids" can be used for the Columbus™ pork because it contains more than 6 g omega-3 fatty acids per kg of meat, which is the lower limit for this claim indicated in the Regulation EC no. 1924/2006 (European Parliament and Council Directive No 1924/200620062006, 2006). The PUFA content found in omega-3 pork marketed in Belgium is consistent to what was described in the literature in controlled experiments were pigs were fed with 5% linseed oil containing feed, resulting in LNA content between 8.5% and 9.1% of total fatty acid in pork muscle, with a n-6/n-3 ratio close to 1 (Nurnberg et al. 1999). Other authors reported for the same kind of experiment a LNA content of 7.3% of total fatty acids in pork and a n-6/n-3 ratio of about 2 (Botsoglou et al. 2012d).Some fatty acids were specified as not detected in pork samples used for this study. However, some of these fatty acids were detected and quantified in egg samples used in this manuscript or other meat

Table 3. Fatty acid composition (g/100 g) of standard and n-3 enriched (Columbus™) raw pork.

	Fatty acid content	
	Columbus™ pork	Standard pork
10:0	ND	ND
12:0	ND	ND
13:0	ND	ND
14:0	**1.3 ± 0.03**	**1.5 ± 0.1**
16:0	**20.0 ± 0.5**	**25.4 ± 0.3**
17:0	ND	ND
18:0	**10.3 ± 0.2**	**13.5 ± 0.2**
20:0	ND	ND
22:0	ND	ND
24:0	ND	ND
16:1 (n-7)	2.2 ± 0.1	2.4 ± 0.1
17:1 (n-7)	ND	ND
18:1 (n-9)	**38.7 ± 0.2**	**46.9 ± 0.5**
18:2 (n-6) (LA)	**15.0 ± 0.5**	**9.7 ± 0.3**
20:2 (n-6)	**1.0 ± 0.04**	**0.7 ± 0.1**
18:3 (n-3) (LNA)	**10.4 ± 0.2**	**ND**
18:3 (n-6)	ND	ND
20:3 (n-3)	**1.2 ± 0.1**	**ND**
18:4 (n-3)	ND	ND
20:4 (n-6) (ARA)	ND	ND
20:5 (n-3) (EPA)	ND	ND
22:5 (n-3) (DPA)	ND	ND
22:6 (n-3) (DHA)	ND	ND
Σ SFA	**31.5 ± 0.7**	**40.3 ± 0.4**
Σ MUFA	**40.9 ± 0.1**	**49.2 ± 0.4**
Σ PUFA	**27.6 ± 0.7**	**10.4 ± 0.3**
n-6 fatty acids	**16.0 ± 0.4**	**10.4 ± 0.3**
n-3 fatty acids	**11.6 ± 0.2**	–
n-6/n-3	**1.4 ± 0.02**	–

Mean ± standard deviation (SD) of seven different meat samples from two different batches. Significant differences comparing Columbus™ and standard pork are indicated in bold ($P < 0.05$). SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; ND, not detected.

or food samples used in other studies, in quantities as low as 0.1%. What is more, other studies working with pork did not mention long chain PUFA when presenting the fatty acid profile of "standard" meat (Ramírez et al. 2004, 2005; Paiva-Martins et al. 2009; Cardenia et al. 2011).

Pork cooking experiment

Evolution of the core temperature in pork during cooking

The maximum core temperatures recorded at the end of the cooking time (in oven) in the standard and Columbus™ pork were 79.8 ± 1.0°C and 80.1 ± 0.7°C, respectively, ($n = 4$) (data not shown).

Fatty acids composition of pork before and after cooking

Pork cooking without culinary fat

Standard pork cooked in the oven and in the pan without the use of fat showed no significant change in the content of total SFA, MUFA, or PUFA, compared to raw meat (Table 4). For Columbus™ pork, pan frying without fat leaded to a significant decrease in the C20:2(n-6) content but did not significantly affect the n-3 fatty acids (LNA and C20:3) or the sum of PUFA (Table 4). Oven cooking (without fat) of the Columbus™ meat showed a larger impact on the fatty acid content of meat, with a significant decrease of LA, LNA, and C20:3(n-3) proportion (14.2% vs. 15.0%, 9.9% vs. 10.4%, and 1.1% vs. 1.2%, in cooked and raw Columbus™ meat, respectively), and a subsequent significant increase of SFA proportion (33.0% vs. 31.6% in cooked and raw Columbus™ meat, respectively) (Table 4). This could be due to the high temperature reached in the core of the meat during oven cooking, affecting the PUFA content of Columbus™ meat which is more than two times higher than in standard meat. The same effect was observed for eggs cooked in the oven (Custard and Savoy cake).

Pork cooking with culinary fat

As expected, meat cooking including the use of culinary fat induced a larger change in the fatty acid profile of both standard and Columbus™ pork than cooking without the use of fat. Table 4 mentions the global fatty acid composition of the culinary fats used in the cooking experiment, according to the Belgian NUBEL food composition table (Nubel 2009). In standard pork cooked with culinary fat, the significant changes in the fatty acid profile are according to the culinary fat used, with an increase of SFA when meat is cooked with butter or margarine and a decrease of PUFA only for meat cooked with butter. When meat is cooked with peanut or sesame oil, a decrease of SFA was observed and an increase of MUFA only for meat cooked with peanut oil (Table 4).

In Columbus™ meat, the same observations are valid for SFA and MUFA: increase of SFA and decrease of MUFA when the meat is cooked with butter or margarine and decrease of SFA and increase of MUFA when the meat is cooked with peanut or sesame oil. The PUFA decreased in any case of Columbus™ pork cooked with culinary fat, except for sesame oil (Table 4). While looking at the detailed fatty acid profile of cooked Columbus™ pork, it is observed that pan frying with butter, compared to raw meat, resulted in an increase of saturated myristic (C14:0), stearic (C16:0), and palmitic acids (C18:0) from 1.3% to 3.1%, 20.0% to 22.6% for C16:0, and 10.3% to 11.4% for

Table 4. Effect of cooking on the fatty acids composition (g/100 g) of standard and Columbus™ pork.

		Σ SFA		Σ MUFA		Σ PUFA		n-3		n-6		C18:3(n-3) LNA		C20:3(n-3)		C18:2(n-6) LA		C20:2(n-5)		n-6/n-3	
Meat type	Condition	LSM	SE	LSM	SE	LSM	SE	LSM	SE	LSM	SE	LSM	SE	LSM	SE	LSM	SE	LSM	SE	LSM	SE
Standard	Raw	40.3	0.3	49.2	0.3	10.4	0.1	ND		10.4	0.1	ND		ND		9.7	0.1	0.7	0.03	–	
	Oven (no fat)	40.6	0.3	49.1	0.3	10.3	0.1	ND		10.3	0.1	ND		ND		9.5	0.1	0.7	0.03	–	
	Pan (no fat)	40.7	0.4	49.0	0.4	10.3	0.1	ND		10.3	0.1	ND		ND		9.8	0.1	0.6	0.04	–	
	Pan (butter)	**44.9***	0.4	**45.8***	0.4	**9.4***	0.1	ND		**9.4***	0.1	ND		ND		**8.8***	0.1	0.6	0.04	–	
	Pan (margarine)	**42.8***	0.4	**46.5***	0.4	10.8	0.1	ND		10.8	0.1	ND		ND		**10.1***	0.1	0.7	0.04	–	
	Pan (peanut oil)	**38.0***	0.4	**50.7***	0.4	**11.2***	0.1	ND		**11.2***	0.1	ND		ND		**10.6***	0.1	0.7	0.04	–	
	Pan (sesame oil)	**38.0***	0.4	49.0	0.4	**13.0***	0.1	ND		**13.0***	0.1	ND		ND		**12.4***	0.1	0.6	0.04	–	
Columbus™	Raw	31.5	0.3	40.9	0.2	27.6	0.2	11.6	0.1	16.0	0.2	10.4	0.1	1.2	0.04	15.0	0.1	1.0	0.04	1.4	0.02
	Oven (no fat)	**33.0***	0.3	40.9	0.2	**26.1***	0.2	**11.0***	0.1	**15.1***	0.2	**9.9***	0.1	**1.1***	0.04	**14.2***	0.1	0.9	0.04	1.4	0.02
	Pan (no fat)	31.1	0.5	41.4	0.2	27.5	0.3	11.6	0.2	15.9	0.2	10.4	0.1	1.2	0.1	15.1	0.2	**0.8***	0.1	1.4	0.03
	Pan (butter)	**37.9***	0.5	**38.4***	0.2	**23.7***	0.3	**9.8***	0.2	**13.9***	0.2	**8.9***	0.1	**0.9***	0.1	**13.2***	0.2	**0.8***	0.1	1.4	0.03
	Pan (margarine)	**35.2***	0.5	**39.2***	0.2	**25.5***	0.3	**10.1***	0.2	15.5	0.2	**9.1***	0.1	**1.0***	0.1	14.8	0.2	**0.7***	0.1	**1.5***	0.03
	Pan (peanut oil)	30.4	0.5	**44.3***	0.2	**25.4***	0.3	**9.7***	0.2	15.7	0.2	**8.6***	0.1	**1.0***	0.1	15.1	0.2	**0.7***	0.1	**1.6***	0.03
	Pan (sesame oil)	**30.2***	0.5	**42.1***	0.2	27.6	0.3	**9.4***	0.2	**18.2***	0.2	**8.5***	0.1	**1.0***	0.1	**17.5***	0.2	**0.7***	0.1	**1.9***	0.03
Butter[1]		60.7		35.3		4.1		1.2		2.9											
Margarine[1]		42.2		36.3		22.1		3.2		18.9											
Peanut oil[1]		17.2		56.2		26.8		0.2		26.6											
Sesame oil[1]		16.0		42.0		42.0		0.0		42.0											

Least-square means (LSM) and Standard errors (SE) of seven different samples for raw meat (from two different batches), four different samples for oven cooked meat and two different samples for each other cooking condition. Significant differences comparing to the raw condition are indicated by asterisks (*) and in bold ($P < 0.05$). SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; n-3, n-3 fatty acids; n-6, n-6 fatty acids; ND, not detected.

[1]From Nubel 2009.

C18:0, respectively (data not shown). A decrease of the percentage of linoleic acid (C18:2n-6) from 15.0% to 13.2% and α-linolenic acid (C18:3n-3) from 10.4% to 8.9% was also observed (Table 4). Those variations are due to the fact that butter contains high amounts of SFA (more than 50% of total fatty acids) and very few polyunsaturated acids (4.1% only of total fatty acids). When the Columbus™ meat is cooked with vegetable oil, the percentage of oleic acid (C18:1) increases in its fatty acid profile (42.4% with peanut oil and 40.4% with sesame oil vs. 38.7% in raw meat), because of the contribution of oleic acid coming from the oil (data not shown). In all cases of pan-frying experiments with culinary fat, compared to the raw meat, the LNA percentage showed a significant decrease, from 10.4% of total fatty acids in raw meat to 9.1% (cooking with margarine), 8.9% (cooking with butter), and 8.6 and 8.5% (cooking with sesame and peanut oils, respectively) (Table 4). This decrease seems not to be due to a loss of LNA, as its percentage remained unchanged when pan frying was performed without fat, but to a dilution effect by the fatty acids coming from the culinary fat, containing very low amount of LNA compared to the Columbus™ meat.

When the meat was cooked with fat, it appeared clearly, that the composition of the culinary fats had a greater influence on its fatty acid profile than the cooking process itself, in both meat types. This was corroborated by the observations of Haak et al. (2007) and Ramírez et al. (2005) who reported that long chain PUFA were not significantly lost by the frying process and that the fatty acids composition of fried pork tended to become similar to that of the culinary fat, as a result of the exchange between culinary fat and meat. The same conclusions were reported by Sioen et al. (2006) for pan-fried fish and by Candela et al. (1998) for deep-fried sardines.

Pork storage experiment

Columbus™ and standard pork were stored raw for 6 weeks at +4°C or 10 weeks at −20°C. Results obtained at day 0 were compared to results obtained at different times of storage in order to estimate the fatty acid profile evolution. No variation in fatty acids composition was observed after storage for both type of meat. What is more, in Columbus™ meat, the PUFA content remained stable during the whole storage experiment, whatever the temperature of conservation (data not shown).

Conclusion

A GC–MS method has been developed for the analysis of fatty acids in food matrices and was applied to determine the omega-3 fatty acid profile of Belgian eggs and pork rich in omega-3 fatty acids (Columbus™ eggs or pork), in order to determine to which extent the omega-3 fatty acids resist to storage or cooking. We can conclude that the omega-3 fatty acids remained unchanged in the ready-to-eat product, except for some specific cooking processes (eggs cooked in custard and meat cooked in oven), where a slight statistically significant loss of PUFA in both Columbus™ eggs or pork was observed. As expected, when Columbus™ pork is cooked with culinary fat, its fatty acid profile is modified according to the nature of the fat used.

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Conflict of Interest

None declared.

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2.1. Supplementary information about the publication of Douny *et al.*, 2015a

Results and Discussion

Performances of the GC-MS analytical method

In this work, the measurement uncertainty was evaluated as the reproducibility relative standard deviation.

The following table was added:

Table 0: Certified values compared to measured values for fatty acids composing CRM BCR-162R (soya-maize oil blend), expressed as g FA/100g total FA.

Fatty acid			CRM BCR-162R (soya-maize oil blend, g FA/100g total FA)					
			Certified Values		Measured values (n=39)			
			FA content (g/100g FA)	Uncertainty (g/100g FA)	FA content (g/100g FA)	Repeatability (RSD_r, %)	Intermediate precision (RSD_R, %)	Recovery (%)
palmitic acid	C16:0	-	10.74	0.16	11.38	7.9	11.5	105.9
stearic acid	C18:0	-	2.82	0.04	2.94	5.6	11.7	104.1
oleic acid	C18:1	n-9	25.40	0.40	27.41	6.7	4.3	107.9
linoleic acid	C18:2	n-6	54.13	0.25	54.10	5.4	4.2	99.9
α-linolenic acid	C18:3	n-3	3.35	0.05	3.35	7.3	15.8	103.0

The titles of the tables in the publication should be:

Table 1: Fatty acid composition (g/100g fatty acids) of standard and n-3 enriched (Columbus™) raw eggs.

Table 2: Effect of cooking on the fatty acid composition (g/100g fatty acids) of standard and Columbus™ eggs.

Table 3: Fatty acid composition (g/100g fatty acids) of standard and n-3 enriched (Columbus™) raw pork.

Table 4: Effect of cooking on the fatty acids composition (g/100g fatty acids) of standard and Columbus™ pork.

CHAPTER 3.
STUDY OF THE OXIDATIVE STABILITY OF LINSEED OIL

3.1. Validation of the analytical procedure for the determination of malondialdehyde and three other aldehydes in vegetable oil using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) and application to linseed oil

For eggs and pork, no or very low significant difference was observed between the fatty acids profiles of raw samples compared to samples obtained from the different storage (various temperatures, exposition to light or not and so on) or cooking conditions (taking into account the measurement uncertainty of the GC-MS method).

This proves that the n-3 fatty acid content announced in a food product is almost the same in that food product after storage or cooking, which is important for the consumer. However, n-3 fatty acids are known to be easily oxidized in secondary oxidation products such as malondialdehyde and other aldehydes. Those are known to be toxic and carcinogenic because they react with DNA to produce adducts. According to Fang and coworkers, the adduct level was 3.6-fold higher in blood of subjects on a diet containing more n-3 fatty acids (rapeseed oil) than that for subjects on a diet containing more n-6 fatty acids (sunflower oil), when considering all individuals (both sexes and all ages) (Fang *et al*., 1996).

Since a low amount of polyunsaturated fatty acids is sufficient to lead to the formation of a significant amount of degradation products, it is essential to measure those degradation products in food. That is why this work was oriented to the development of analytical tools to evaluate the presence of aldehydes in food as secondary oxidation products coming from PUFA. It was decided to focus on linseed oil because it is an oil particularly interesting in nutrition, for diets rich in n-3 fatty acids, due to its high content in α-linolenic acid (more than 50% of the fatty acids).

Some aldehydes being known to be toxic to human health, it was estimated that an analytical method able to dose individually each aldehyde formed during fatty acid oxidation would give more specific information than the TBARS. At this stage, we can insist on the fact that, while the methods described in the literature are developed to measure carbonyl compounds (aldehydes and ketones) in ambient air, in environmental matrices such as water and petroleum, or in biological matrices (Zwiener *et al*. 2002; Brombacher *et al*. 2002; Lord *et al*., 2009), few are dedicated to food matrices (Tsaknis *et al*., 1998 & 1999; Papastergiadis *et al*., 2014a; Jung *et al*., 2016). In addition, most of these methods focused

either on the analysis of a single compound (MDA) or either on other aldehydes but scarcely MDA with other aldehydes in the same run. Therefore, an analytical method involving separation by liquid chromatography (HPLC) coupled to the detection by tandem mass spectrometry (MS/MS) has been developed to evaluate the concentration of malondialdehyde (MDA) and three other aldehydes (4-hydroxy-2E-nonenal (4-HNE), 4-hydroxy-2E-hexenal (4-HHE) and 2,4-decadienal) in linseed oil.

The method has then been validated according to the criteria and procedure described in international standards. The validation parameters were evaluated based on arbitrary criteria that were less severe than those usually considered for compounds having a regulatory limit. They were adapted to both the European Commission Decision (Commission of the European Communities, 2002) and the AFNOR protocol NF V 03-110 (AFNOR, 2010). Indeed, while no official levels are fixed for aldehydes in food, acceptance limits were selected arbitrarily at 50 %. This was decided to stay close to the analytical objectives set for the developed methods but also to the philosophy of those two documents. The criteria were evaluated during experiments planned on different non-consecutive days with a “blank” matrix (coconut oil has been used as model matrix) and that “blank” matrix spiked at different levels of concentration.

Once developed and validated, the method has been applied to the analysis of samples of linseed oil submitted to accelerated oxidation in order to estimate the ideal shelf life of this oil in the case of use in human diet.

The method development and validation and the results obtained from linseed oil oxidation were published in Food Analytical Methods (Douny *et al*., 2015b).

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Validation of the Analytical Procedure for the Determination of Malondialdehyde and Three Other Aldehydes in Vegetable Oil Using Liquid Chromatography Coupled to Tandem Mass Spectrometry (LC-MS/MS) and Application to Linseed Oil

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Abstract Secondary oxidation products of fatty acids, mainly aldehydes, are susceptible to cause significant deterioration in chemical, sensory and nutritional food properties, as well as adverse health effects. An analytical method involving separation by liquid chromatography coupled to the detection by tandem mass spectrometry (LC-MS/MS) has been developed to evaluate the concentration of four aldehydes in oil samples: malondialdehyde (MDA), 4-hydroxy-2-nonenal (4-HNE), 4-hydroxy-2-hexenal (4-HHE) and 2,4-decadienal (2,4-DECA). The optimisation of the extraction, derivation, detection and quantification has been finalised for coconut oil, used as a model of vegetable oils. The method has been validated according to the criteria and procedure described in international standards. The evaluated parameters include specificity/selectivity, recovery, precision, accuracy, uncertainty, limits of detection and quantification, using the concept of accuracy profiles. These parameters have been evaluated during experiments planned on different non-consecutive days with coconut oil spiked at different levels of concentration. The validation of the developed analytical method showed that it is possible to analyse MDA, 4-HHE, 4-HNE and 2,4-decadienal in oil samples, in the same run, with a very good accuracy for MDA, and a defined accuracy at specified concentrations for the three other aldehydes. The accuracy profile of MDA showed a recovery rate of 100 % (±1) and a maximum coefficient of variation for the intermediate precision of 14 % at 0.15 mg kg^{-1}. For the three other aldehydes, recovery rates ranged between 79 and 101 % and coefficient of variation for the intermediate precision between 13 and 23 %. In first pressure linseed oil, stored for several days at 60 °C according to the Schaal oven test, it was shown that 4-HHE was the most produced aldehyde, reaching levels of 85 and 382 μmol kg^{-1} after 12 and 24 days, respectively, versus levels of 18 and 28 μmol MDA kg^{-1} of oil, respectively, and 17 and 51 μmol 4-HNE kg^{-1} of oil.

Keywords Aldehydes · Liquid chromatography · Tandem mass spectrometry · Linseed oil · Validation · Accuracy profile

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Introduction

Linseed (*Linum usitatissimum*), also known as flax, is widely cultivated for its fibre for the textile industry or its seeds for the production of linseed oil, in Canada, China and India. Due to its high α-linolenic acid content (more than 50 %), linseed oil is also particularly interesting in human nutrition, to increase the n-3 polyunsaturated fatty acids (PUFA) intake. This high polyunsaturated fatty acids level also contribute to its rapid oxidation (Belitz et al. 2004; Guillén and Uriarte 2012; Uriarte and Guillén 2010). PUFA oxidation leads to the formation of hydroperoxydes (primary oxidation products), while the

secondary degradation compounds are mainly aldehydes. These aldehydes are relatively stable and have been shown to be cytotoxic and genotoxic by reacting with proteins and nucleic acids (Esterbauer et al. 1991; WHO 2003; Reed 2011; Vander Jagt et al. 1997).

Among aldehydes, four compounds were chosen as relevant markers to evaluate the lipid oxidation of linseed oil: malondialdehyde (MDA), 4-hydroxy-2-nonenal (4-HNE), 4-hydroxy-2-hexenal (4-HHE) and 2,4-decadienal. The malondialdehyde is known to be formed from the degradation of unsaturated fatty acids and thus can be found in vegetable oils (Tsaknis et al. 1998; Wei et al. 2011). 4-HNE and 4-HHE are α,β-unsaturated aldehydes mainly formed during the oxidation of n-6 and n-3 fatty acids, respectively (Guichardant et al. 2006; Han and Csallany 2009; Vankuijk et al. 1990). 2,4-Decadienal, an aldehyde associated with a frying odour, according to Seppanen and Csallany (2001), is one of the major compounds reported to be formed by the autoxidation of linoleic acid. In addition, MDA and 4-HNE have been recently considered, in an advice of the Belgian Superior Health council, of major concern for human health (Belgian Superior Health Council 2011). Until now, no maximum levels have been established for aldehydes in food, mainly because of the lack of data in the literature about toxic levels, quantities contained in food and quantities of these oxidation products actually ingested by a consumer.

Usually, aldehydes are detected by fast and simple enzymatic or colorimetric methods, such as the thiobarbituric acid reactive species (TBARS) method (Papastergiadis et al. 2012; Ulu 2004; Pikul et al. 1989). However, these methods lack of specificity because they measure the total content of aldehydes able to react with thiobarbituric acid, expressed in malondialdehyde content. That is why more recently chromatographic methods have been developed to fill that gap. Those techniques are used to separate, identify and quantify individual compounds, for instance aldehydes (Papastergiadis et al. 2014; Kölliker et al. 2001; Deighton et al. 1997; Zwiener et al. 2002; Wang et al. 2014). A lot of the methods cited in the literature are developed to measure carbonyl compounds (aldehydes and ketones) in ambient air, in environmental matrices such as water and petroleum, or in biological matrices (Zwiener et al. 2002; Brombacher et al. 2002; Lord et al. 2009), but very few are dedicated to food matrices. In addition, most of these methods focused either on the analysis of a single compound (MDA) or either on other aldehydes but scarcely MDA with other aldehydes in the same run. Consequently, it is important to set up more specific analytical methods to assess the quantity of those secondary oxidation products in the same analysis in order to better characterise the oxidation products in foods with high PUFA content, such as linseed oil. Therefore, a liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) method has been developed to evaluate the concentration of malondialdehyde, 4-HHE, 4-HNE and 2,4-decadienal in linseed oil samples (Fig. 1). The developed method used methylmalondialdehyde (MeMDA) as internal standard (Claeson et al. 2001; Sim et al. 2003; Paroni et al. 2002).

The second objective of this work was to set up a validation protocol of the developed method based on various performance parameters. As there is no specific validation criteria for aldehydes in food matrices, our work was based primarily on the Decision of the European Commission 2002/657/EC (Commission of the European Communities 2002), a guideline used for the analysis of residues of veterinary drugs, which presents requirements concerning chromatographic techniques and mass spectrometry that can be applied here. Other parameters were also assessed according to the recommendations of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH 2005). The analytical method presented in this work brings something new compared to what is commonly found in the literature since this method allows to quantify, in the same run, MDA and three other aldehydes: 4-HHE, 4-HNE and 2,4-decadienal, in food samples, while most of the other methods developed and described in the literature are not validated for food matrices (Surh and Kwon 2003; Alghazeer and Howell 2008; Mathew et al. 2011).

Finally, the developed method was applied to monitor the four aldehydes in first pressure linseed oil, stored for several days at 60 °C, according to the Schaal oven test.

Materials and Methods

Chemical Reagents and Materials

1,1,3,3-Tetraethoxypropane (TEP), 2,4-dinitrophenylhydrazine (DNPH, 97 %), 2,4-decadienal and butylated hydroxytoluene (BHT, 99 %) were purchased from Sigma-Aldrich (Milan, Italy). Stock solutions of 4-hydroxy-2-hexenal (4-HHE) and 4-hydroxy-2-nonenal (4-HNE) in ethanol (10 mg ml^{-1}) were obtained from Cayman Chemicals (Michigan, USA). Methylmalondialdehyde (MeMDA) was synthetised from (dimethylamino)-2-methyl-2-propenal (DMP, Sigma-Aldrich) according to the method described by Syslová et al. (2009).

Chloroform and water were of Chromanorm quality, while acetic acid 100 % was of Normapur quality. They were all provided by VWR International (West Chester, Pennsylvania, USA). Ethanol absolute for analysis, tricholoroacetic acid and hydrochloric acid 37 % were from Merck (Darmstadt, Germany). LC-MS-grade acetonitrile was obtained from Biosolve (Valkenswaard, The Netherlands).

The filters were hydrophilic single use syringe filters (0.2 μm pore size, Minisart) from Sartorius (Goettingen,

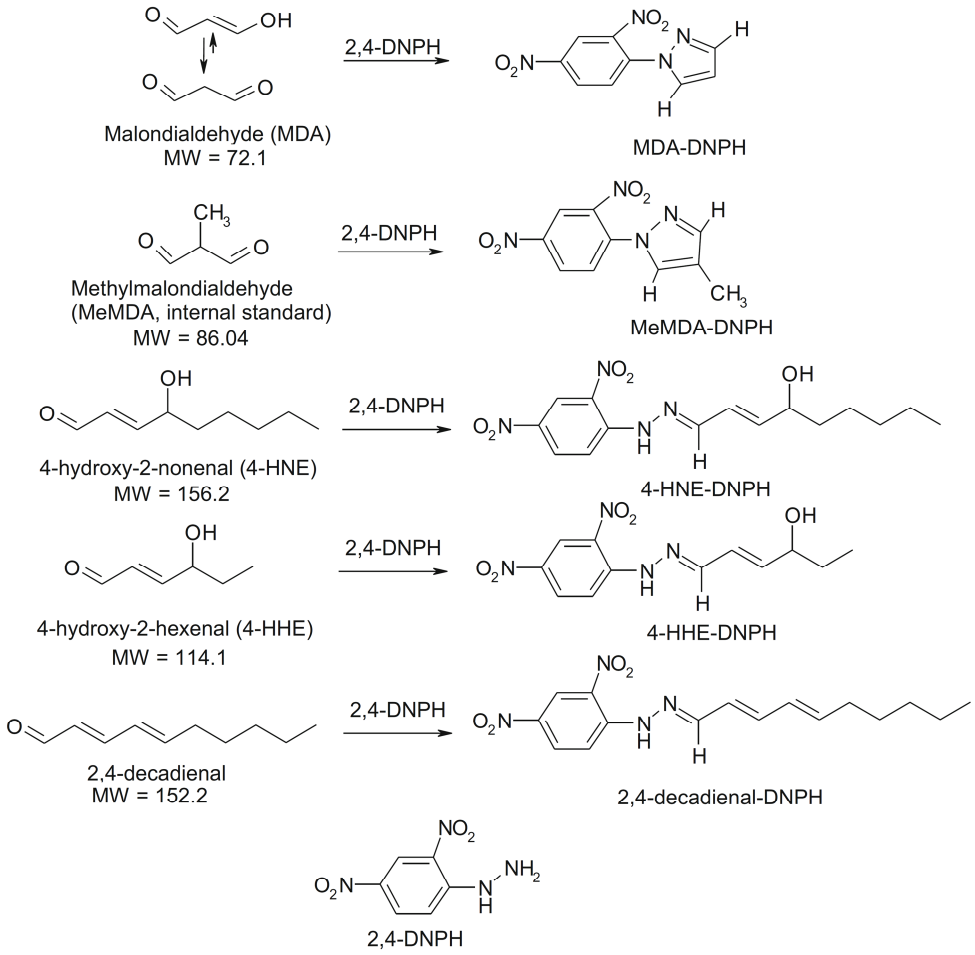

Fig. 1 Structures of four aldehydes, internal standard and their DNPH derivatives

Germany). Fifteen-millilitre Falcon polypropylene graduated conical tube with cap was commercially available from Greiner Bio-One (Germany).

Stock solution of malondialdehyde 1 mg ml^{-1} (MDA) was obtained by hydrolysis of 1,1,3,3-tetraethoxypropane (TEP) in tricholoroacetic acid 5 %. Stock solution of 2,4-decadienal (1 mg ml^{-1}) was prepared by dissolving the compound in water/ethanol 50:50 (*v*/*v*). A mixture of MDA, 4-HHE, 4-HNE and 2,4-decadienal at a concentration of 20, 8, 8 and 24 ng μl^{-1} was obtained by diluting in water/ethanol 50:50 (*v*/*v*) in a 20-ml volumetric flask 400, 16, 16 and 480 μl of each stock solution of the four standards, respectively. A solution of MeMDA at 10 ng μl^{-1} was obtained by diluting the synthetised crystals in water/ethanol 50:50 (*v*/*v*) and was used as internal standard. All of the standard solutions were kept for maximum 6 months at +4 °C.

Sample Preparation

Two grams of oil was weighted in a test tube, additioned with 200 μl BHT (1 mg ml^{-1} in chloroform), 100 μl MeMDA (10 ng μl^{-1}) and 1,900 μl water/ethanol 50:50 (*v*/*v*). The tube was vortexed for 15 s and then centrifuged at 3,700 g for 5 min on a REAX Top vortex from Heidolph (Germany) and a Minifuge T centrifuge from Heraeus (Germany), respectively. This extraction was repeated a second time with 2 ml water/ethanol 50:50 (*v*/*v*), and supernatants were then filtered through a hydrophilic filter.

Dinitrophenylhydrazone derivatives were prepared by adapting the method from Fenaille et al. (2001): 250-μl DNPH solution (0.05 M in ethanol/HCl 12 M 9:1 (*v*/*v*)) was added to 750 μl of extract in an injection vial and reaction took place for 2 h at 60 °C.

LC-MS/MS Analysis

Separation and detection of aldehydes as dinitrophenylhydrazone derivatives were performed using a ThermoFinnigan Spectra System P4000 HPLC system and a ThermoFinnigan LCQ Deca ion trap mass spectrometer, equipped with an electrospray source. Separation was achieved on an Atlantis T3 C_{18} column (3 μm, 2.1 × 150 mm), with an Atlantis guard column T3 C_{18} (3 μm, 2.1 × 10 mm), both from Waters Corporation (Milford, MA,

USA). The solvent flow was 0.25 ml min^{-1}, column temperature was set at 40 °C and the injection volume was 20 μl. The mobile phase was acetonitrile (solvent A) and acetic acid solution at pH=3.55 (solvent B). The gradient elution conditions were from 40 to 65 % of solvent A in 9.6 min and from 65 to 100 % in 4.4 min; then, conditions were held for 1 min and the contribution of solvent A was decreased to 40 % over 5 min and maintained for another 8 min reconditioning. The analysis with the mass spectrometer was performed in MS/MS mode, with positive ionisation for MDA and MeMDA and negative ionisation for the other compounds.

Derivatised standard solutions were synthesised in our laboratory and infused directly in the mass spectrometer to optimise the MS tune parameters. The capillary temperature was set at 300 °C. The derivative forms of the four aldehydes and the internal standard were detected using the multiple reaction monitoring (MRM) mode, with two transitions for each compound (Table 1). Methylmalondialdehyde was used as internal standard for quantification. Results were calculated using Xcalibur Software (ThermoFinnigan).

Samples Used for the Calibration Curves and Validation of the Method

For the validation of the method, organic coconut oil (AmanPrana brand, Noble House, Belgium) was bought in a supermarket. Indeed, linseed oil is so sensitive to oxidation that it can contain quite high amounts of aldehydes if it is not really fresh and stored in appropriate conditions. So, it was decided to use coconut oil, which is highly saturated and thus not sensitive to oxidation, to be sure to use stable oil for calibration curves. Using the developed method, coconut oil was evaluated as "blank" regarding its aldehyde content. Indeed, malondialdehyde was not detected in coconut oil, while 4-HHE, 4-HNE and 2,4-decadienal were sometimes detected in coconut oil, but their chromatographic peak areas were close to those of the background noise peaks. Coconut oil was then used as blank samples and spiked quality control (QC) samples for validating the developed method and for the calibration curves.

Calibration Curves

Seven samples of coconut oil spiked with the internal standard at a concentration of 0.5 mg kg^{-1} and with concentrations ranging from 0.06 to 3.0 mg kg^{-1} for 4-HHE and 4-HNE, 0.15 to 7.5 mg kg^{-1} for MDA and 0.18 to 9.0 mg kg^{-1} for 2,4-decadienal were extracted simultaneously with the samples. The concentration range of the calibration curve was chosen to cover the range of concentrations usually observed for each aldehyde analysed in oxidised oil samples. The extracts of these seven samples were used to construct the calibration curves: The response (ratio between each aldehyde and the internal standard peak areas) was plotted versus standard concentrations. Calibration points were injected before each series of samples, and the extract spiked at a concentration corresponding to the central point of the calibration curve was injected one more time after all the samples. The choice of the best regression model was studied using the statistic *F*-test, which is also known as Mandel fitting test (Mandel 1964). A quadratic regression was used, and no "fit weighting" was applied. The correlation coefficients R^2 associated with those curves were higher than 0.98. It was also established that only one point of the curve can deviate from the curve by more than 20 % of the corresponding calculated value.

Confirmation Criteria

Each aldehyde was considered as positively identified in samples if the ratio between the chromatographic retention time of the analyte and that of the internal standard, i.e. the relative retention time (RRT) of the analyte, corresponded to that of the average retention time of the calibration solutions within a ±2.5 % tolerance interval and the peak area ratio of the two transitions of the native analyte corresponded to that of the averaged transition ratio of the calibration solutions within the tolerances set by the Commission Decision 2002/657/EC (Commission of the European Communities 2002).

Validation of the Method

The validation was performed according to the Commission Decision 2002/657/EC and following the most commonly used approaches (Willetts and Wood 2000; Feinberg 1996). The Commission Decision 2002/657/EC promotes the use of Certified Reference Materials (CRM) to validate an analytical method. Since no CRM of aldehyde is available in oil, coconut oil fortified with standard solutions of aldehydes was used to assess the performance of the method.

Extractions of samples and injections of samples extracts were performed on five non-consecutive days. The "low", "medium" and "high" levels of concentrations tested are detailed in Table 2.

Data generated during the validation of the method were processed with e-Noval 2.0 software, from Arlenda (Liège, Belgium).

Linseed Oil Oxidation Conditions

First pressure linseed oil (1PLO), free of any added antioxidant, was kindly supplied by Vandeputte (Mouscron, Belgium).

Samples of 1PLO (20 g) were distributed in tinted glass bottles (100 ml, diam. 4 cm) left open and heated for up to 24 days at 60 °C in a ventilated oven (ULM800, Memmert,

Table 1 Ionisation mode, mass of precursor and daughter ions, spray voltage and normalised collision energies followed in MS/MS for each compound analysed

Compounds	ESI mode	m/z	Spray voltage (kV)	Normalised collision energy (%)
MDA-DNPH	+	235>159 235>189	6.0	30
MeMDA-DNPH	+	249>173 249>203	6.0	32
4-HHE-DNPH	−	293>163 293>167	5.0	29
4-HNE-DNPH	−	335>167 335>288	4.0	32
2,4-decadienal-DNPH	−	331>230 331>284	1.5	34

Germany), according to the Schaal oven test. Temperature in oven was monitored during the test with temperature probe from VWR International (Radnor, Pennsylvania), with a measurement uncertainty of 0.5 °C. After 0, 3, 4, 6, 12 and 24 days, bottles were removed from the oven, flushed with nitrogen, closed and stored at −80 °C until analysis. Samples were analysed in triplicate on three independent experiments, with three different batches of 1PLO, leading to nine samples per day of storage and a total of 54 samples.

Statistical Analysis

JMP® 7.0.2 software (SAS Institute, Cary, North Carolina, USA) was used for statistical analysis. Significant differences of aldehyde levels between days of accelerated oxidation were tested using the analysis of the variance and generalised linear models (GLM) procedure of JMP® software. Levels of significant effects were compared using least square means and associated standard error (significant for $P<0.05$).

Results and Discussion

Method Development

Optimisation of the Derivation Process

Many publications mention the use of 2,4-dinitrophenylhydrazine to derivatise carbonyl compounds.

Table 2 Validation parameters of the LC-MS/MS method for the quantification of four aldehydes in vegetable oil

Aldehyde	Targeted spiking levels (mg kg^{-1})	Mean introduced concentrations (mg kg^{-1})	LOD (mg kg^{-1})	LOQ (mg kg^{-1}) lower-upper	Repeatability (RSD_r, %)	Intermediate precision (RSD_R, %)	Recovery (%)	Relative expanded uncertainty (%)	Linearity (R^2)	Calibration curve equations
MDA	*0.15–3.00–7.50*	0.15–3.02–7.48	0.05	0.15–7.50	12–11–4	14–12–4	101–101–100	29–24–9	0.9946	$y=-0.0304+2.8074*x-0.0380*x^2$
4-HHE	0.06–1.20–3.00	0.04–1.26–2.90	0.14	0.70–3.00	11–9–11	20–14–14	90–98–97	44–30–29	0.9938	$y=-0.0073+0.6572*x-0.0343*x^2$
4-HNE	0.06–1.20–3.00	0.06–1.09–2.38	0.02	0.06–2.34	18–10–11	19–15–13	99–91–79	40–33–27	0.9953	$y=-0.0116+0.5757*x-0.0457*x^2$
2,4-Deca	0.18–3.60–9.00	0.17–3.63–7.96	0.12	1.54–9.00	21–14–12	23–14–13	95–101–88	49–30–28	0.9985	$y=-0.0060+0.0969*x-0.0022*x^2$

The three values for repeatability, intermediate precision, recovery, linearity and expanded uncertainty are respective to the three mean introduced concentration values ($n=5$)

MDA malondialdehyde, *4-HHE* 4-hydroxy-2-hexenal, *4-HNE* 4-hydroxy-2-nonenal, *2,4-Deca* 2,4-decadienal

Among them, Stafiej et al. (2006) mentioned the fact that a lot of different and diverging derivation protocols are applied. Incubation time can last a few minutes to several hours or even beyond 24 h. In addition, the stability problems of the hydrazones formed are scarcely mentioned. Indeed, the area of the chromatographic peak of hydrazones derivatives of malondialdehyde was stable, while the area of the chromatographic peak of the derivatives formed from other compounds decreased over time (4-HHE, 4-HNE) or showed two chromatographic peaks rather than one (MeMDA) (data not shown).

Concerning the duplication of the chromatographic peaks, according to Lin et al. (2009) and Uchiyama et al. (2003), this phenomenon was probably due to the formation of isomers during the reaction of the aldehydes with 2,4-DNPH. As the aromatic ring of MeMDA contains a methyl group (Fig. 1), it could indeed form a geometric isomer. A reduction of the amine of DNP hydrazone using 2-picoline borane could transform the double C=N bond in a simple bond C–N (Uchiyama et al. 2009; Sato et al. 2004; Uchiyama et al. 2011). The reduced compound formed would be more stable because of the saturated bonds and would therefore suffer no conformation change. A single isomer would therefore be present, which would improve the accuracy of analysis. Various concentrations of 2-picoline borane combined with different additions of acid, time and temperature of derivation were tested without any improvement. This could be explained by the fact that methyl-malondialdehyde cannot undergo this double bond reduction because its C=N double bond is included in an aromatic ring.

In order to solve this problem of MeMDA peak duplication, the optimisation of the conditions of the derivation reaction to switch the reaction equilibrium towards the most complete formation of the desired product was investigated. To do this, the DNPH concentration (0.05, 0.2, 0.5 M), the solvent (water, acetonitrile, ethanol) and the acid concentration in the DNPH solution have been tested. A single chromatographic peak was obtained for methyl-malondialdehyde with a DNPH concentration of 0.05 M in ethanol/HCl 12 M (9:1, *v*/*v*).

The observed decreasing concentration of the 4-HHE and 4-HNE derivatised compounds over time could be explained by the formation of precipitates of aldehydes-2,4-dinitrophenylhydrazones. Those could be solubilised in a greater extent in the solution by reducing the moisture content of the DNPH solution used. In addition, time (0, 1, 2, 3, 4, 5 h), derivatisation temperature (25, 40, 60 °C) and the use of an agitation have been tested.

The larger chromatographic peak areas and stability of these areas were obtained with a reaction time of 2 h at 60 °C. The molar ratio between DNPH and aldehydes was ranging from 1,828 to 14 for the first and last points of calibration curve, respectively.

Optimisation of the Extraction

Despite the derivation optimisation, the 2,4-decadienal chromatographic peak was very small, following an aqueous extraction. This compound is quite non-polar and is eluted from the HPLC column after more than 20 min. Therefore, the water extraction applied in a first trial was not the best choice for 2,4-decadienal, while it was perfectly suitable for the more polar compounds (MDA, 4-HHE, 4-HNE). Therefore, in order to extract the four aldehydes, the extraction in various solvents has been tested: water/acetonitrile mixtures (100:0, 90:10, 75:25, 50:50, 25:75, 0:100; *v*/*v*) and water/ethanol (75:25, 50:50; *v*/*v*). In addition, water and ethanol acidified with trichloroacetic acid (TCA) 5 % were also tested.

The best compromise was obtained with water/ethanol 50:50 (*v*/*v*). Figure 2 shows the chromatogram of a QC spiked with the four aldehydes at a medium level, corresponding to a coconut oil sample spiked with 1.2 mg kg^{-1} 4-HHE and 4-HNE, 3.0 mg kg^{-1} MDA and 3.6 mg kg^{-1} 2,4-decadienal.

Method Validation

For the validation, seven coconut oil samples were spiked with increasing concentrations of aldehydes to be used for each calibration curve. In addition, 20 coconut oil samples were spiked only with internal standard to be used as blank samples. Sixty-six coconut oil samples were spiked with internal standard and three different concentration levels (low, medium, high; n=22 for each concentration level) of aldehydes (Table 2), which correspond to the first, the fourth and the sixth point of the calibration curve, respectively. Samples were extracted, derivatised and analysed in five independent series. Raw response data, without any correction, obtained for both calibration standards and spiked samples were computed using the validation software e-Noval.

The parameters relevant to evaluate the performances of the developed method were selectivity/specificity, linearity, recovery, precision (repeatability and intermediate precision), accuracy, limit of detection (LOD) and limit of quantification (LOQ).

Validation was performed using the concept of accuracy profile (Boulanger et al. 2003; Hubert et al. 2004; Brasseur et al. 2007; Feinberg et al. 2004; Feinberg and Laurentie 2006).

Selectivity and specificity were demonstrated by showing the absence of significant peak in the blanks and the presence of quantifiable peaks in the QCs. When a peak was detected in the blanks, it was shown that it fulfilled the confirmation criteria detailed in “Validation of the Method” section (data not shown).

Accuracy profiles computed for the four aldehydes are shown in Fig. 3. Accuracy refers to the closeness of agreement between the test result and the accepted reference value (FDA

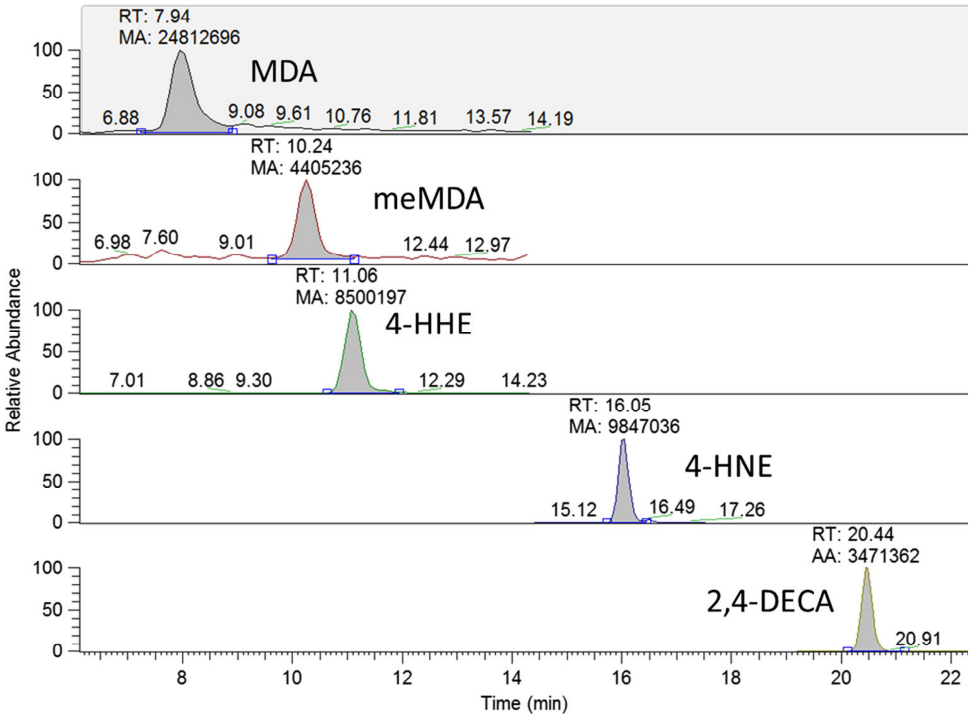

Fig. 2 Chromatogram of aldehyde DNPH-derivatives analysed by LC-MS in a QC at medium level of aldehydes concentration, corresponding to a coconut oil sample spiked with 1.2 mg kg^{-1} 4-HHE and 4-HNE, 3.0 mg kg^{-1} MDA and 3.6 mg kg^{-1} 2,4-decadienal

1995). The accuracy takes into account the total error, related to the test results. While no official levels are fixed for aldehydes in food, acceptance limits (dotted external lines) were selected arbitrarily at 50 %. The solid central line represents the bias, and the dots correspond to the relative back-calculated concentrations, plotted with respect to their targeted concentrations (expressed in percentage of variation from the targeted concentration). Internal dashed lines represent the 95 % β-expectation tolerance interval (the result expected for a level included in the dosing range will fall in these limits, with a risk of 5 % of being outside). The analytical procedure is considered as valid within the working range if the limits of the accuracy profile (dashed lines) are narrower than the acceptance limits (external dotted lines). For the MDA, the 95 % tolerance interval is located between the acceptance limits, demonstrating the validity of the method for the whole analytical range tested. For the 4-HHE and 2,4-decadienal, the 95 % tolerance interval is higher than the acceptance limits of 50 % for the low level of concentration: 65.5 and 62.2 %, respectively. For 4-HNE, this interval is slightly higher than the acceptance limit for the high level of concentration (51.4 %).

Target spiking levels, mean introduced concentrations (average of the real concentrations for all experiments), LOD, LOQ, repeatability, intermediate precision, recovery, linearity (R^2), calibration curve equations and uncertainty are summarised in Table 2.

Limits of detection (LODs) (the smallest quantity of the targeted substance that can be detected but not accurately quantified in the sample) are calculated within the e-Noval software by adding three times the residual standard deviation obtained from an ANOVA test to the *y* intercepts of the calibration curves. The LODs were found below the lowest spiked level for all compounds, except for 4-HNE, and ranged from 0.02 to 0.14 mg kg^{-1} (Table 2). The lower *limit of quantification* (LOQ) is defined here as the smallest quantity of the targeted substance in the sample that can be assayed under experimental conditions with well-defined accuracy (FDA 1995). The definition can also be applicable to the upper LOQ which is the highest quantity of the targeted substance in the sample that can be assayed under experimental conditions with well-defined accuracy. It is computed by the validation software as the smallest and highest concentrations beyond which the β-expectation limits go outside the acceptance limits. Depending of the compounds, the lower and upper LOQ range respectively from 0.06 to 1.54 and 2.34 to 9 mg kg^{-1} (Table 2).

The *precision* (closeness of agreement between independent test results) of the method is evaluated through its repeatability (variation of the results obtained in a same series), expressed as a relative standard deviation or RSD_r, and its intermediate precision (variation of the results obtained in different series), also expressed as relative standard deviation, and is often called RSD_R. The repeatability was between 4 and 21 %, and the intermediate precision was between 4 and 23 % for the four aldehydes (Table 2).

The *recovery* (the proportion of the amount of analyte, added to the analytical portion of the test material, which is extracted and presented for measurement) ranged from 88 to 101 %, being for MDA close to 100 % for the three levels of

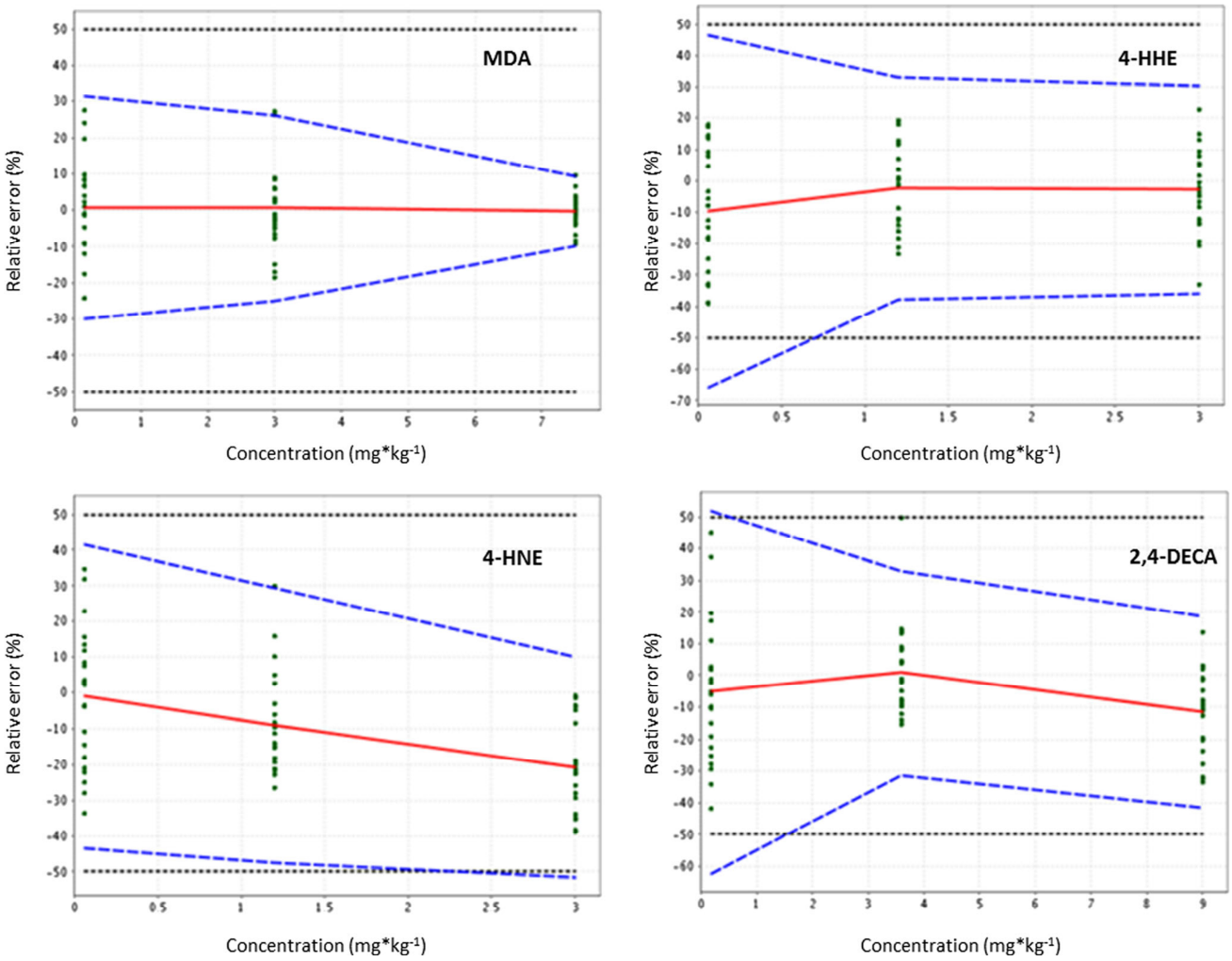

Fig. 3 Accuracy profile of the four aldehydes, computed with e-Noval 2.0 (Arlenda). The *dotted external lines* represent the acceptance limits. The *solid central line* represents the bias, and the *dots* correspond to the relative back-calculated concentrations, plotted with respect to their targeted concentrations (expressed in percentage of variation from the targeted concentration). *Internal dashed lines* represent the 95 % β-expectation tolerance interval

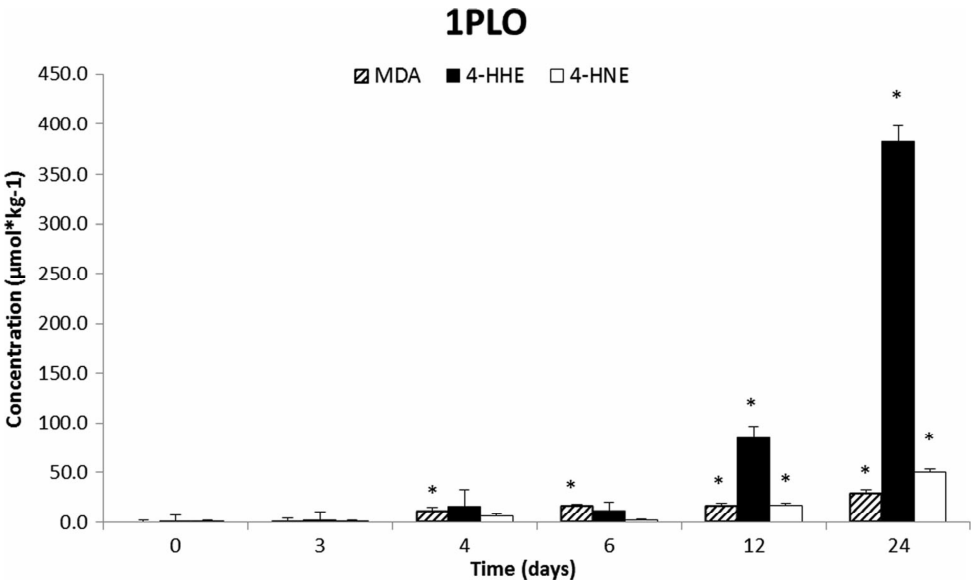

Fig. 4 Least-square means (LSM) and standard errors (SE) of concentration expressed in micromoles per kilogram of malondialdehyde (*MDA*), 4--hydroxy-2-hexenal (*4-HHE*) and 4-hydroxy-2-nonenal (*4-HNE*) for 1PLO stored at 60 °C for up to 24-day samples (n=9 per day of storage). Significant differences comparing day 0 and other time of storage are indicated by asterisks (*) (P<0.05). LOQ, expressed, in micromoles per kilogram, are 2 μmol kg^{-1} for MDA, 6 μmol kg^{-1} for 4-HHE and 0.4 μmol kg^{-1} for 4-HNE

concentrations tested (Table 2). All recoveries met the criteria of the European decision 2002/657/EC (Commission of the European Communities 2002), which is set between 80 and 110 %.

The *measurement uncertainty* (MU) (a parameter that characterises the dispersion of the values that could reasonably be attributed to the measurand) is expressed here as a relative expanded uncertainty (a percentage of the result) and is calculated from the total variance of the β-expectation tolerance interval, using a coverage factor of 2. Relative expanded uncertainties ranged from 9 to 49 % (Table 2).

Evaluation of Linseed Oil Oxidation

First, linseed oil was spiked with various concentrations of aldehydes to check the absence of potential interferences coming from the matrix at the retention times of the target compounds. Then, the four aldehydes were quantified in first pressure linseed oil (1PLO) submitted to the Schaal oven test ("Statistical Analysis" section). Malondialdehyde (MDA), 4-hydroxy-2-hexenal (4-HHE), 2,4-decadienal and 4-hydroxy-2-nonenal (4-HNE) were measured in the oil collected after 0, 3, 4, 6, 12 and 24 days of storage at 60 °C. Least-square means (LSM) and standard errors (SE) of the results are presented in Fig. 4. Concentrations are expressed in micromoles aldehyde per kilograms linseed oil. The 2,4-decadienal was not detected in any sample at any time of storage. At day 0, MDA was not detected in oil, and 4-HHE was below the LOQ, while quantifiable but low levels of 4-HNE were found (1 μmol kg^{-1}). At the fourth day of storage at 60 °C, MDA levels became significantly different from those at day 0, but this was not the case for 4-HHE and 4-HNE, which remain at the same level from day 0 to day 6. At the 12th day of storage at 60 °C, the three aldehydes were significantly higher than at day 0, with similar levels for MDA and 4-HNE (18 and 17 μmol kg^{-1}) and about five more times 4-HHE (85 μmol kg^{-1}). After 24 days at 60 °C, the largest amounts were found for 4-HHE (382 μmol kg^{-1}), followed by 4-HNE (51 μmol kg^{-1}) and MDA (28 μmol kg^{-1}).

Guillén and Uriarte (2012) observed that 4-HHE as a major compound formed when virgin linseed oil was fried at 190 °C for 3.6 and 20 h. Guichardant et al. (2006), and Han and Csallany (2009) reported as well that 4-HNE and 4-HHE are α,β-unsaturated aldehydes mainly formed during the oxidation of n-6 and n-3 fatty acids, respectively. This corroborates our results, showing that 4-HHE and 4-HNE are the major aldehyde found in oxidised linseed oil, a vegetable oil containing more than 50 % of alpha-linolenic fatty acid and approximately 14 % of linoleic acid.

Conclusion

The quantification of aldehydes is a good indicator of the oxidation level of the polyunsaturated fatty acids in food matrices. The analytical method presented in this work brings something new compared to what is commonly found in the literature since this method allows to quantify, in the same run, MDA and three other aldehydes: 4-HHE, 4-HNE and 2,4-decadienal, in food samples, while most of the other methods developed and described in the literature are not validated for food matrices.

The validation of the developed analytical method showed that it is possible to analyse MDA, 4-HHE, 4-HNE and 2,4-decadienal in oil samples, in the same run, with a very good accuracy for MDA, and a defined accuracy at specified concentrations for the three other aldehydes.

In linseed oil subjected to storage at high temperature (60 °C), it was shown that 4-HHE was the most produced aldehyde after 12 days of storage, with high levels (above 80 μmol kg^{-1}), indicating that 4-HHE is a relevant marker of linseed oil oxidation. More research is needed to assess the risk linked to the possible ingestion of 4-HHE through oxidised fatty food.

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Conflict of Interest Caroline DOUNY declares that she has no conflict of interest. Angélique Tihon declares that she has no conflict of interest. Pierre Bayonnet declares that he has no conflict of interest. François Brose declares that he has no conflict of interest. Guy Degand declares that he has no conflict of interest. Eric Rozet declares that he has no conflict of interest. Jérôme Milet declares that he has no conflict of interest. Laurence Ribonnet declares that she has no conflict of interest. Loranne Lambin declares that she has no conflict of interest. Yvan Larondelle declares that he has no conflict of interest. Marie-Louise Scippo declares that she has no conflict of interest. This article does not contain any studies with human or animal subjects.

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3.2. Linseed oil presents different patterns of oxidation in real-time and accelerated aging assays

A comprehensive study of the oxidation phenomenon is often complex to perform and, to do this, a wide choice of methods exist, each with its advantages and limits based on the lipid matrix and oxidation products formed. In addition, a study conducted at room temperature and in the real conditions of the food storage requires a lot of time, which is incompatible with the quality control at an industrial scale. Therefore, some methods use forced oxidation conditions, such as the Storage Stability Schaal Oven Test, also known as the AOCS Cg 5-97 method and realized at 60 °C (American Oil Chemists' Society, 1998).
Concerning the Schaal Oven Test, the American Oil Chemists' Society (1997) described the protocol as an accelerated aging of oils or fats at 60 °C. This temperature leads to a moderate oxidative stress in the samples and retains the same oxidation mechanism at lower temperature conditions but without the problems of long storage periods (fatty acid polymerization, cyclization of compounds, bad solubilization of oxygen in oil….). According to literature, some authors use a temperature between 22 to 26 °C to simulate room temperature oxidation of fatty acids, while a temperature of 60 or 65 °C is used to simulate a moderate oxidation of fatty acids (Evans *et al*., 1973; Abou-Gharbia *et al*., 1996; Khan *et al*., 2001). If accelerated aging at 60 °C seems to be an acceptable approach, we must be aware that it does not exactly simulate oxidation at room temperature. Indeed, according to Frankel and coworkers, the kinetics of oil oxidation during accelerated aging is linear, whereas it is exponential in room temperature aging (Frankel *et al*., 1994).

The question then arises on the accuracy of these accelerated test results and their extrapolation to the daily operating conditions of food. According to Frankel and coworkers, each accelerated test must be adapted to the type of fat and stick as close as possible to the real conditions in which will the oxidation reactions take place (Frankel *et al*., 2005). This is why we decided in this thesis to verify the hypothesis proposed by different authors that, for some oils, an aging of 1 day at 65 °C was equivalent to an aging of 1 month at 25 °C (Khan *et al*., 2001). To do that, an accelerated (at 60 °C) and a real-time aging of linseed oil (at 20 °C) were performed with linseed oil supplemented or not with myricetin or butyl-hydroxytoluene (BHT) as antioxidants. Myricetin is a natural and powerful antioxidant due to its pyrogallol group and we aimed to compare its efficacy to protect linseed oil from oxidation with the efficacy of BHT, a synthetic antioxidant. Primary

products of PUFA oxidation were evaluated with the determination of conjugated dienes and peroxide value while secondary products were evaluated with the determination of the *para*-anisidine value and aldehyde concentrations.

The results obtained from linseed oil aging were published in Food Chemistry (Douny *et al*., 2016a).

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Short communication

Linseed oil presents different patterns of oxidation in real-time and accelerated aging assays

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ABSTRACT

This study aimed at verifying if the hypothesis that one day at 60 °C is equivalent to one month at 20 °C could be confirmed during linseed oil aging for 6 months at 20 °C and 6 days at 60 °C using the "Schaal oven stability test". Tests were conducted with linseed oil supplemented or not with myricetin or butyl-hydroxytoluene as antioxidants. Oxidation was evaluated with the peroxide and *p*-anisidine values, as well as the content in conjugated dienes and aldehydes. All four indicators of oxidation showed very different kinetic behaviors at 20 and 60 °C. The hypothesis is thus not verified for linseed oil, supplemented or not with antioxidant. In the control oil, the conjugated dienes and the peroxide value observed were respectively of 41.8 ± 0.8 Absorbance Unit (AU)/g oil and 254.3 ± 5.8 meq.O_2/kg oil after 6 months at 20 °C. These values were of 18.2 ± 1.3 AU/g oil and 65.2 ± 20.3 meq.O_2/kg after 6 days at 60 °C.

1. Introduction

Linseed (*Linum usitatissimum*) oil has a high α-linolenic acid (ALA) content and is therefore interesting in nutrition. Indeed, the consumption of n-3 polyunsaturated fatty acids (PUFA) is necessary for various physiological reasons and has been associated with a decrease of the incidence of inflammatory and cardiovascular pathologies (Simopoulos, 2008). Unfortunately, the high PUFA content of linseed oil also contributes to its rapid oxidation (Guillén & Uriarte, 2012). To prevent it, supplementation with antioxidants is required. Synthetic antioxidants commonly used in food include butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), ethoxyquin and ascorbyl palmitate (Reische, Lillard, & Eitenmiller, 2008). These antioxidants show an effective protection of the PUFA (Anwar, Siddiq, Iqbal, & Asi, 2007), but serious doubts concerning their safety oriented research towards the exploration of natural antioxidants (Chirinos, Huaman, Betalleluz-Pallardel, Pedreshi, & Campos, 2011; Martín et al., 2014), such as flavonoids, tocopherols and carotenoids (Pelli & Lyly, 2003). Michotte et al. (2011) showed that myricetin, a flavonol present in many plants, is an effective protector against autoxidation of PUFA in linseed oil. Other studies demonstrated that myricetin can also protect sunflower (Marinova, Toneva, & Yanishlieva, 2008) and rapeseed oils (Chen, Chan, Ho, Fung, & Wang, 1996).

The AOCS Cg 5-97 method, also known as the *Schaal oven stability test*, is an oven storage test used to simulate the real-time aging of oils (Michotte et al., 2011). This test should be conducted with oils as little oxidized as possible, at 60 °C in the dark. This method shows a good correlation with real-time stability studies. In the literature, some authors showed that the oxidation parameters obtained from aging realized at 60 °C and at a temperature close to ambient temperature are linearly linked. Indeed, Abou-Gharbia, Shehata, Youssef, and Shahidi (1996) indicated that when Tehina, a paste obtained with ground sesame seeds, was aged 2 days at 65 °C, the *p*-anisidine (PA) value and peroxide value

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(PV) were similar to the values obtained with Tehina aged for 60 days at 22 °C. Similarly, soybean and cotton oils aged in closed bottles had equivalent «flavor scores» after 4 days at 60 °C and 4 months at 26 °C (Evans, List, Moser, & Cowan, 1973). Those works were reported by Khan and Shahidi (2001) and led to the hypothesis that an aging of 1 day at 65 °C was equivalent to an aging of 1 month at 25 °C. A linear relationship would mean that the results obtained at 60 °C during a short time experiment could be extrapolated to real-time (room temperature) aging of oil. Therefore, the objective of our study was to verify this hypothesis during linseed oil aging, without added antioxidant and with BHT or myricetin. In each aging experiment, primary products of PUFA oxidation were evaluated with the determination of conjugated dienes and PV; secondary products were evaluated with the determination of the PA value and aldehyde concentrations.

2. Materials and methods

2.1. Standards and reagents

1,1,3,3-tetraethoxypropane, 2,4-dinitrophenyl-hydrazine (97%), 2,4-decadienal, BHT (99%), PA were purchased from Sigma-Aldrich (St. Louis, Missouri). Stock solutions of 4-hydroxy-2-hexenal (4-HHE) and 4-hydroxy-2-nonenal (4-HNE) were obtained from Cayman Chemicals (Ann Arbor, Michigan). Myricetin was obtained from Extrasynthese (Genay, France).

Chloroform and water were of Chromanorm quality, while acetic acid 100% was of Normapur Quality, all provided by VWR International (Radnor, Pennsylvania). Ethanol absolute, trichloroacetic acid and hydrochloric acid 12 M were from Merck (Darmstadt, Germany). LC–MS-grade acetonitrile was obtained from Biosolve (Valkenswaard, The Netherlands).

2.2. Sample preparation and oxidation conditions

Refined linseed oil (RLO) free of synthetic antioxidant was kindly supplied by Vandeputte (Mouscron, Belgium). RLO was collected in a 1 L plastic bottle, inerted and stored at 4 °C until use. Samples of RLO (20 g), with added antioxidant and without (controls), were prepared as described by Michotte et al. (2011): BHT and myricetin were solubilized in a limited quantity of ethanol so that it did not exceed 4% of the weight of the final solution. Then, RLO was added in order to obtain the desired concentration of 555 µmol antioxidant/kg oil for each compound. The solution was strongly mixed with a glass rod for 10 min and flushed with nitrogen for 3 min to remove ethanol. The different mixtures were stored at 4 °C until the start of the experiments. The mixtures were then strongly shaken and distributed (20 g) in left open tinted glass bottles (100 ml, diam. 4 cm), which were stored for 6 days at 60 °C (accelerated aging) or 6 months at 20 °C (real-time aging) in two ventilated ovens (ULM800, Memmert, Schwabach, Germany). Both oven temperatures were monitored during the test with temperature probe from VWR International (Radnor, Pennsylvania). For the 60 °C condition, bottles were removed from the oven every 12 h for the two first days, then every 24 h until 6 days. For the 20 °C condition, bottles were removed from the oven every 2 weeks for the two first months, then every month until 6 months. Then, bottles were flushed with nitrogen, closed and stored at −70 °C until random analysis.

2.3. Conjugated dienes

Conjugated dienes were evaluated in 25 mg RLO diluted with 100 mL hexane by measuring the absorbance at 232 nm with a UV–vis Cecil 2041 spectrophotometer (Cecil Instruments Limited, Cambridge, UK) (Dieffenbacher, 1992).

2.4. Peroxide value

The hydroperoxides were measured using the iodometric titration method (AOAC, 1995). Five g of RLO were diluted in 30 mL acetic acid/chloroform solution (3/2, v/v) then 0.5 mL of a saturated potassium iodide solution were added and a titration was realized with a 0.1 N $Na_2S_2O_3$ solution.

2.5. p-anisidine value

The PA value was determined by diluting 0.5 g RLO with 25 mL isooctane and adding PA (0.25% in glacial acetic acid) (American Oil Chemists' Society, 1998). PA value was measured at 350 nm.

2.6. Aldehyde measurement by LC-MS/MS

The aldehyde measurement was performed according to Douny et al. (2015). Briefly, 2 g of oil, added with BHT and methylmalondialdehyde as internal standard were extracted two times with water/ethanol 50/50 (v/v). Dinitrophenylhydrazone derivatives were prepared by adding a 2,4-dinitrophenyl-hydrazine solution (0.05 M in ethanol/HCl 12 M 9:1 (v/v)) and incubating for 2 h at 60 °C.

Separation and detection of aldehydes as derivatives were performed using a Spectra System P4000 HPLC system and a LCQ Deca ion trap mass spectrometer, with an Electrospray source (ThermoQuest Finnigan, San Jose, California).

2.7. Statistical analysis

Statistical Analysis System (SAS Institute, 2000) was used to check the data for normal distribution with a Shapiro–Wilk test and when variables were not normally distributed, a logarithmic transformation was performed. Significant differences between day 0 and other days/months ($p < 0.05$) were tested using the general linear model procedure. The non-parametric test of Kruskal Wallis has been used when normality was not respected despite a logarithmic transformation.

3. Results

The determination of oxidation products obtained from RLO with and without added antioxidants aged at a temperature of 60 °C or 20 °C are presented in Fig. 1. Conjugated dienes (Fig. 1I and II), PV (Fig. 1III and IV) and PA value (Fig. 1V and VI) are expressed as mean ± standard deviation (SD) of three independent experiments, with measurements performed twice on each sample, leading to a total of 6 values per condition. MDA content (Fig. 1VII and VIII) is expressed as the mean concentration ± SD of three independent experiments, with measurements performed once on each sample, leading to a total of 3 values per condition.

3.1. Primary oxidation products

3.1.1. Conjugated dienes

For the dienes, the absorbance value reached 41.8 ± 0.8 AU/g oil after 6 months at 20 °C, for the control, and 28.0 ± 0.6 AU/g oil, for the RLO samples supplemented with BHT, corresponding to an increase of respectively nearly 150% and 75%, compared to the absorbance value measured at day 0 (16.4 ± 0.2 AU/g oil for the control RLO and 15.9 ± 0.6 AU/g oil for RLO supplemented with BHT) (Fig. 1.I). At this temperature, conjugated diene values

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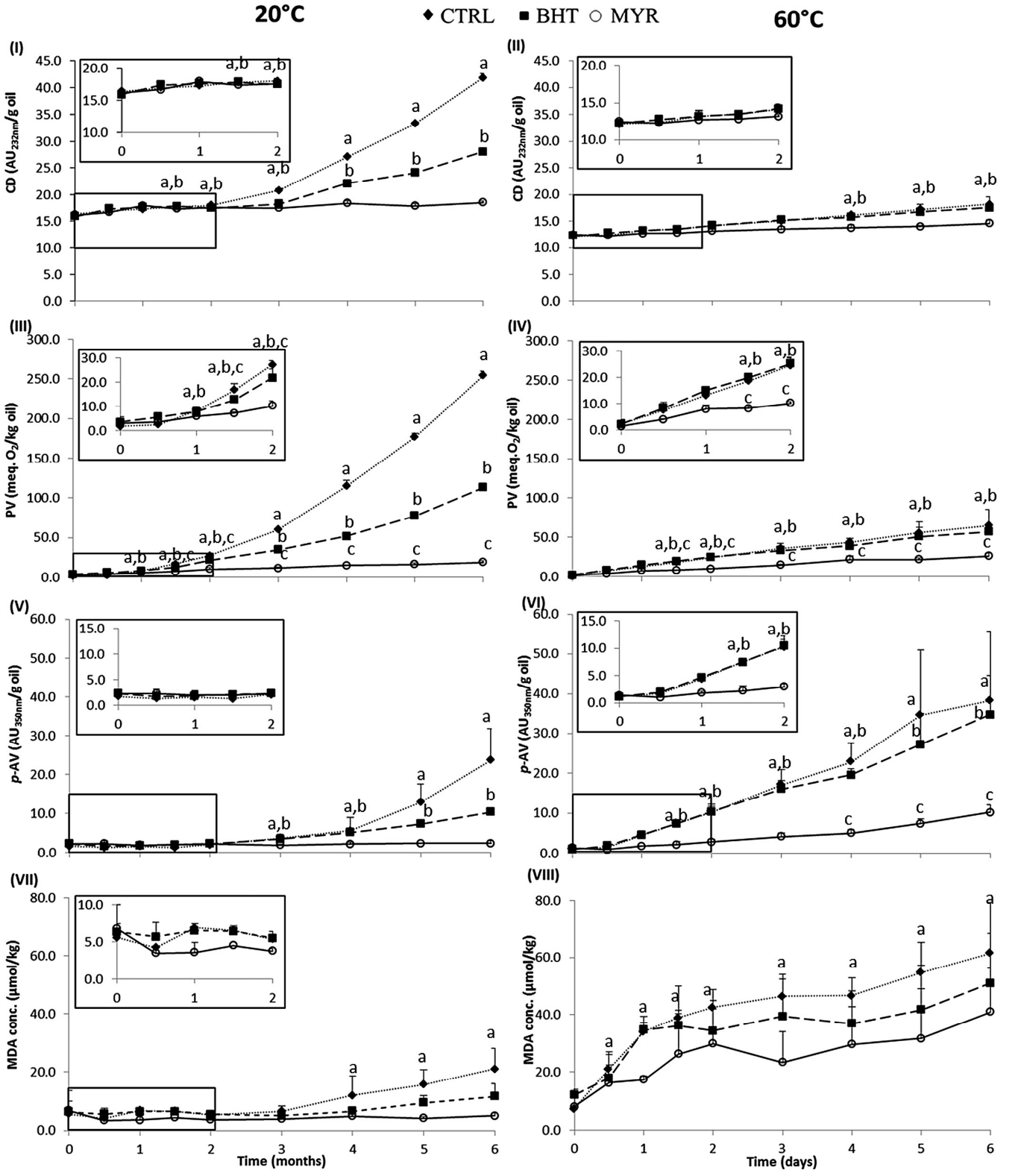

Fig. 1. Evolution of the products of oxidation expressed as conjugated dienes (CD, AU_{232nm}/g oil) (I, II), peroxide value (PV, mequiv.O_2/kg oil) (III, IV), p-anisidine value (*p*-AV, AU_{350nm}/g oil) (V, VI) and malondialdehyde (MDA) (VII, VIII) concentration (µmol/kg oil) in refined linseed oil during real-time aging at 20 °C for 6 months (I, III, V, VII) or accelerated aging at 60 °C for 6 days (II,IV,VI,VIII). Mean ± standard deviation (SD) of three independent experiments. Significant differences comparing day 0 to other times of storage are indicated by a letter ($P < 0.05$): a for control, b for BHT and c for myricetin. AU stands for Absorbance Unit.

became significantly different from those at day 0 ($p < 0.05$) after 1.5 month of storage for both control and RLO containing BHT, while no significant difference compared to day 0 was recorded for RLO containing myricetin (Fig. 1.I). At 60 °C, the absorbance value reached 18.2 ± 1.3 AU/g oil for the control aged 6 days and 17.5 ± 0.5 AU/g oil for the samples containing BHT, corresponding

to an increase of respectively nearly 50% and 43% compared to the absorbance value measured at day 0 (12.2 ± 0.4 AU/g oil for the control RLO and 12.2 ± 0.2 AU/g oil for RLO supplemented with BHT) (Fig. 1II). At 60 °C, conjugated diene values became significantly different from those at day 0 ($p < 0.05$) after 4 days of storage for both control RLO and RLO containing BHT, while, as it was observed at 20 °C, no significant difference compared to day 0 was recorded for RLO containing myricetin (Fig. 1II).

3.1.2. Peroxide value

At 20 °C, PV increased drastically in control RLO from 1.8 ± 0.3 meq.O_2/kg oil at day 0 to 254.3 ± 5.8 meq.O_2/kg oil after 6 months (Fig. 1III). In RLO samples supplemented with BHT or myricetin, the PV, after 6 months at 20 °C, was respectively 113.2 ± 3.4 and 18.9 ± 1.7 meq.O_2/kg oil. At this temperature, PV became significantly different from those at day 0 ($p < 0.05$) after 1 month of storage for both control and RLO containing BHT, and 1.5 month in RLO containing myricetin (Fig. 1III). After 6 days of storage at 60 °C, PV increased from 2.4 ± 1.5 meq.O_2/kg oil at day 0 to 65.2 ± 20.3 meq.O_2/kg oil in RLO without any added antioxidant, and to 57.2 ± 8.9 and 26.4 ± 3.6 meq.O_2/kg oil in RLO samples containing BHT and myricetin, respectively (Fig. 1IV). After 1.5 day of storage at 60 °C, PV became significantly different from those at day 0 ($p < 0.05$) for the control RLO, as well as for RLO containing BHT or myricetin (Fig. 1.IV).

3.2. Secondary oxidation products

3.2.1. p-anisidine value

At 20 °C, after 6 months of storage, PA values reached 23.7 ± 8.1 AU/g oil and 10.3 ± 0.8 AU/g oil in control and BHT supplemented RLO, respectively, while it remained close to the value measured at day 0 (i.e. 2.2 ± 0.5 AU/g oil) in RLO supplemented with myricetin (Fig. 1V). Compared to day 0, the PA values were significantly higher ($p < 0.05$) in both the control and the RLO containing BHT from three and six months of storage at 20 °C, respectively (Fig. 1V). At 60 °C, a significant accumulation of aldehydes could be observed after 1.5 day of storage when looking at the PA values for the control and the RLO supplemented with BHT, and after 4 days of storage for the RLO containing myricetin (Fig. 1VI). The observed values increased from 1.1 ± 0.5 AU/g oil (at day 0) to 38.3 ± 17.3 AU/g oil for the control and to 34.7 ± 9.9 and 10.2 ± 1.9 AU/g oil for the RLO containing BHT and myricetin, respectively, after 6 days of accelerated aging.

3.2.2. Aldehyde levels

At 20 °C, in the control, the MDA content increased from 5.6 ± 1.8 µmol/kg oil (day 0) to 21.1 ± 4.6 µmol/kg oil after 6 months of storage (Fig. 1VII). MDA level became significantly different ($p < 0.05$) from day 0 after 4 months of storage for the control while no significant difference compared to day 0 was observed for the RLO containing BHT or myricetin (Fig. 1VII).

At 60 °C, for the control, the MDA concentration increased from 7.3 ± 2.6 µmol/kg oil (day 0) to 61.5 ± 6.8 µmol/kg oil after 6 days of storage (Fig. 1VIII). As it was observed at 20 °C, a significant increase of MDA level was only observed in the control after 0.5 day of storage, while no significant difference compared to day 0 was observed in the RLO supplemented with BHT or myricetin (Fig. 1VIII).

Concerning the other aldehydes analyzed, 4-HNE and 2,4-decadienal were not detected in any sample at any time of aging, while 4-HHE was sporadically detected but always remained below the limit of quantification of the method (i.e. 6.1 µmol/kg oil).

4. Discussion

Without antioxidant, the conjugated dienes (Fig. 1I and II) and PV (Fig. 1III and IV) in RLO presented different patterns of increase, depending on the temperature. Indeed, they increased in a linear way at 60 °C while their increase was exponential at 20 °C. Moreover, the values obtained for these two parameters were nearly 3 times higher after 6 months at 20 °C than after 6 days at 60 °C, while the opposite trend has been observed for secondary oxidation products with aldehyde contents higher in RLO stored at 60 °C than in RLO stored at 20 °C. These observations suggest that the primary oxidation products accumulate in oil during real-time aging while they are transformed into other compounds during accelerated aging, mainly secondary oxidation products. As mentioned by Vieira and Regitano D'Arce (2001), conjugated dienes and hydroperoxides are very unstable at high temperature. They are then able to interact with other compounds (Cho, Endo, Fujimoto, & Kaneda, 1989) and they are rapidly transformed into secondary oxidation products like MDA (Zacheo, Cappello, & Perrone, 1998). Regarding MDA levels in the control during the accelerated aging, MDA formation was clearly faster at 60 °C and the final concentration (after 6 months at 20 °C or 6 days at 60 °C) was higher in RLO stored at 60 °C.

Whatever the measured oxidation products, myricetin was always better to prevent RLO from oxidation than BHT, at both temperatures. Regarding BHT, after 6 days of storage at 60 °C, the values of parameters measured in RLO containing this antioxidant were similar to those measured in the control. In contrast, during the aging experiment at 20 °C, the values of parameters measured in RLO containing BHT were lower than those observed for the control but higher than for the oil with myricetin. These findings could be explained by the fact that BHT could lose its antioxidant activity by reacting with secondary oxidation products (Chirinos et al., 2011). Concerning myricetin, this study confirms its high protective effect against lipid oxidation already shown in studies concerning ALA (Michotte et al., 2011), methyl linoleate (Pekkarinen, Heinonen, & Hopia, 1999) or lipid peroxidation in rat hepatocytes (Nuutila, Puupponen-Pimiä, Aarni, & Oksman-Caldentey, 2003).

5. Conclusion

When measuring PUFA oxidation, the difference in the behavior of RLO submitted to two different aging treatments, 60 °C or 20 °C, was visible after 3, 4 or 6 days or months of aging, respectively, depending on the oxidation products considered. It clearly appeared that the accelerated aging at 60 °C underestimates the primary oxidation products, as compared to a real-time aging at 20 °C, while the secondary products are overestimated at 60 °C.

In conclusion, the hypothesis that 1 day at 60 °C is equivalent to 1 month at 20 °C is not verified for RLO, with or without antioxidant.

Conflict of interest

All others should have no conflict of interest.

This article does not contain any studies with human or animal subjects.

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CHAPTER 4. DEVELOPMENT OF AN LC-MS/MS ANALYTICAL METHOD FOR THE SIMULTANEOUS MEASUREMENT OF ALDEHYDES FROM POLYUNSATURATED FATTY ACIDS DEGRADATION IN ANIMAL FEED

4. DEVELOPMENT OF AN LC-MS/MS ANALYTICAL METHOD FOR THE SIMULTANEOUS MEASUREMENT OF ALDEHYDES FROM POLYUNSATURATED FATTY ACIDS DEGRADATION IN ANIMAL FEED

Animal feed rich in n-3 fatty acids may contain extruded flax seeds, linseed oil or fish oil. Considering that, animal feed may be oxidized during storage because storage is performed at room temperature and generally lasts several months. The oxidation of fatty acids present in animal feed may thus lead to the production of primary oxidation products, such as hydroperoxides, and secondary oxidation products, such as aldehydes and ketones. Produced aldehydes include those for which a LC-MS/MS quantification method has been developed for linseed oil previously in the laboratory (Douny *et al.*, 2015b) but, according to information collected in Table 2, other aldehydes could be pertinent to be analyzed in view of their important presence as products of degradation of mono- and polyunsaturated fatty acids. Then, the LC-MS/MS method developed for the analysis of four aldehydes in linseed oil was subsequently extended to four other aldehydes considered as relevant: crotonaldehyde, hexanal, 2,4-nonadienal and benzaldehyde. Thus, virtually, all of the existing families of aldehydes are covered by the analytical method. Indeed, in each aldehyde family encountered in Table 2, at least one compound (between brackets) is studied: alkanals (hexanal), alkenals (crotonaldehyde), hydroxyalkenals (4-hydroxy-2E-hexenal, 4-hydroxy-2E-nonenal), alkadienals (2,4-decadienal), dialdehydes (malondialdehyde) and cyclic aldehydes (benzaldehyde).

The development of the method mainly consisted of an optimization of the parameters of the extraction of aldehydes out of samples of pet food (amount of sample, extraction solvent, volumes and time of extraction...). While new standards and internal standards (methylcrotonaldehyde, benzaldehyde-^{13}C, 4-HNE-D_3 and hexanal-D_{12}) were added to the method, the parameters of derivatization (DNPH concentration, solvent used...) as well as the nature of the post-derivatization solvent had also to be optimized. Finally, the optimized method has been validated according to the criteria and procedure described previously and applied to various samples of animal feed available in the laboratory.

The method development and validation in animal feed were published in Drug Testing and Analysis (Douny *et al.*, 2016b).

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Development of an LC-MS/MS analytical method for the simultaneous measurement of aldehydes from polyunsaturated fatty acids degradation in animal feed

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Knowing that polyunsaturated fatty acids can lead to the formation of potentially toxic aldehydes as secondary oxidation products, an analytical method using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) detection was developed to measure the concentration of eight aldehydes in animal feed: malondialdehyde (MDA), 4-hydroxy-2-nonenal (4-HNE), 4-hydroxy-2-hexenal (4-HHE), crotonaldehyde (CRT), benzaldehyde (BNZ), hexanal (HXL), 2,4-nonadienal, and 2,4-decadienal. The developed method was validated according to the criteria and procedure described in international standards. The evaluated parameters were specificity/selectivity, recovery, precision, accuracy, uncertainty, limits of detection and quantification, using the concept of accuracy profiles. These parameters were determined during experiments conducted over three different days with ground Kellogg's® Corn Flakes® cereals as model matrix for animal feed and spiked at different levels of concentration. Malondialdehyde, 4-HHE, 4-HNE, crotonaldehyde, benzaldehyde, and hexanal can be analyzed in the same run in animal feed with a very good accuracy, with recovery rates ranging from 86 to 109% for a working range going from 0.16 to 12.50 mg/kg. The analysis of 2,4-nonadienal and 2,4-decadienal can also be performed but in a limited range of concentration and with a limited degree of accuracy. Their recovery rates ranged between 54 and 114% and coefficient of variation for the intermediate precision between 11 and 25% for these two compounds. Copyright © 2016 John Wiley & Sons, Ltd.

Keywords: aldehydes; liquid chromatography; tandem mass spectrometry; animal feed; validation

Introduction

Nowadays, many nutritional and health studies recommend a higher consumption of fat composed of polyunsaturated fatty acids (PUFA), mainly n-3 polyunsaturated fatty acids.[1,2] Increasing the amount of n-3 fatty acids in animal feed is a way to increase human intake of those compounds through the consumption of food from animal origin other than fatty fish, the major natural dietary source of long chain n-3 fatty acids.[3–5] Consequently, many products enriched with n-3 fatty acids can be found in the market: meat, milk and dairy products, eggs, etc. A good way to increase the amount of n-3 fatty acids in animal feed is to use linseed (also known as flax or *Linum usitatissimum*) for its high α-linolenic acid content (more than 50% of the fatty acids)[3,6,7] or fish oil and microalgae for their eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) content.[8,9] Unfortunately, at these high polyunsaturated fatty acids levels there is rapid oxidation to other less desirous productsd[10–12]; consequently, it is important to monitor this potential oxidation pathway in animal feed because it can lead to less polyunsaturated fatty acids in meat, milk, or eggs than expected. The formed aldehydes, including malondialdehyde (MDA), 4-hydroxy-2-nonenal (4-HNE), 4-hydroxy-2-hexenal (4-HHE), crotonaldehyde (CRT), benzaldehyde (BNZ), hexanal (HXL), 2,4-nonadienal and 2,4-decadienal can be relatively stable and some of them have been shown to be cytotoxic and genotoxic by reacting with proteins and nucleic acids.[13–16] What is more, in 2011, in an advisory note from the Belgian Superior Health Council, MDA, crotonaldehyde, and 4-HNE have been considered of major concern for human health.[17] Until now, no maximum levels have been established for aldehydes in food or animal feed, mainly because of the lack of data in the literature about toxic concentrations, quantities contained in food or feed, and quantities of these oxidation products actually ingested.

Most of the published methods described to measure aldehydes in animal feed use a colourimetric method called the TBARS (thiobarbituric acid reactive species) method.[18–20] However, this method lacks specificity because it measures the total content of aldehydes able to react with thiobarbituric acid, expressed in malondialdehyde content, instead of measuring each compound individually.

Eight aldehydes that can be formed after PUFA oxidation were chosen to evaluate the lipid oxidation from linseed in animal feed (MDA, 4-HNE, 4-HHE, CRT, BNZ, HXL, 2,4-nonadienal and 2,4-decadienal (Figure 1)) and a liquid chromatography coupled

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to tandem mass spectrometry detection (LC-MS/MS) method was developed to measure their concentration as dinitrophenylhydrazone derivatives in animal feed. The developed method used methylmalondialdehyde (MeMDA), methylcrotonaldehyde (MeCRT), benzaldehyde-^{13}C, hexanal-D_{12} and 4-HNE-D_3 as internal standards. Subsequently, a validation protocol for the developed method was set-up, based on various performance parameters. As there is no specific validation criteria for aldehydes in food or feed matrices, our work was based primarily on the Decision of the European Commission 2002/657/EC,[21] a guideline used for the analysis of residues of veterinary drugs, which presents requirements concerning chromatographic techniques and mass spectrometry that can be applied here. Other parameters were also assessed according to the recommendations of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use.[22]

Materials and methods

Chemical reagents and materials

1,1,3,3-Tetraethoxypropane (TEP), 2,4-dinitrophenyl-hydrazine (DNPH), crotonaldehyde, benzaldehyde, hexanal, 2,4-nonadienal, 2,4-decadienal, methylcrotonaldehyde, benzaldehyde-^{13}C, hexanal-D_{12} and butylated hydroxytoluene (BHT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of 4-hydroxy-2-hexenal (4-HHE), 4-hydroxy-2-nonenal (4-HNE) and 4-hydroxy-2-nonenal-D_3 (4-HNE-D_3) were obtained from Cayman Chemicals (Ann Harbor, MI, USA). Methylmalondialdehyde (MeMDA) was synthetized from (dimethylamino)-2-methyl-2-propenal (DMP, Sigma-Aldrich, St. Louis, MO, USA) according to the method described by Syslovã *et al.*[23]

Water was of Chromanorm quality, while acetic acid 100% was of Normapur Quality and were both provided by VWR International (West Chester, PA, USA). Ethanol absolute for analysis and trichloroacetic acid were from Merck (Darmstadt, Germany). LC-MS grade acetonitrile was obtained from Biosolve (Valkenswaard, the Netherlands). Picograde hexane was from LGC Standard (Wesel, Germany). The filters were hydrophilic single use syringe filters (0.2 μm pore size, 25 mm, Chromafil) from Macherey-Nagel (Düren, Germany).

Stock solution of malondialdehyde 1 mg/mL (MDA) was obtained by hydrolysis of 1,1,3,3-Tetraethoxypropane (TEP) in trichloroacetic acid 5%. Stock solutions of the other aldehydes were prepared by dissolving the compound in water/ethanol 50/50 (*v/v*). A mixture of the 8 aldehydes of interest at a concentration of 8 (4-HHE and 4-HNE), 25 (hexanal) and 20 ng/μL (for the 5 other aldehydes) was prepared in water/ethanol 50/50 (*v/v*). Methylcrotonaldehyde (MeCRT), benzaldehyde-^{13}C, hexanal-D_{12}, 4-HNE-D_3 and methylmalondialdehyde (MeMDA) were used as internal standards in the quantitative analysis. A mixture of the six internal standards was prepared at a concentration of 25 (MeMDA, benzaldehyde-^{13}C and hexanal-D_{12}), 12 (MeCRT) and 0.8 ng/μL (4-HNE-D_3) in water/ethanol 50/50 (*v/v*). All of the standard solutions were kept for maximum 6 months at + 4°C.

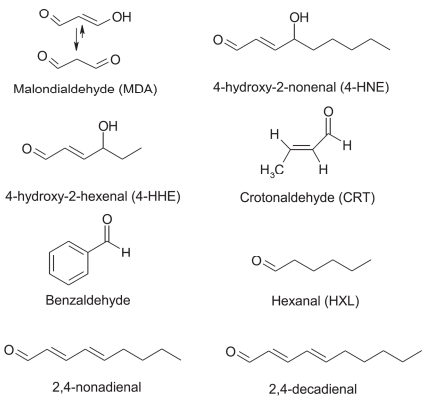

Figure 1. Structures of the 8 aldehydes analyzed: malondialdehyde (MDA), 4-hydroxy-2-nonenal (4-HNE), 4-hydroxy-2-hexenal (4-HHE), crotonaldehyde (CRT), benzaldehyde (BNZ), hexanal (HXL), 2,4-nonadienal and 2,4-decadienal.

Sample preparation

One gram (1 g) of mixed sample was weighed in a test tube, spiked with 200 μL of BHT solution (1 mg/mL in ethanol), 100 μL of the solution containing the six internal standards and 2200 μL water/ethanol 50/50 (*v/v*). The tube was vortexed for 15 s and then centrifuged at 3700 g for 10 min on a REAX Top vortex from Heidolph (Schwabach, Germany) and a Minifuge T centrifuge from Heraeus (Hanau, Germany), respectively. One mL of the supernatant was collected and filtered through a hydrophilic filter. The extraction was repeated a second time with 2.5 mL water/ethanol 50/50 (*v/v*) and supernatants were grouped and homogenized.

Dinitrophenylhydrazone derivatives were prepared by adding 750 μL DNPH solution (0.05 M in acetonitrile/acetic acid 9:1 (*v/v*)) to 750 μL of extract in an injection vial and reaction took place for 2h at 60°C. After the derivatization, 2 mL of water was added and the solution was vortexed. The derivatives formed were extracted with 4 x 1 mL hexane. The solvent was then evaporated to dryness in a SpeedVac concentrator (SVC100, Savant Instruments Inc., Farmingdale, NY, USA) and 100 μL of 0.03% acetic acid solution/acetonitrile 60/40 (*v/v*) was added to the tube. The solution was vortexed and transferred in an injection vial containing an insert.

LC-MS/MS analysis

Separation and detection of aldehydes as dinitrophenylhydrazone derivatives were performed using a ThermoFinnigan Spectra System P4000 HPLC and a ThermoFinnigan LCQ Deca ion trap mass spectrometer, equipped with an electrospray source. Separation was achieved on an Atlantis T3 C_{18} column (3 μm, 2.1 x 150 mm), with an Atlantis guard column T3 C_{18} (3 μm, 2.1 x 10 mm), both from Waters Corporation (Milford, MA, USA). The solvent flow was 0.25 mL/min, column temperature was set at 40°C and the injection volume was 20 μL. The mobile phase was acetonitrile (solvent A) and 0.03% acetic acid solution (solvent B). The gradient elution conditions were from 40 to 65% of solvent A in 8.5 min and from 65 to 100% in 4 min; then, conditions were held for 7.5 min and the contribution of solvent A was decreased to 40% over 2 min and maintained for another 6 min reconditioning. The analysis with the mass spectrometer was performed in MS/MS mode, with positive ionization for MDA and MeMDA and negative ionization for the other compounds.

Derivatized standards were synthesized in our laboratory according to the derivation protocol explained in Section Sample preparation and infused directly in the mass spectrometer to optimize the MS tune parameters. The capillary temperature was set at 300 °C. The derivative forms of the aldehydes and the internal standards were detected using the multiple reaction monitoring (MRM) mode, with two transitions for each compound (Table 1). Results

were calculated using Xcalibur Software (Thermo Fisher Scientific, Waltham, MA, USA).

Samples used for the calibration curves and validation of the method

For validation purposes, it was decided to use ground Kellogg's® Corn Flakes® cereals to mimic animal feed in calibration curves and for the validation of the method, knowing that this cereal contains 0.9% fat only and is thus poorly sensitive to oxidation. Kellogg's® Corn Flakes® cereals were bought in a local supermarket.

Calibration curves

Six samples of ground Kellogg's® Corn Flakes® cereals were spiked with the internal standards solution and five of them with concentrations ranging from 0.16 to 4.0 mg/kg for 4-HHE and 4-HNE, 0.15 to 7.5 mg/kg for MDA, crotonaldehyde, benzaldehyde, 2,4-decadienal and 2,4-nonadienal and 0.5 to 12.5 mg/kg for hexanal. Those six spiked samples were extracted simultaneously with the unknown samples, in order to build a calibration curve. The concentration range of the calibration curve was chosen to cover the range of concentrations observed previously in our laboratory for each aldehyde analyzed in oxidized animal feed containing high amounts of PUFAs. The response (ratio between each aldehyde and the internal standard peak areas) was plotted versus standard concentrations. Calibration points were injected before each series of unknown samples and the extract spiked at a concentration corresponding to the central point of the calibration curve was injected one more time after all the samples. A linear regression was used and no 'fit weighting' was applied. As quality control parameters, the correlation coefficients R^2 associated with those curves had to be higher than 0.98, and only one point could deviate from the curve by more than 20% of the corresponding calculated value.

Confirmation criteria

Each aldehyde was considered as positively identified in samples if the ratio between the chromatographic retention time of the analyte and that of the internal standard, i.e., the relative retention time (RRT) of the analyte, corresponded to that of the average retention time of the calibration solutions within a ±2.5% tolerance interval and the peak area ratio of the two transitions of the native analyte corresponded to that of the averaged transition ratio of the calibration solutions within the tolerances set by the Commission Decision 2002/657/EC.[21]

Validation of the method

The validation was performed according to the Commission Decision 2002/657/EC and following the most commonly used approaches.[24,25] The Commission Decision 2002/657/EC promotes the use of Certified Reference Materials (CRM) to validate an analytical method. Since no CRM of aldehyde is available in animal feed, Kellogg's® Corn Flakes® cereals ground and fortified with standard solutions of aldehydes were used to assess the performance of the method. As illustrated in Figure 2, the developed method was applied in ground Kellogg's® Corn Flakes® cereals and they were evaluated as 'blank' regarding their malondialdehyde, 4-HHE and 2,4-nonadienal contents. Indeed, those three compounds were either not detected or detected with their chromatographic peak areas close to those of the background noise peaks in Corn Flakes®. The five other aldehydes were detected in Corn Flakes® cereals but their chromatographic peak areas were smaller than those of the corresponding peak in the first point of the calibration curve (Figure 2).

Extractions of samples and injections of samples extracts were performed on three days. The low, medium, and high levels of concentrations tested are detailed in Table 2. Data generated during the validation of the method were processed with e-Noval 3.0 software, from Arlenda (Liège, Belgium).

Results and discussion

Method development

The method used in the present study was adapted from earlier work where 4 aldehydes (MDA, 4-HNE, 4-HHE and 2,4-decadienal) were analyzed in linseed oil, using MeMDA as the internal

Table 1. Ionization mode, mass of precursor and transition product ions, spray voltage and normalized collision energies followed in MS/MS for each compound analyzed

Compounds	ESI mode	m/z	Spray volage (kV)	Normalized collision energy (%)	Associated internal standard
MDA-DNPH	+	235>159 235>189	6.0	30	MeMDA-DNPH
4-HHE-DNPH	-	293>163 293>167	5.0	29	
Crotonaldehyde-DNPH	-	249>152 249>179	4.0	28	Methyl-crotonaldehyde-DNPH
Benzaldehyde-DNPH	-	285>163 285>238	4.0	28	Benzaldehyde-13C-DNPH
4-HNE-DNPH	-	335>167 335>288	4.0	32	4-HNE-D3-DNPH
Hexanal-DNPH	-	279>163 279>249	1.0	25	Hexanal-D12-DNPH
2,4-nonadecadienal-DNPH	-	317>230 317>270	1.5	30	
2,4-decadienal-DNPH	-	331>230 331>284	1.5	34	

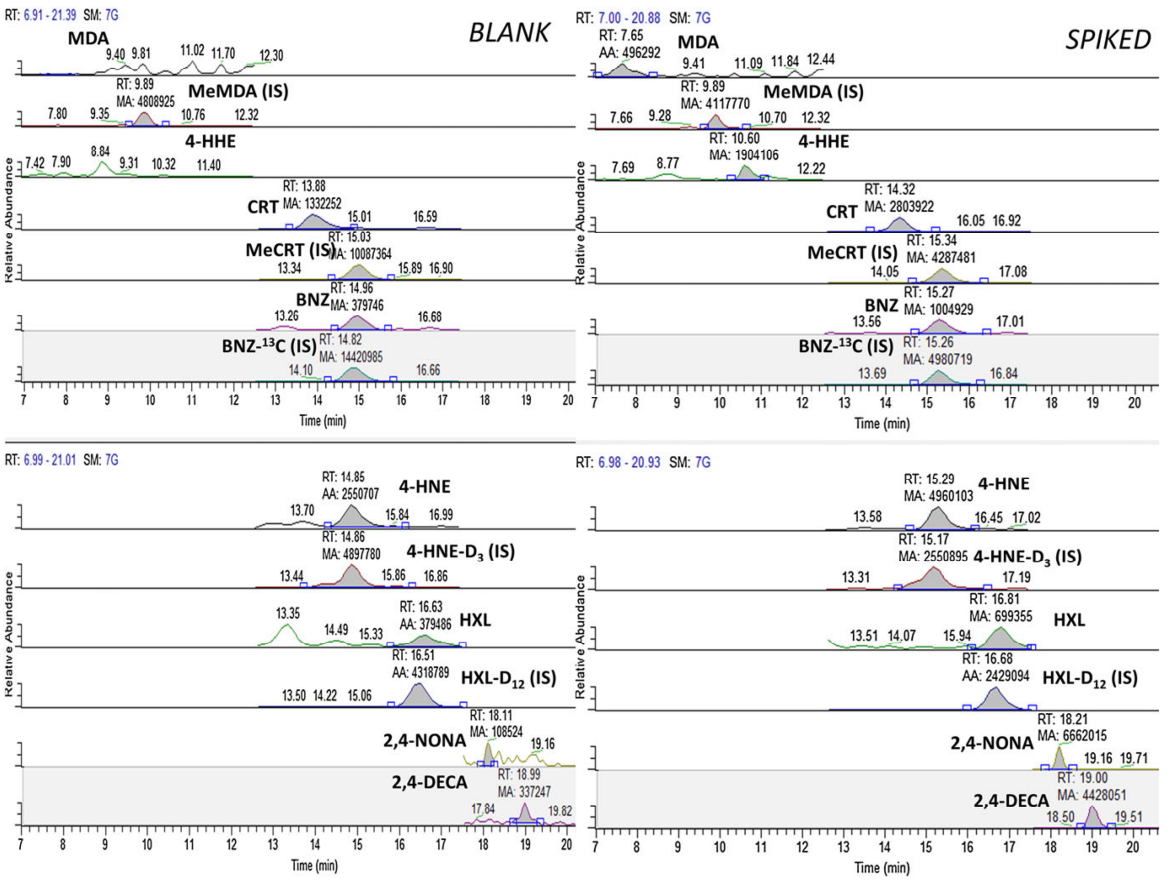

Figure 2. Chromatograms of aldehyde DNPH-derivatives analyzed by LC-MS in a blank Corn Flakes® cereal sample spiked with internal standards and a Corn Flakes® cereal sample spiked with internal standards and 0.4 mg*kg-1 malondialdehyde (MDA), Crotonaldehyde (CRT), benzaldehyde (BNZ), 2,4-nonadienal and 2,4-decadienal, 0.16 mg*kg-1 4-HHE and 4-HNE and 0.5 mg*kg-1 hexanal (HXL). These concentrations correspond to the first point of the matrix matched calibration curve.

standard.[26] Four aldehydes (CRT, HXL, BNZ and 2,4-nonadienal) and three internal standards (methylcrotonaldehyde (MeCRT), benzaldehyde-^{13}C, hexanal-D_{12} and 4-HNE-D_3) were added to the previously developed method for oil (Table 1). Unfortunately, no isotopic analogues of the 2,4-nonadienal or 2,4-decadienal were commercially available, therefore those two compounds were quantified using hexanal-D_{12}, the internal standard with the closest retention time. Because the cost of the stable isotope analogue of 4-HHE was prohibitively high, 4-HNE-D_3 was bought instead and used for 4-HHE and 4-HNE quantification. However, it was decided to quantify 4-HNE using 4-HNE-D_3 and 4-HHE using MeMDA instead of 4-HNE-D_3, according to preliminary results which were better when MeMDA was used as internal standard to quantify 4-HHE.

Increasing the number of aldehydes analyzed in a single run implied a consequent increase in the 2,4-dinitrophenylhydrazine (DNPH) concentration used during the derivatization step, leading to some DNPH solubility problem. In order to solve this problem, the optimization of the concentration of DNPH, the amount and type of acid and the solvent to be used were investigated. The DNPH concentration (0.0125 M, 0.025 M, 0.05 M, 0.1 M, and 0.15 M), the solvent (acetonitrile, ethanol) and the type of acid (hydrochloride, acetic, sulphuric, and phosphoric acids) in the DNPH solution were tested. The best compromise between solubility and chromatographic peak areas was obtained for the 8 aldehydes with a DNPH concentration of 0.05 M in acetonitrile/acetic acid (9/1, *v/v*). After checking that they were still suitable, the derivatization time and temperature were maintained at 60°C for 2 h, as previously optimized.[26]

To avoid the quick clogging of the chromatographic and spectrometric systems, an extraction of the dinitrophenylhydrazone derivatives obtained was considered. Prior to extraction, the addition of a post-derivatization solvent to increase the aqueous phase was investigated. No solvent or 2 mL of water, ethanol, acetonitrile, ethanol/water 50/50 (*v/v*), and acetonitrile/water 50/50 (*v/v*) were tested. The best condition was obtained when 2 mL water were used as post-derivatization solvent. According to several authors[27–29] the malondialdehyde hydrazones could be extracted with two or four times 1 mL hexane. The proposed extractions were tested to extract the dinitrophenylhydrazone derivatives of the eight aldehydes of interest and five internal standards and four times 1 mL hexane showed the best results. Indeed, Figure 2 shows the chromatograms of aldehyde DNPH-derivatives analyzed by LC-MS in a blank Corn Flakes® cereals sample spiked with internal standards and a Corn Flakes® cereals sample spiked with internal standards and 0.4 mg/kg malondialdehyde (MDA), Crotonaldehyde (CRT), benzaldehyde (BNZ), 2,4-nonadienal and 2,4-decadienal, 0.16 mg/kg 4-HHE and 4-HNE and 0.5 mg/kg hexanal (HXL), which

Table 2. Validation parameters of the LC-MS/MS method for the quantification of aldehydes in animal feed. The three values for repeatability, intermediate precision, recovery, linearity and expanded uncertainty are respective to the three mean introduced concentration values, corresponding to the low, medium, and high level, respectively

Aldehyde	Targeted spiking levels ($mg*kg^{-1}$)	Mean introduced concentrations ($mg*kg^{-1}$)	LOD ($mg*kg^{-1}$)	LOQ ($mg*kg^{-1}$) lower-upper	Repeatability (RSDr, %)	Intermediate precision (RSD_R, %)	Recovery (%)	Relative expanded uncertainty (%)	Linearity (R^2)	Calibration curve equations
MDA	0.40 – 3.00 – 10.00	0.45 - 3.00 – 10.35	0.12	0.40 – 10.00	8 – 7 – 4	11 – 8 – 4	109 – 100 – 104	25 – 16 – 7	0.9966	Y = -0.0214 + 1.0350 X
4-HHE	0.16 – 1.20 – 4.00	0.17 – 1.23 – 3.44	0.05	0.16 – 3.14	11 – 7 –7	12 – 7 – 15	104 – 102 – 86	25 – 15 – 34	0.9492	Y = 0.1125 + 0.8391 X
4-HNE	0.16 – 1.20 – 4.00	0.16 – 1.26 – 4.10	0.05	0.16 – 4.00	14 – 7 – 6	14 – 8 – 6	103 – 105–103	28 – 17 – 13	0.9919	Y = 0.0129 + 1.0230 X
CRT	0.40 – 3.00 – 10.00	0.43 – 3.00 – 10.11	0.12	0.40 – 10.00	7 – 4 – 4	7 – 5 – 4	106 – 100 – 101	14 – 11 – 8	0.9970	Y = -0.0046 + 1.0110 X
BNZ	0.40 – 3.00 – 10.00	0.40 – 2.99 – 10.24	0.12	0.40 – 10.00	6 – 3 – 2	6 – 4 – 3	100 – 100 – 102	12 – 8 – 7	0.9980	Y = -0.0479 + 1.0270 X
HXL	0.50 – 3.75 – 12.50	0.52 – 3.71 – 12.65	0.15	0.50 – 12.5	7 – 5 – 2	8 – 5 – 2	104 - 99 – 101	16 – 11 – 4	0.9987	Y = -0.03039 + 1.0130 X
2,4-Nona	0.40 – 3.00 – 10.00	0.46 – 3.45 - 7.87	0.12	0.40 – 6.36	13 – 9 – 5	18 – 16 – 20	114 – 115 –79	40 – 35 – 45	0.8866	Y = 0.6092 + 0.7420 X
2,4-Deca	0.40 – 3.00 – 10.00	0.39 – 3.09 – 5.35	0.13	0.43 – 4.80	14 – 11 – 10	19 – 11 – 25	97 – 103 – 54	40 – 22 – 56	0.6665	Y = 0.8122 + 0.4766 X

MDA = Malondialdehyde ; 4-HHE = 4-hydroxy-2-hexenal ; 4-HNE = 4-hydroxy-2-nonenal; CRT = crotonaldehyde; BNZ = Benzaldehyde; HXL= hexanal ; 2,4-Nona= 2,4-Nonadienal; 2,4-Deca = 2,4-Decadienal

are the concentrations of the respective aldehydes in the first point of the matrix matched calibration curve.

Method validation

During the three days of the validation, 20 ground cereal samples were spiked only with internal standards to be used as 'blank' samples and 60 ground cereal samples were spiked with internal standards and three different concentrations (low, medium, high; n=20 for each concentration) of aldehydes (Table 2), which correspond to the first, the fourth, and the sixth point of the calibration curve, respectively. In addition, during each of the three days of the validation, six ground Kellogg's® Corn Flakes® cereal samples were spiked with increasing concentrations of aldehydes to be used as calibration curves, leading to a total of 98 ground cereal samples. Samples were extracted, derivatized and analyzed in three independent series. Raw response data, without any correction, obtained for both calibration standards and spiked samples were computed using the validation software e-Noval. The parameters relevant to evaluate the performances of the developed method were selectivity/specificity, linearity, recovery, precision (repeatability and intermediate precision), accuracy, limit of detection (LOD), and limit of quantification (LOQ). Validation was performed using the concept of accuracy profile.[30,31]

Selectivity and specificity were demonstrated by showing the absence of significant peak or chromatographic peak areas smaller than those of the corresponding peak in the first point of the calibration curve in the blanks and the presence of quantifiable peaks in the spiked samples. When a peak was detected, it was shown that it fulfilled the confirmation criteria detailed in Section Confirmation criteria (Figure 2).

Accuracy profiles computed for the aldehydes are shown in Figure 3. Accuracy refers to the closeness of agreement between the test result and the accepted reference value.[32] The accuracy takes into account the total error, related to the test results. While no official regulatory standards have been set for aldehydes in food or animal feed, acceptance limits (dotted external lines) were selected arbitrarily at 50% for all aldehydes except for 2,4-nonadienal where the acceptance limits were selected at 70%. The solid central line represents the bias and the dots correspond to the relative back–calculated concentrations, plotted with respect to their targeted concentrations (expressed in percentage of variation from the targeted concentration). Internal dashed lines represent the 95% β-expectation tolerance interval (the result expected for a level included in the dosing range will fall in these limits, with a risk of 5% of being outside). The analytical procedure is considered as valid within the working range if the limits of the accuracy profile (dashed lines) are narrower than the acceptance limits (external dotted lines). For the MDA, 4-HNE, crotonaldehyde, benzaldehyde and hexanal, the 95% tolerance interval is located between the acceptance limits, demonstrating the validity of the method for the whole analytical range tested. For the 4-HHE, 2,4-decadienal and 2,4-nonadienal, the lower 95% tolerance interval is higher than the acceptance limit of 50% for the "high" level of concentration (66.3, 139.2, and 108.5%, respectively). These observations can be explained by the fact that 4-HHE, 2,4-decadienal and 2,4-nonadienal are the three aldehydes quantified by using an internal standard which is not their isotopic analogue or the same molecule with an added methyl group and which has a behaviour different from the one of the analyzed aldehydes. Target spiking levels, mean introduced concentrations (average of the real concentrations for all experiments), LOD, LOQ, repeatability, intermediate precision,

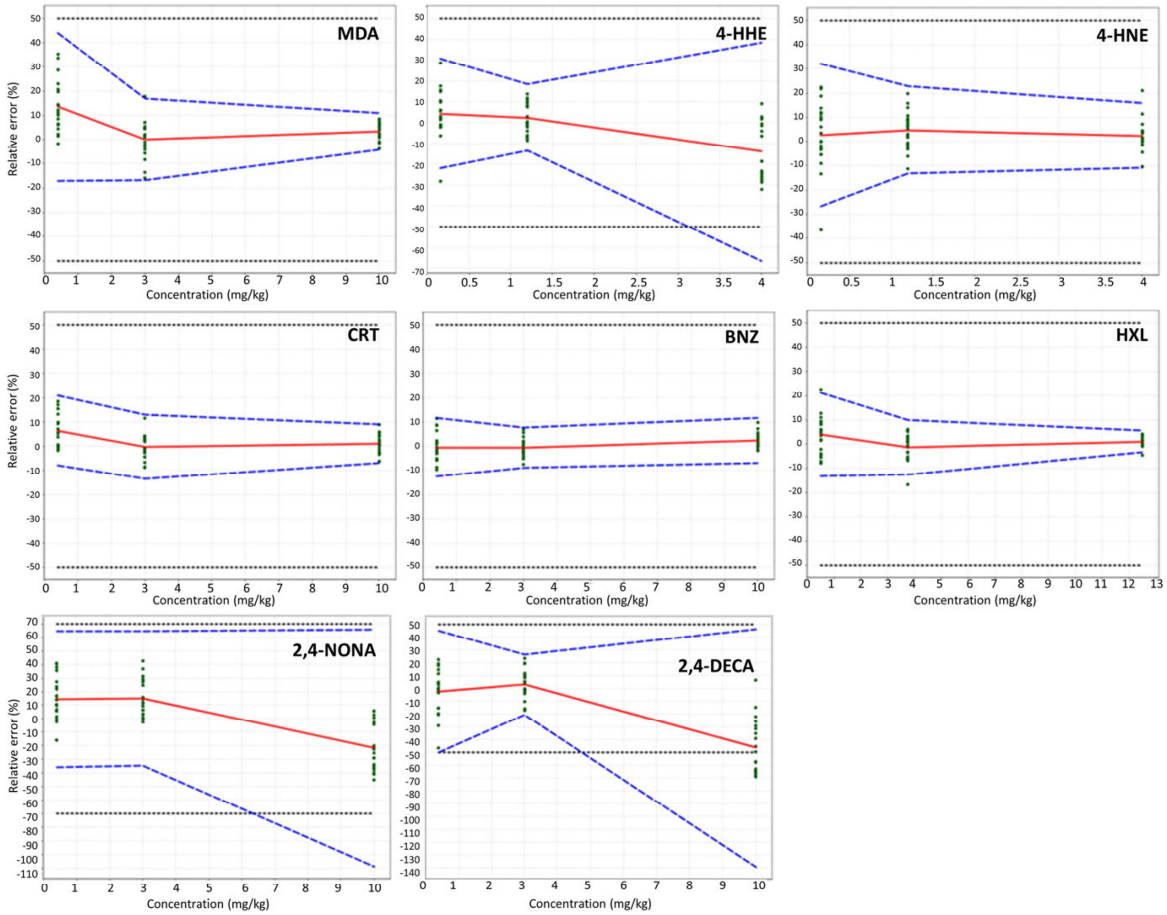

Figure 3. Accuracy profile of the 8 aldehydes, computed with e-Noval 3.0 (Arlenda). The dotted external lines represent the acceptance limits. The solid central line represents the bias and the dots correspond to the relative back–calculated concentrations, plotted with respect to their targeted concentrations (expressed in percentage of variation from the targeted concentration). Internal dashed lines represent the 95% β-expectation tolerance interval.

recovery, linearity (R^2), calibration curve equations, and uncertainty are summarized in Table 2.

LODs (the smallest quantity of the targeted substance that can be detected but not accurately quantified in the sample) are calculated within the e-Noval software by either dividing the lower LOQ by 3.3 or by adding three times the residual standard deviation obtained from an ANOVA test to the y intercepts of the calibration curves. The software chooses the smaller value obtained. The LODs were found below the lowest spiked level for all compounds (Table 2). The LOQ is defined here as the smallest quantity of the targeted substance in the sample that can be assayed under experimental conditions with well-defined accuracy.[32] The definition can also be applicable to the upper LOQ which is the highest quantity of the targeted substance in the sample that can be assayed under experimental conditions with well-defined accuracy. It is computed by the validation software as the smallest and highest concentrations beyond which the β-expectation limits go outside the acceptance limits. Depending on the compounds, the lower and upper LOQ ranged respectively from 0.16 to 0.50 and 3.14 to 12.5 mg/kg (Table 2).

The precision (closeness of agreement between independent test results) of the method is evaluated through its repeatability (variation of the results obtained in a same series), expressed as a relative standard deviation or RSD_r, and its intermediate precision (variation of the results obtained in different series), also expressed as relative standard deviation, and is often called RSD_R. The repeatability was between 2 and 14% and the intermediate precision was between 2 and 25% for the 8 aldehydes (Table 2).

The recovery (the proportion of the amount of analyte, added to the analytical portion of the test material, which is extracted and presented for measurement) ranged from 54% to 115% (Table 2). Recoveries met the criteria of the European decision 2002/657/EC,[18] which is set between 80 and 110% for MDA, 4-HHE, 4-HNE, crotonaldehyde, benzaldehyde and hexanal. The values obtained for 2,4-nonadienal and 2,4-decadienal ranged between 54 and 115%.

The measurement uncertainty (MU) (a parameter that characterizes the dispersion of the values that could reasonably be attributed to the measurand) is expressed here as a relative expanded uncertainty (a percentage of the result) and is calculated from the total variance of the β-expectation tolerance interval, using a coverage factor of 2. Relative expanded uncertainties ranged from 4% to 56% (Table 2).

Conclusion

An LC-MS/MS method was developed for the analysis of potentially toxic aldehydes as markers of the lipid oxidation in animal feed. The analytical method presented in this paper is novel compared to what is commonly found in the literature since this method allows to quantify in the same run, MDA and 7 other aldehydes: 4-HNE, 4-HHE, CRT, BNZ, HXL, 2,4-nonadienal and 2,4-decadienal, in animal feed. Most of the other published methods described in the literature were not validated and demonstrated to be fit-for-use for food or feed matrices.

The validation of the developed analytical method showed that MDA, 4-HHE, 4-HNE, CRT, BNZ, and HXL can be analyzed in a single run in animal feed with very good accuracy.

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CHAPTER 5.
GENERAL DISCUSSION, PERSPECTIVES AND CONCLUSIONS

5.1. General discussion

5.1.1. Effect of storage and cooking on fatty acids profile of omega-3 enriched eggs and pork meat marketed in Belgium

Nowadays, there is a will to promote healthy diets. In that field, a lot of nutritional studies and advices can be found regarding the fatty acid content of food and more specially the n-3 fatty acid content. A diet enriched with n-3 fatty acids is usually the advice proposed by scientists to decrease the n-6 to n-3 ratio in the food intake. In 2006, a Wagralim project called Walnut 20 was initiated in Wallonia. The goal of this project was, on one hand, to develop, validate or improve research tools to highlight the functionality of an ingredient and, on the other hand, to discover new compounds or mixtures of functional compounds by using these same tools to develop a range of nutri-functional products. At this period, different food enriched with n-3 fatty acids such as eggs, pork or chicken were available. Producers of pork and eggs enriched with n-3 fatty acids were among the 20 partners of this large project, explaining why eggs and pork were particularly studied in this thesis.

Despite all the recommendations promoting the consumption of polyunsaturated fatty acids, and more particularly of n-3 fatty acids, an important issue is to be taken into consideration: the oxidation of unsaturated fatty acids, resulting in the formation of toxic compounds, and thus possibly transforming a healthy food into a toxic food if it is not appropriately stored or transformed. The main objective of this work was the study of markers of lipid alteration in food using different analytical methods. These methods were dedicated to the study of fatty acids themselves but also aldehydes as degradation products in n-3 enriched food matrices such as eggs, pork, animal feed and linseed oil.

To study lipid alteration, our first choice was to follow the fatty acid content in food submitted to various cooking and storage conditions. To do that, a GC-MS method was developed to detect and quantify more than 20 fatty acids from C10:0 to C24:0 in fat extracted from various food matrices (eggs, meat, milk, oil …). The developed method fulfills the performance criteria required for a confirmatory method. Indeed, different parameters like retention time and ion ratios, selectivity, specificity, repeatability and reproducibility were checked with the help of the calibration curves prepared and the Certified Reference Material available (CRM, soya-maize oil blend).

The developed method was applied to determine the n-3 fatty acid profile of Belgian eggs and pork rich in n-3 fatty acids (Columbus™ eggs or pork), in order to determine to which extent the n-3 fatty acids resist to storage or cooking. The analysis confirmed that both n-3 enriched food products contained about 10 % of *alpha*-linolenic acid in their fat while, in this study, we measured no n-3 PUFA in standard pork fat and standard eggs contained around 2 % of n-3 polyunsaturated fatty acids. The piece of meat (shoulder) used for the study was relatively fat, leading to a high amount of triglycerides. Since long chain n-3 PUFA are mainly located in phospholipids, this can explain why no or few n-3 PUFA were detected in standard meat or eggs. What is more, the solvent of extraction being hexane, few phospholipids must have been extracted from the samples.

The conclusion of this study was that the n-3 fatty acids remained unchanged in the ready-to-eat product, except for some specific cooking processes (eggs cooked in custard and meat cooked in oven), where a slight statistically significant loss of PUFA in both Columbus™ eggs or pork was observed. Those results are corroborated by various studies found in the literature for eggs (Van Elswyk *et al.,* 1992; Botsoglou *et al*., 2012c; Cortinas *et al*., 2003) and pork (Janiszewski *et al*., 2016;) or other matrices like fish or lamb (Neff *et al*., 2014; Flakemore *et al*., 2017). As expected, when Columbus™ pork was cooked with culinary fat, its fatty acid profile was modified depending on the nature of the fat used, according to what was previously shown in the literature (Haak *et al*., 2007; Ramírez *et al*., 2005). That observation was also made by Neff and coworkers (2014) in Canadian freshwater fish fried with canola oil. Indeed, they observed that cooking treatments had little effect on n-3 fatty acid content; however, fried fish generally had higher n-6 and MUFA content, which is likely a result of the cooking oil used (Neff *et al*., 2014).

We observed a good stability of n-3 fatty acids during the experiments of storage and cooking. If we consider that a Columbus™ egg of 55 g contains 9 % fat, each Columbus™ egg contains 5 g of fat, among which there is 1.5 % or 0.075 g DHA. According to the Belgian Food Consumption Survey realized in 2014 (Federal Public Service - Public Health, Safety of the food chain and Environment, 2014), Belgian people consume an average of 10 g of egg per day, which corresponds to 0.014 g DHA. The Belgian Superior Health Council recommends a maximum consumption of 3 eggs per week, if Columbus™ eggs are consumed, it can lead to 0.225 g DHA per week or 0.032 g DHA per day. Since an adult is supposed to ingest between 0.25 and 0.5 g of EPA+DHA each day (Belgian Superior

Health Council, 2016), the consumption of Columbus™ eggs instead of standard eggs could be an easy way to increase the intake in LC n-3 PUFA.

As suggested in different studies (Onal *et al*., 2002; Botsoglou *et al*., 2012a; Jones *et al.*, 2015), the stability of n-3 fatty acids to cooking or storage could be explained by the protective effect of *alpha*-tocopherol. Indeed, *alpha*-tocopherol is naturally present in raw eggs (Murcia *et al*., 1999) and is also brought by the Columbus™ feed (150 mg *alpha*-tocopherol/kg feed) (Biron, personal communication). The *alpha*-tocopherol content of the food submitted to cooking or storage was not evaluated in this work.

5.1.2. Study of the oxidative stability of linseed oil

Since we noticed that the fatty acid profile was quite stable in food submitted to oxidation, the study of degradation products instead of fatty acid profile was found more suitable. Because hydroperoxides are known to be unstable and to be rapidly converted into secondary oxidation products (Vieira *et al*., 2001; Gotoh *et al*., 2007), among those compounds, the choice was made to study aldehydes such as malondialdehyde, 4-HNE, 4-HHE …. Two LC-MS/MS methods were developed to detect and quantify aldehydes: one in linseed oil and one in animal feed.

The developed methods were validated using the concept of accuracy profile (Boulanger *et al*., 2003; Hubert *et al*., 2004; Feinberg *et al.,* 2004 & 2006). They fulfilled the performance criteria required for a confirmatory method. Indeed, different parameters like retention time and ion ratios, selectivity, specificity, repeatability and reproducibility were checked with the help of matrix-matched calibration curves and spiked samples of coconut oil to mimic linseed oil and ground Kellogg's® Corn Flakes® cereals to mimic animal feed.

The validation of the method developed for linseed oil showed that it is possible to analyze MDA, 4-HHE, 4-HNE and 2,4-decadienal in oil samples, in the same run, with a very good accuracy for MDA, and a defined accuracy at specified concentrations for the three other aldehydes. When linseed oil samples subjected to storage at high temperature (60 °C) using the ‘‘Schaal oven stability test" during 24 days were analyzed, 4-HHE was the most produced aldehyde with high levels (above 80 µmol*kg−1), indicating that 4-HHE is a relevant marker of linseed oil oxidation. Long and coworkers conducted experiments designed to test the neurotoxic potential of 4-HHE. They observed that 4-HHE and 4-HNE were toxic to primary cultures of cerebral cortical neurons with LD50's of 23 and 18

µmol/L, respectively (Long *et al.,* 2008). This means that 4-HHE could be potentially toxic at the levels found in linseed oil after storage of several days at 60 °C.

The results obtained are corroborated by Guillén and coworkers (2012) who found 4-HHE as a major compound formed when virgin linseed oil was fried at 190 °C for 3.6 and 20 h. Other authors (Surh *et al*., 2005; Guichardant *et al.,* 2006a; Han *et al.*, 2009) reported as well that 4-HNE and 4-HHE are α,β-unsaturated aldehydes mainly formed during the oxidation of n-6 and n-3 fatty acids, respectively. These 2 compounds showed also their importance in other matrices. Indeed, in 2009, Guillén and Uriarte studied sunflower oil submitted to 190 °C in a domestic fryer and they observed that some secondary oxidation compounds such as aldehydes are generated very early, among them, the genotoxic and cytotoxic 4-hydroxy-trans-2-alkenals such as 4-HNE and 4-HHE (Guillén *et al*., 2009).

Since lipoxygenase is present in a wide variety of plants and vegetable oils, long storage of linseed oil can lead to lipolysis and high amounts of free fatty acids which are known to be more sensitive to oxidation. This can explain the high levels of 4-HHE that were detected in linseed oil. However, as reported by Buranasompob and coworkers (2007), LOX in dry pinto beans lost 100 % activity after 15 sec exposure to 100 °C and 93 % of the initial activity after a 10 min exposure to 65 °C. Another publication reported that LOX activity of the crude extracts of almonds was lost after 10 min exposure to 80 °C (Zacheo *et al*., 2000). As literature lacks information about the effect of time and temperature on lipoxygenase activity in linseed oil, it could be interesting to study it.

Surh and coworkers evaluated the 4-hydroxyalkenal content and fatty acid compositions in various oils (sesame, perilla, olive, corn, soybean, pepper seed, sunflower, safflower, canola and rice bran), fish and shellfish (Surh *et al*., 2010). They observed strong correlations between 4-HNE contents and n-6 PUFA or PUFA. However, in their study, a low quantitative correlation was observed between 4-HHE and its source fatty acids, namely the n-3 PUFA.

The analytical method developed for linseed oil was also used to verify if the hypothesis that an accelerated aging of oil of one day at 60 °C is equivalent to a real-time aging of one month at 20 °C. Indeed, this hypothesis is often used for various oils (Khan *et al.*, 2001; Cao *et al*., 2014). To do that, the comparison of aging of linseed oil for 6 months at 20 °C and for 6 days at 60 °C using the ‘‘Schaal oven stability test” was done. In this study, oxidation was evaluated with the peroxide value and *para*-anisidine value as well as with

the content in conjugated dienes and aldehydes. All four indicators of oxidation showed very different kinetic behaviors at 20 and 60 °C. Indeed, conjugated dienes and peroxide value increased in a linear way at 60 °C while their increase was exponential at 20 °C. Moreover, the values obtained for these two parameters were nearly 3 times higher after 6 months at 20 °C than after 6 days at 60 °C, while the opposite trend was observed for secondary oxidation products with aldehyde contents higher in oil stored at 60 °C than at 20 °C. These observations suggest that the primary oxidation products accumulate in oil during real-time aging while they are transformed into other compounds during accelerated aging, mainly secondary oxidation products. The hypothesis that an aging of one day at 60 °C is equivalent to an aging of one month at 20 °C was thus not verified for linseed oil.

5.1.3. Development of an LC-MS/MS analytical method for the simultaneous measurement of aldehydes from polyunsaturated fatty acids degradation in animal feed

Because most of the published methods described to measure aldehydes in animal feed use the TBARS method (Sheehy *et al.,* 1994; Yang *et al*., 2012; Song *et al*., 2013; Shurson *et al*., 2015), it was necessary to develop a more specific method, capable of quantifying aldehydes individually. The method used in the present study was adapted from earlier work where 4 aldehydes (MDA, 4-HNE, 4-HHE and 2,4-decadienal) were analyzed in linseed oil, using MeMDA as the internal standard (Douny *et al*., 2015b). Four aldehydes (crotonaldehyde, hexanal, benzaldehyde and 2,4-nonadienal) and four internal standards (methylcrotonaldehyde (MeCRT), benzaldehyde-^{13}C, hexanal-D_{12} and 4-HNE-D_3) were added to the previously developed method for oil. The validation of the developed method based on the accuracy profile showed that the accuracy of the method was good when MDA, 4-HNE, crotonaldehyde, benzaldehyde and hexanal were analyzed in animal feed, demonstrating the validity of the method for the whole analytical range tested. On the other hand, 4-HHE, 2,4-decadienal and 2,4-nonadienal showed a restricted accuracy when analyzed in animal feed with the developed method. These observations can be explained by the fact that 4-HHE, 2,4-decadienal and 2,4-nonadienal are the three aldehydes quantified by using an internal standard which is not their isotopic analogue or the same molecule with an added methyl group and which has a behavior different from the one of the analyzed aldehyde.

5.2. Future perspectives

As future perspectives to complete this work, the assessment of the consumer exposure to these aldehydes from food should be further developed. Indeed, there are a limited number of studies published concerning the dietary exposure of humans to these potentially toxic aldehydes.

Concerning crotonaldehyde, very few exposure studies from food are available in the literature. In studies realized between the 1960s and the 1980s, strongly varying concentrations of crotonaldehyde are reported to occur in food, e.g., in fish (71–1000 µg * kg^{-1} (Yurkowski *et al.,* 1965; Yoshida *et al.,* 1984)), in meat (10–270 µg*kg^{-1} (Noleau *et al.,* 1986; Cantoni *et al*., 1969)), and in fruits and vegetables (1–100 µg*kg^{-1} (Winter *et al*., 1964; Linko *et al*., 1978)). According to Eder and coworkers, these crotonaldehyde levels are relatively low compared to those found in cigarette smoke (Eder *et al.,* 2001).

A study was conducted in Korea, where 4-HHE and 4-HNE were determined in 56 commercially available PUFA-fortified foods, including infant formulas and baby foods but also soymilks, cereal powders, yoghurts, milks, fruit juices, fish sticks, tuna cans and snacks (Surh *et al*., 2007). 4-HHE and 4-HNE were respectively present at <10-77 and 41–132 µg kg^{-1} in the infant formulas (n=12) and at <10-52 and 36–116 µg kg^{-1}, in the baby foods (n=7), all coming from Korean supermarkets. Low correlations were observed between fortified amounts of PUFA and the concentrations of 4-hydroxy-2E-alkenals for the infant formulas and baby foods, and no correlations for the other samples. According to the authors, there are several possible reasons to account for the lack of strong correlation between the aldehyde products and their source fatty acids in real food lipids:
(i) a difference in the contents of antioxidative nutrients (tocopherol, ascorbic acid…) or pro-oxidative metals (iron, zinc, copper…); (ii) a difference in the fatty acids composition depending on each food (iii) a difference in manufacturing process (iv) the decomposition of 4-hydroxy-2-alkenals following accumulation of the initially formed 4-hydroxy-2-alkenals (v) further interaction of 4-hydroxy-2-alkenals with other constituents in the samples (Surh *et al*., 2007).
The authors concluded that the generation of 4-hydroxy-2E-alkenals appeared to be governed not only by the addition of PUFA, but also by PUFA originally present in foods. Based on the results observed and the manufacturer's instructions for infant formulas and

baby foods, babies' exposure was estimated at a maximum of 20.2 and 12.0 mg * kg^{-1} body weight per day for a 3-month-old and a 1-year-old, respectively, which was two orders of magnitude higher than that of Korean adults.

Recently, Papastergiadis and coworkers realized an exposure assessment of malondialdehyde, 4-hydroxy-2E-nonenal and 4-hydroxy-2-E-hexenal through specific foods available in Belgium (Papastergiadis *et al*., 2014b). Due to lack of specific toxicological data, the concept of Threshold of Toxicological Concern (TTC) was applied. This concept is a useful tool for preliminary risk characterization of chemicals of known structure to which humans are exposed at low levels, but for which no specific toxicity data are available (Rennen *et al*., 2011; EFSA, 2016). In the study of Papastergiadis and co-workers (2014b), a TTC of 30 µg/kg body weight per day was accepted for MDA and 1.5 µg/kg body weight per day for 4-HNE and 4-HHE. A total of 390 samples (from plant oils, dry nuts, biscuits, salmon, milk, cheese, ready-to-eat meals…) were purchased from Belgian supermarkets, except for French fries and fried snacks that were purchased from local fast food shops. The authors concluded that consumers of the studied food categories are mainly exposed to MDA and less to 4-HNE. Exposure to 4-HHE was not found to be significant. The results presented in this paper show that the consumer exposure to MDA, 4-HNE and 4-HHE is lower than their respective TTC and suggest thus that no risk could be identified related to the consumption of these foods for the vast majority of the consumers.

In the study from Papastergiadis and coworkers, the Belgian food samples that were studied were not particularly rich in n-3 fatty acids, except for salmon (Papastergiadis *et al*., 2014b). As mentioned above, ten years ago, began the Walnut-20 project, in the framework of Wagralim, the Walloon Agro-industry cluster. Walnut-20 focused on n-3 fatty acid enriched products, which are still presented to consumers, nowadays, as "healthy" products (e.g. n-3 pork promoted by Delhaize under the label "Mieux pour tous" or "Better for everyone"). The research done in this thesis should be completed with extensive studies about aldehyde formation during storage and cooking in these enriched n-3 foods from animal origin. These studies will allow to assess comprehensively the exposure to these aldehydes from Belgian food, which could be higher if n-3 fatty acid enriched food is considered, like Columbus™ eggs or pork, and not only "standard food".

5.3. Conclusions

The analytical tools developed in this thesis (i.e. GC-MS method to analyze fatty acids in oil and LC-MS/MS method to analyze aldehydes in food and feed) showed good performances.

In particular, the LC-MS/MS analytical methods presented in this work for aldehydes bring something new compared to what is usually published since our methods allow quantifying, in the same run, MDA and other aldehydes in oil or feed samples while most of the methods found in literature focus either on the analysis of a single compound (MDA) or on other aldehydes, but scarcely MDA with other aldehydes in the same analysis. Moreover, the published methods are not validated for food matrices.

The developed methods settled during this thesis aimed to measure up to 8 aldehydes in food and feed; they presented some limitations for 3 aldehydes due to the availability or price of internal standards, but were suitable to evaluate the major markers of lipid alteration in n-3 fatty acid enriched food.

Knowing that these aldehydes show the capacity to form DNA-adducts in human after n-3 rich food intake (see section 3.1.) and thus are possible mutagenic compounds, it is of very high importance to be able to measure these compounds in food and feed but also on biological fluids (blood, urine, feces), in order to perform biomonitoring studies.

As very little is known about aldehydes absorption and metabolism in human, the analytical methods developed here could help for example to study absorption and metabolism in *in vitro* models such as intestinal Caco2 cells or the SHIME (Simulator of the Human Intestinal Microbial Ecosystem) model.

In food industry, another use of the developed methods would be as tools for the monitoring or kinetics of oxidation of oils or other n-3 PUFA rich food in a quality control approach.

Even if analytical methods are available to analyze and quantify specifically the potentially toxic aldehydes present in food, toxicological data and exposure evaluation are still lacking and should be conducted in various countries and on various kinds of food to allow a global evaluation on the situation of aldehydes in food.

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LIST OF PUBLICATIONS OF CAROLINE DOUNY

The full list of publications of Caroline Douny can be accessed at:

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