To Mari for her patience

A contribution to the risk assessment in relation to the formation of toxic aldehydes in foods as a result of lipid oxidation

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Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences: Chemistry

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Dutch translation of the title :

Een bijdrage tot de risicobeoordeling van de vorming van toxische aldehyden in levensmiddelen als gevolg van lipide oxidatie

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INTRODUCTION AND OBJECTIVES

INTRODUCTION AND OBJECTIVES

Lipid oxidation occurring in foods, especially those containing poly unsaturated fatty acids (PUFAs), results in the contamination of foods with secondary lipid oxidation products. A portion of these secondary lipid oxidation products consists of aldehydes with a potential toxicity. However, the dietary exposure of consumers to these compounds and the potential risk this exposure represents has not been well studied. Therefore, the main goal of this PhD was to contribute to the risk assessment in relation to the dietary intake of hazardous aldehydes present in foods available in the Belgian market as a result of lipid oxidation. The objectives set to reach this goal are discussed further.

The study was focused on particular aldehydes and their selection was based on the available information concerning their occurrence in foods and potential health hazards could introduce to humans. The selected aldehydes included malondialdehyde (MDA), 4-hydroxy-2(E)-nonenal (HNE), 4-hydroxy-2(E)-hexenal (HHE) and 2-butenal. MDA is a well-known secondary lipid oxidation product present in foods and has been used in several studies as a marker of lipid oxidation. The presence of hydroxylated unsaturated aldehydes such as HNE and HHE in foods has recently attracted the interest of researchers. However, as for MDA, the levels of dietary exposure of these compounds has not been studied and any potential risk remains unknown. 2-butenal is known to be a secondary oxidation product of polyunsaturated fatty acids but there is a lack of information related to the levels this compound is present in a free form in foods. The interest of monitoring this compound in foods is closely associated to its high chemical reactivity and therefore its potential hazardous nature to humans.

Knowledge of the origin of the studied compounds, as well as their chemical properties is an essential requirement. The lipid oxidation mechanism, proposed pathways of formation of the studied aldehydes and a description of risk assessment are presented in *Chapter 1*. Assessing the exposure to the studied aldehydes through the diet requires accurate and reliable data of the occurrence of these compounds in consumed foods. Thus, availability of reliable analytical methods to achieve this aim is essential. However, the reliability and practicality of the analytical methods available in the literature was found to be questionable. Thus, a systematic evaluation,

and when necessary, development of new reliable methods was considered essential. The **first objective** of this research was the evaluation of the available analytical methods for determination of the studied aldehydes and the development and validation of new methods.

The ability of the spectrophotometric Thiobarbituric Acid Reactive Substances (TBARS) test traditionally used for determination of MDA in foods was evaluated (*Chapter 2*). This study indicated the non-specific nature of this method towards determination of MDA in various food matrices. Therefore, a more specific liquid chromatographic method involving fluorescence detection was suggested.

With regard to the determination of HNE and HHE in foods, the analytical methods available in the literature were not found reliable and appropriate for analyzing large amounts of samples in a relatively short period. Thus, a reliable stable isotope dilution assay using GC-MS for the simultaneous determination of HNE and HHE in various food matrices was developed and validated (*Chapter 3*).

Concerning quantification of 2-butenal in various food matrices, a reliable stable isotope dilution assay using HS-GC-MS was developed and validated (*Chapter 4*). For this reason 2-butenal *d6*, a deuterated form of 2-butenal, was synthesized in the lab and was used as an internal standard. All the analytical methods developed and validated were the foundation of the following objectives.

Investigation of the factors influencing the formation and the behavior of the studied aldehydes was the **second objective** of this research. Even though the fatty acids responsible for the production of these aldehydes have been identified and are well understood, little is known about the effect intrinsic and extrinsic parameters may have in the formation of these compounds in foods during processing and storage. Thus there is a particular interest in understanding how factors such as heating, storage period, exposure to light could affect the formation of the studied aldehydes. In *Chapter 4* results related to the formation of 2-butenal in oils subjected to frying temperatures and the effect of different fatty acid profiles are presented. Furthermore, the formation of 2-butenal in foods during frying was investigated. In *Chapter 6* the formation of MDA, HNE, HHE and 2-butenal in oil-in water emulsions during auto- and photo-oxidation as well as the effect of different fatty acid profiles was investigated.

The **third objective** of this research was the dietary exposure assessment of the Belgian population to the studied aldehydes and the evaluation of the potential risk (*Chapter 5*). The aldehydes involved in this study have been used as indicators for lipid oxidation in food matrices, oils as well as biological samples. However, the levels of dietary exposure remain largely unknown and are not acknowledged. Given the hazardous nature of these compounds and the limited knowledge related to the levels of exposure, a systematic evaluation of any potential risk was performed. For this reason, the levels of these aldehydes were determined in selected food categories purchased from the local market and data related to the consumption of these products were obtained. Probabilistic and deterministic approaches were applied for exposure assessment. The Threshold of Toxicological Concern (TTC) was applied for the characterization of the potential risk.

The research performed to achieve the aforementioned objectives and reach the goal of this PhD study are presented throughout the six chapters of this dissertation. In *Chapter 7* the conclusions and future perspectives of this research are presented. The outline of this research is illustrated in ***Figure I***.

The work presented in this PhD thesis was part of a research funded by Service of Contractual Research of the Belgian Public Service of Public Health, Safety of the Food Chain and Environment. In this project also other aspects related to the presence of reactive secondary oxidation products in foods were studied by the author of this thesis, which are however not elaborated in the experimental chapters. In particular these aspects related to experiments with respect to the analytics of acrolein and the behavior of selected aldehydes in protein containing emulsions. For the sake of completeness of the general discussion presented in *Chapter 7* however, reference will be made on some occasions with respect to the observations made in these particular experiments.

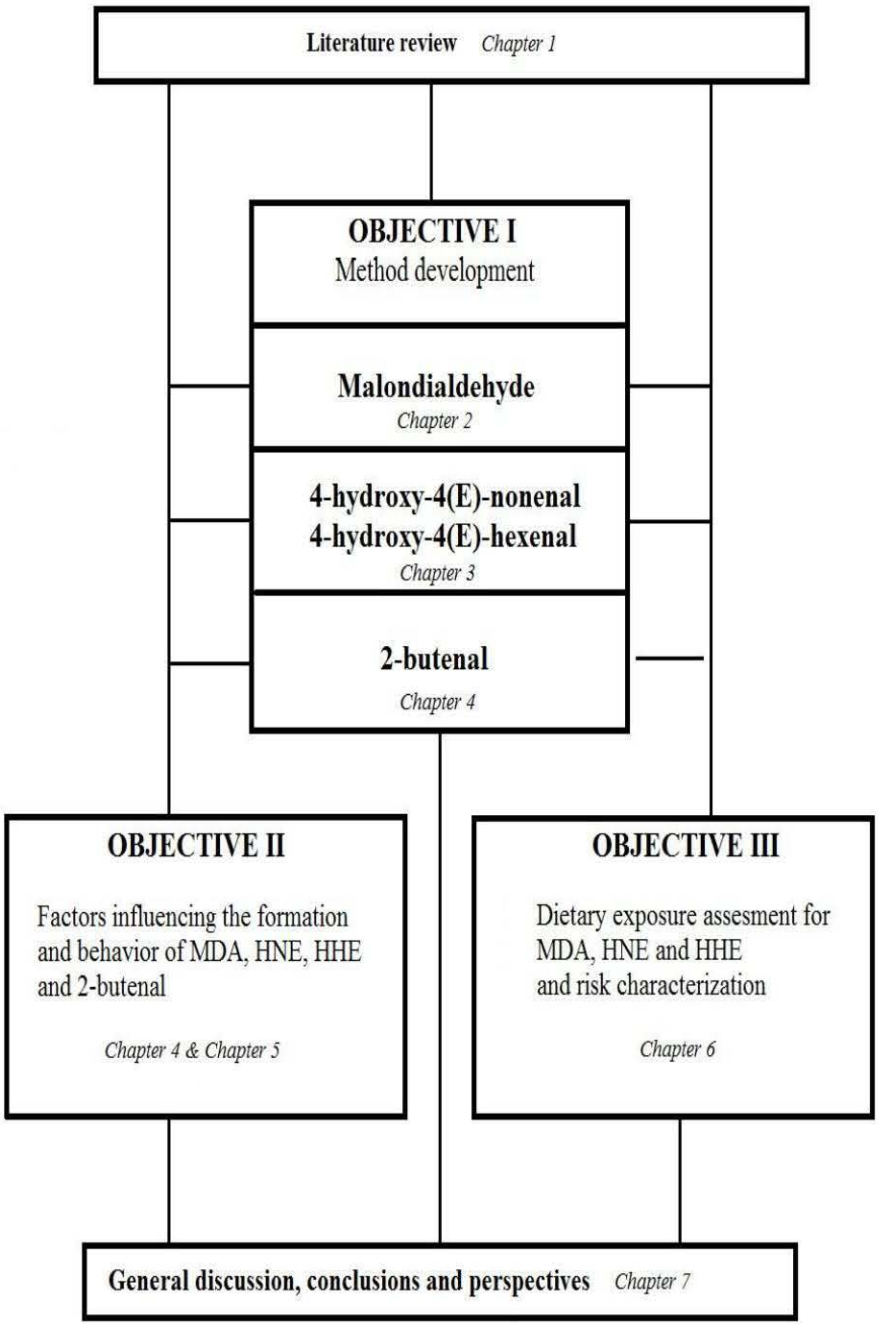

FIGURE I Schematic outline of the objectives of the PhD study

CHAPTER 1

LITERATURE REVIEW

RISK ASSESSMENT AND CHEMISTRY OF MALONDIALDEHYDE , 4-HYDROXY-2-(E)-NONENAL, 4-HYDROXY-2-(E)-HEXENAL AND 2-BUTENAL IN FOODS

CHAPTER 1. LITERATURE REVIEW: RISK ASSESSMENT AND CHEMISTRY OF MALONDIALDEHYDE OF 4-HYDROXY-2-(E)-NONENAL AND 4-HYDROXY-2-(E)-HEXENAL AND 2-BUTENAL IN FOODS

1.1 INTRODUCTION

Lipid oxidation is one of the major causes of chemical deterioration of foods. The mechanisms of hydroperoxides formation have been well studied and understood within the last fifty years and the secondary lipid oxidation products have also been identified up to an extent (Frankel, 2005). Several modern analytical techniques have been applied in order to gain the profile of the lipid oxidation related compounds responsible for alterations in flavors of foods . This direction in research is of great importance since it gives a valuable insight on the impact of lipid oxidation on the consumers perception toward foods. However, possible health implications related to consumption of oxidized foods should also be studied in detail. In the current status there are a few studies investigating possible adverse health effects related to the consumption of oxidized foods (Surh & Kwon, 2005; Del Rio, Stewart, & Pellegrini, 2005).

Risk assessment is a powerful tool that can be applied for evaluation of the risk consumers may face due to consumption of oxidized foods. Initially the potential hazard has to be identified (Vinci et al., 2012). With respect to lipid oxidation related compounds present in foods, this implies that extensive screening should be performed in order to have a good knowledge of which compounds are present. This is a dynamic field, since up to date new compounds resulting from lipid oxidation are reported. Once the compounds are identified, their potential hazard, if not known, should be evaluated. In the present study MDA, HNE, HHE and 2-butenal were chosen among other secondary lipid oxidation products and this decision was based on scientific evidence indicating their potential toxicity. The interest in the research presented in this dissertation was exclusively pointed towards the aldehydes present in the food matrix in a free form, not bound to other food components (i.e. proteins).

Further in this chapter an introduction to risk assessment is presented, followed by a short description of lipid oxidation. The formation pathways of the targeted aldehydes are described along with their potential hazards to humans.

1.2 LIPID OXIDATION, FORMATION AND MEASUREMENT OF MALONDIALDEHYDE, 4-HYDROXY-2-(E)-NONENAL, 4-HYDROXY-2-(E)-HEXENAL AND 2-BUTENAL IN FOODS

1.2.1 Lipid oxidation

Lipid oxidation is one of the major causes of chemical deterioration of fat containing foods, especially those containing polyunsaturated fatty acids (PUFA) and can take place during processing and storage of foods (Belitz, Grosh, & Schieberle, 2009) . The autoxidation of unsaturated fatty acids is a process that essentially occurs in three steps: initiation, propagation and termination. The first two steps are illustrated in ***Figure 1.2***. As a result of Initiation a free radical is formed due to a loss of one hydrogen from an unsaturated fatty acid. The free radical is further reacting with molecular oxygen and a lipid peroxyl radical is formed. During the propagation the lipid peroxyl radical, which is an unstable species, can "steal" a hydrogen from another unsaturated fatty acid to form a new lipid peroxyl radical and a lipid hydroperoxide (Kamal-Eldin, Makinen, & Lampi, 2003).

In the last stages of autoxidation the peroxyl radicals have a tendency to accumulate and the last step of termination occurs. Termination leads in the formation of inactive non-radical products which can be produced through various routes. Depending on the conditions non radical products can be formed due to condensation between two peroxyl radicals, an alkoxyl and an alkyl radical or between two alkyl radicals (Kamal-Eldin et al., 2003; Frankel, 2005).

On prolonged oxidation the hydroperoxides of monounsaturated fatty acids and PUFAs are further decomposed to form multiple compounds referred as secondary lipid oxidation products. A complex mixture of volatile, nonvolatile, and polymeric secondary oxidation products is formed including aldehydes, ketones, alcohols, hydrocarbons, volatile organic acids, and epoxy compounds, among others (Shahidi & Zhong, 2005). In the next part, formation of malondialdehyde, hydroxylated α,β unsaturated aldehydes and 2-butenal and analytical methods applied for their detection in foods will be discussed.

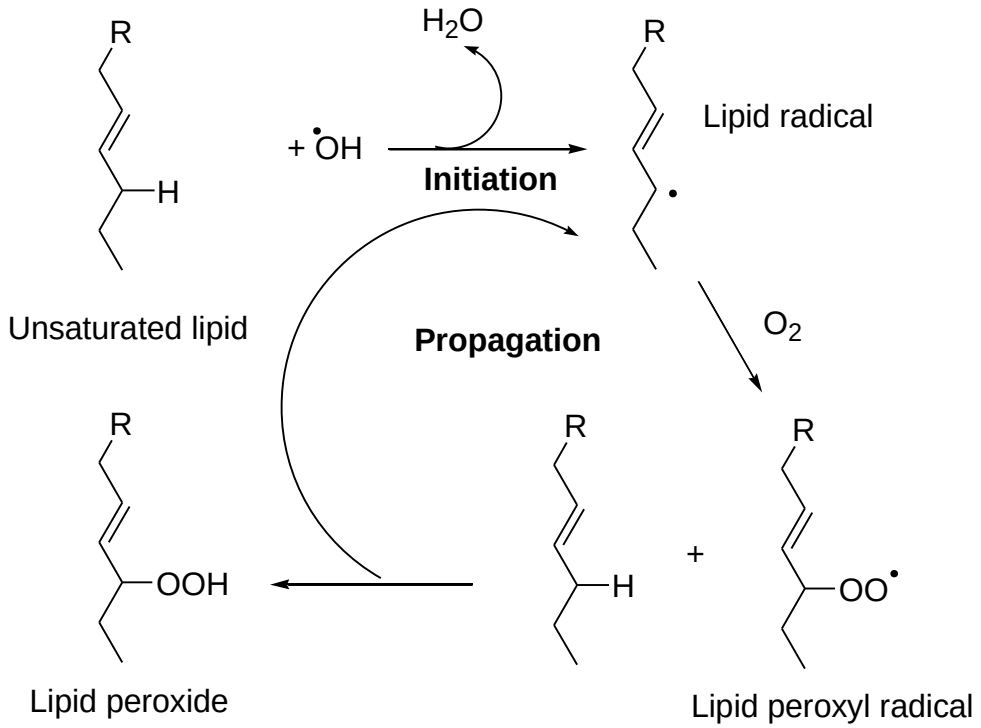

Figure 1.1 Graphical representation of initiation and propagation steps during lipid oxidation

1.2.2 Formation and measurement of Malondialdehyde in foods

Formation of malondialdehyde in foods

Malondialdehyde (MDA) is a three carbon dialdehyde with carbonyl groups at the first and third carbon positions and can be produced due to oxidation of PUFAs (Frankel, 2005). There are several suggested pathways describing the formation of MDA from PUFAs. Hydroperoxy epidioxides of ω-3 and ω-6 PUFAS as well as bicycloendoperoxides have been identified as main precursors of MDA. (Frankel & Neff, 1983; Pryor & Stanley, 1975). The formation of hydroperoxy epidioxides after cyclization of 12- and 13- hydroperoxides of linolenic acid and their decomposition to MDA are shown in ***Figures 1.2*** and ***1.3*** respectively. Interestingly it can be observed that both 12- and 13- hydroperoxides are precursors of MDA. An alternative mechanism explaining formation of MDA from PUFAs with more than three double bonds has also been suggested (Esterbauer, Schaur, & Zollner, 1991). According to this mechanism MDA can be formed due to successive degradation (formation of hydroperoxides followed by beta cleavage) of the fatty acid chain to a hydroperoxy aldehyde, which could then form an

acrolein radical. This radical can further react with an OH-radical to form MDA in the enol form (***Figure 1.4***).

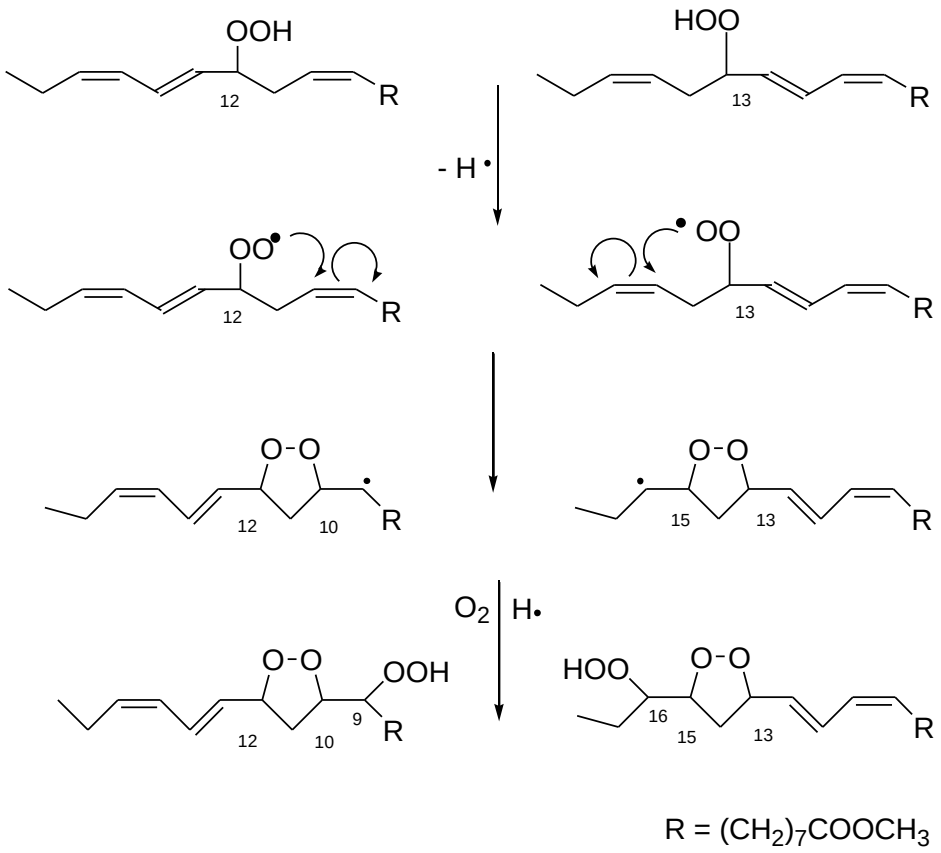

Figure 1.2 Formation of hydroperoxy epidioxides of methyl linolenate. Adapted from Pryor & Stanley, 1975

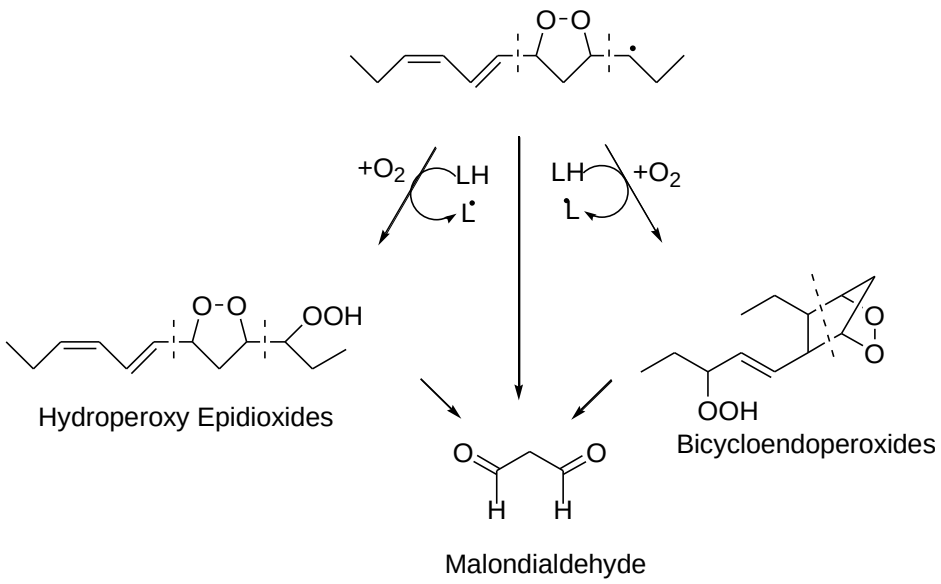

Figure 1.3 Formation of MDA from hydroperoxy epidioxides and bicycle-endoperoxides of methyl linolenate. Adapted from Pryor & Stanley,1975

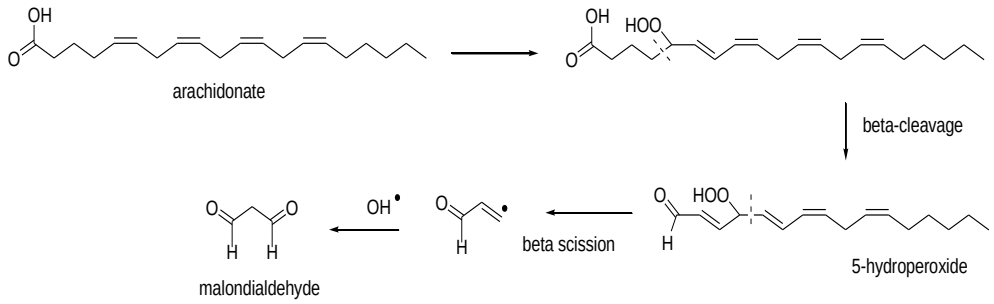

Figure 1.4 Formation of MDA from arachidonate. Adapted from Esterbauer et al. 1991

Measurement of malondialdehyde in foods

The most common method to determine MDA in foods is the spectrophotometric measurement of the pink colored adduct of MDA with 2-thiobarbituric acid (TBA) which gives a maximum absorbance at 532-535nm (Botsoglou et al., 1994). TBA can react directly with the food sample and the TBA-MDA adduct is extracted prior to the analysis. Alternatively MDA can be extracted from the samples either by distillation (Tarladgis, Watts, Younathan, & Dugan, 1960) or solvent extraction (Vyncke, 1975). All the spectrophotometric methods have been criticized due to their unspecificity towards MDA (Wang, Zhu, & Brewer, 1997). It is well accepted that components present in food matrices such as browning reaction products, protein and sugar degradation products participate in the formation of the TBA color complex (Guillen-Sans & Guzman-Chozas, 1998; Du & Bramlage, 1992), thus a more specific analysis of the TBA-MDA complex is required.

Although the distillation method is minimizing the interferences with TBA, there are limitations due to the long analysis time and the possible artefactual formation of MDA during heating (Pikul, Leszczynski, & Kummerow, 1983). Application of High Performance Liquid Chromatography (HPLC) analytical techniques coupled with fluorescence or UV detection has offered better specificity and sensitivity towards MDA determination in foods and biological systems based on the analysis of the MDA-TBA complex (Sakai, Habiro, & Kawahara, 1999; Khoschsorur et al., 2000; Mendes, Cardoso, & Pestana, 2009). Furthermore, there are available HPLC methods based on the analysis of MDA derivatives with hydrazine compounds such as 2,4- dinitrophenyl hydrazine (DNPH) (Cordis, Maulik, & Das, 1995; Rezaei, Jamshidzadeh, Sanati, & Hejazy, 2011), or on the direct measurement of MDA (Wei, Li, Thushara, & Liu, 2011; Tsaknis, Lalas, Hole, Smith, & Tychopoulos, 1998). Information on MDA levels in various foods according to the available literature, as measured by various methods and without considering their reliability, vary between 0.1 μg g-1 of sample and up to 27 μg g^{-1} for heavily oxidized samples.

1.2.3 Formation and measurement of 4-hydroxy-2(E) nonenal and 4-hydroxy-2(E) hexenal in foods

Formation of HNE and HHE in foods

4-hydroxy-2(E) nonenal (HNE) and 4-hydroxy-2(E) hexenal (HHE) are α,β- unsaturated aldehydes that can be formed due to decomposition of PUFAs hydroperoxides. It is well accepted that HNE is formed due to decomposition of ω-6 PUFA hydroperoxides, while HHE is mainly related to the oxidation of ω-3 PUFA (Long & Picklo, 2010; Surh, Lee, & Kwon, 2010; Han & Csallany, 2009; Pryor & Porter, 1990; Guillen & Uriarte, 2012). There are two main mechanisms of HNE formation proposed, involving the 9 and 13 hydroperoxides of ω-6 PUFAs . In ***Figure 1.4*** a proposed mechanism on the formation of HNE from linoleic acid is illustrated. It is notable that in both mechanisms 4-hydroperoxy-2-nonenal is formed which is further reduced to 4-hydroxy-nonenal (Schneider, Tallman, Porter, & Brash, 2001). Even though the formation mechanism of HHE is not studied extensively it has been proposed that it is formed in a similar manner as HNE from ω-6 PUFAs (Long & Picklo, 2010).

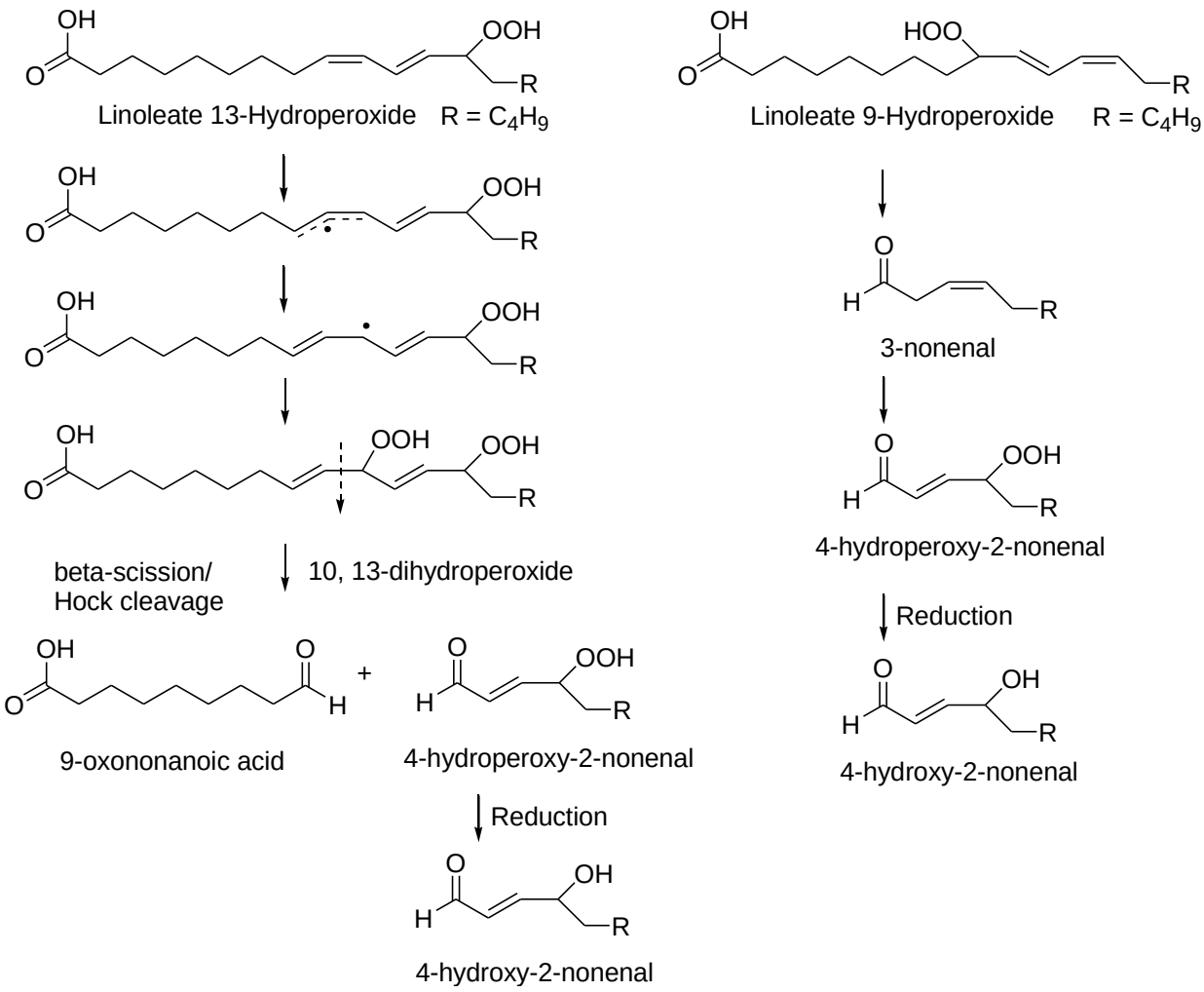

Figure 1.5 Proposed mechanisms of HNE formation from 13 and 9 hydroperoxides of linoleate. Adapted from Schneider et al., 2001

Measurement of HNE and HHE in foods

Several studies have been published on detection and quantification of unbound 4-hydroxy alkenals from foods and oils. Some involve the extraction and derivatization of 4-hydroxyalkenals with DNPH followed by analysis with HPLC and UV detection (Seppanen & Csallany, 2002; Seppanen & Csallany, 2001), direct analysis with HPLC-MS/MS (Zanardi, Jagersma, Ghidini, & Chizzolini, 2002), extraction of the aldehyde, derivatization and analysis with GC-MS (Surh & Kwon, 2002; Surh & Kwon, 2003), direct analysis with usage of HS-SPME-GC-MS(Guillen, Carton, Salmeron, & Casas, 2009; Guillen & Goicoechea, 2008a) or GC coupled with electron capture detection (Santaniello, Repetto, Chiesa, & Biondi, 2007).

The analytical methods presented in the aforementioned studies, with the exception of those applying SPME, involve several extraction steps in which loss of a portion of the analyte can be expected, leading to an underestimation of the actual amount present in the sample. Furthermore, in some cases the extraction techniques applied are time consuming and when complex food matrices are analyzed, interaction between the analytes and particularly proteins are not taken into account. (Han & Csallany, 2012; Han & Csallany, 2009) Among the available techniques in use for determination of aldehydes in foods, GC-MS is the most attractive one. On one hand it has the advantage of the powerful chromatographic separation provided by gas chromatography, and on the other hand it offers the option of using isotopicaly labeled HNE and HHE as internal standards (IS). Addition of IS into the samples prior to the analysis potentially minimizes both underestimation of the analytes and variation between the sample replicates. Such techniques have been previously reported in the analysis of HNE and HHE in biological samples (Rees, Vankuijk, Siakotos, & Mundy, 1995; Vankuijk, Siakotos, Fong, Stephens, & Thomas, 1995) and human milk (Michalski, Calzada, Makino, Michaud, & Guichardant, 2008) using deuterium labeled HNE and HHE. Recently, some authors (LaFond, Jerrell, Cadwallader, & Artz, 2011) have published an analytical method for determination of HNE in frying oils that involves the addition of deuterated HNE as an IS prior to the extraction steps. Levels of HNE in thermally oxidized oils have been reported between 5.6 μg g^{-1} (LaFond et al., 2011) and as high as 40 μg g^{-1} when oils rich in PUFAs were used (Seppanen & Csallany, 2004).

1.2.4 Formation and measurement of 2-butenal

2-Butenal, also referred as crotonaldehyde, is a four carbon α,β-unsaturated aldehyde which is an ubiquitous environmental pollutant and can be also produced from the decomposition of hydroperoxides resulting from oxidation of ω-3 PUFAs (Frankel, 2005). A proposed mechanism of 2-butenal formation from oxidized linolenic acid is illustrated in ***Figure 1.5.*** In this mechanism however formation of 2-butenal is based on the production of 15-hydroperoxide due to singlet oxygen present in photooxidation. Up to date there are limited data on the importance of 2-butenal formation during processing and storage of foods. Concerning short chain α,β-unsaturated aldehydes, recently acrolein has attracted the interest of researchers and the levels it is formed during frying with vegetable oils has been accurately determined (Ewert, Granvogl, & Schieberle, 2011). However, there is no available reliable analytical method for accurate determination of 2-butenal in oils and foods.

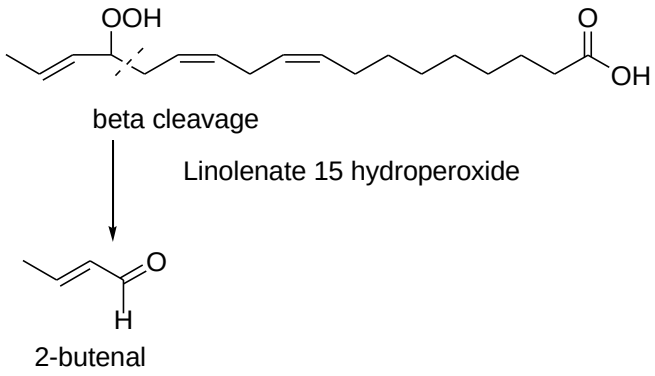

Figure 1.6 2-butenal formation from photosensitized oxidation of linolenic acid. Adopted from Frankel, 2005

1.2.5 Hazard identification and characterization with regard to the dietary intake of MDA, HNE, HHE and 2-butenal

MDA, HNE and HHE have attracted the attention in biological systems due to their potential toxicity to humans which is attributed to their high reactivity with proteins and DNA, consequently leading to structural damage and alteration of their functionality (Esterbauer, 1982; Esterbauer & Cheeseman, 1990; StAngelo, 1996; Uchida, 2003; Guillen & Goicoechea, 2008b; Voulgaridou, Anestopoulos, Franco, Panayiotidis, & Pappa, 2011).

More specifically, it has been confirmed that MDA can modify double-stranded DNA by formation of amino-imino-propen crosslinks between the NH_2 groups of a guanosine base and the NH_2 group of the complementary cytosine base (Esterbauer et al., 1991). Furthermore, it has been reported that MDA can react with NH_2 containing amino acids and it is noteworthy that compared to amino acids, proteins have been found to be more readily modified by MDA in physiological conditions (Nair, Cooper, Vietti, & Turner, 1986). Concerning hydroxylated α,β-unsaturated aldehydes, similar involvement in protein and DNA modification has been reported (Uchida & Stadtman, 1992; Uchida, 2003; Wakita, Honda, Shibata, Akagawa, & Uchida, 2011). The LD50 (Lethal dose 50%) of HNE for mice has been reported as 0.44 mmol kg^{-1} body weight if given intra-peritoneally and of HHE 0.98 mmol kg^{-1} of body weight (Esterbauer et al., 1991). Interestingly, mice which orally received an HNE-rich fraction of oxidized linoleic acid showed severe lymphocyte necrosis in the thymus 24 h later (Oarada et al., 1988). With respect to 2-butenal, data on oral acute toxicity to rats indicate an LD50 equal to 4.2 mmol kg^{-1} of body weight (Esterbauer et al., 1991). However, given the low amounts 2-butenal produced under various oxidizing conditions (see

chapters 4 and 6) and its high reactivity with food components such as proteins, the dietary exposure of free 2-butenal was not incorporated in the current study.

Even though phenomena related to the endogenously formed MDA and α,β-unsaturated aldehydes due to lipid peroxidation are well studied, the role and importance of these aldehydes when absorbed through the diet is not extensively investigated to date apart from a few studies. Some examples include studies related to the bio-accessibility of unsaturated aldehydes (Goicoechea et al., 2008) where it was demonstrated that a certain proportion of these compounds remained unaltered after digestion. Thus it was suggested that they are bio-accessible in the gastrointestinal tract and so could reach the systemic circulation. In another study (Grootveld et al., 1998) it was demonstrated that trans-2-alkenal compounds are readily absorbed from the gut into the systemic circulation in vivo, metabolized (primarily via the addition of glutathione across their electrophilic carbon-carbon double bonds), and excreted in the urine as C-3 mercapturate conjugates in rats. Subsequently it was suggested that the dietary ingestion of oxidized PUFA-rich culinary oils could promote the induction, development, and progression of cardiovascular diseases. It has also been demonstrated by others that consumption of oxidized lipids is resulting in formation of unsaturated aldehydes in the stomach(Kanazawa & Ashida, 1998) .At this point t should also be mentioned that none of these compounds has been classified as carcinogen and there is limited knowledge concerning the dietary exposure of humans to these aldehydes (Surh & Kwon, 2005).

1.3 RISK ASSESSMENT OF CHEMICALS IN FOOD

Risk assessment is a structured scientific evaluation process which is directed to the identification of potential hazards a chemical compound could pose to living organisms. According to its description, risk assessment consists of the following necessary steps: hazard identification, hazard characterization, exposure assessment and risk characterization as it is illustrated in ***Figure 1.5*** (Renwick et al., 2003). Furthermore, it is important to mention that risk assessment explicitly addresses uncertainty (i.e. what is not known about the risk) in a logical, transparent and well-documented manner. Risk assessment can be performed in qualitative or quantitative manner. Qualitative risk assessment is the process of compiling, combining and presenting evidence to support a statement about risk. On the other hand quantitative risk assessment is based on numerical data and analysis. It can be deterministic

or probabilistic and can address risk management questions at a finer level of detail than a qualitative risk assessment (Barlow et al., 2002; Barlow & Schlatter, 2010).

1.2.1 Hazard identification

As already mentioned, risk assessment consists of four distinguished but strongly related steps. Hazard identification involves the identification of a risk source that is potentially capable of causing adverse health effects to humans, together with a qualitative description of the nature of these effects. The potential of a chemical compound to pose a risk is evaluated by reviewing available toxicological data and the biological mechanism responsible for the toxicity (Barlow et al., 2002). Toxicological studies can be performed either in vitro or in vivo and the data can be extrapolated to humans. However, this approach may be itself an important source of uncertainty (Renwick et al., 2003).

1.2.2 Hazard characterization

Hazard characterization consists of the quantitative or semi-quantitative evaluation of the nature of the adverse health effects to humans following exposure to a risk source. A dose-response assessment, when available, should be included (European Commission, 2000). The dose-response assessment is the determination of the relationship between the magnitude of exposure to a chemical (dose) and the severity and frequency of the associated health effects (response) to humans.

Many of the non-carcinogenic adverse effects observed in animal or humans are characterized by a threshold dose, below which no adverse effects are observed (Kuiper-Goodman, 2004). The no observed adverse effect level (NOAEL) is such a threshold dose below which no toxic effects are observed. For these effects, the level of exposure without significant adverse effect can be estimated and health-based guidance values such as a tolerable daily intake (TDI) or acceptable daily intake (ADI) can be derived (Barlow et al., 2006). Regarding carcinogens, a dose without a potential effect cannot be defined, unless it can be clearly established that the mode of action involves an indirect mechanism that may have a threshold (Barlow et al., 2006; Kuiper-Goodman, 2004).

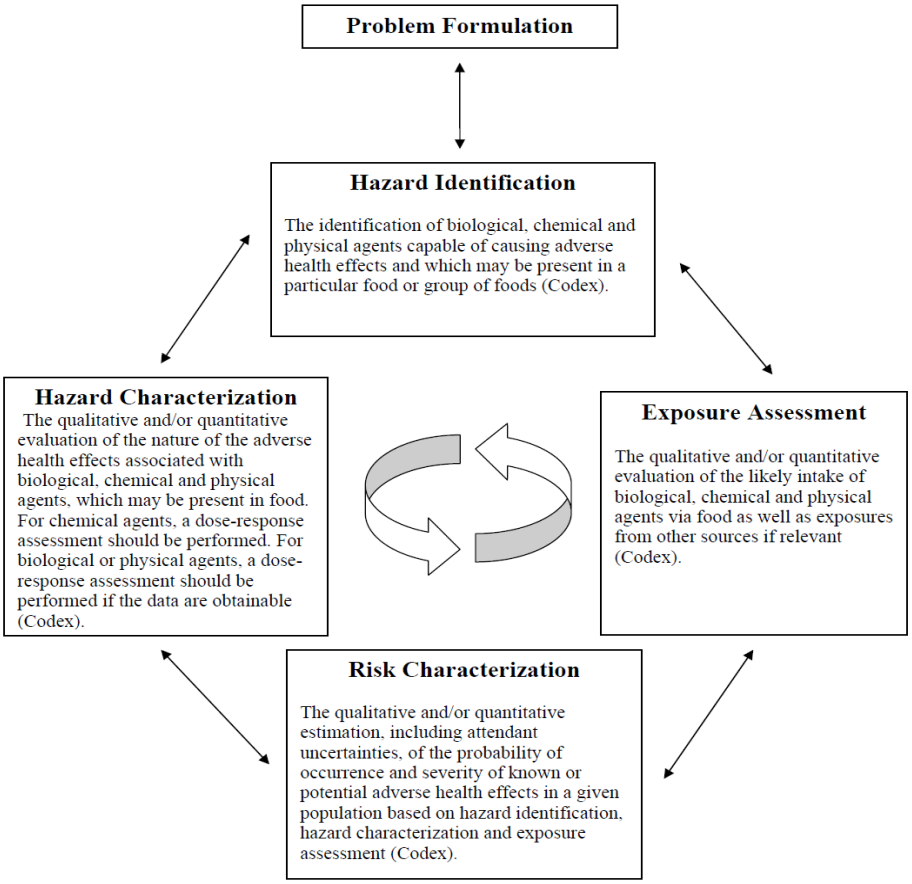

Figure 1.7 Steps in the risk assessment process Adopted from FAO/WHO, 2005

1.2.3 Exposure assessment

Exposure assessment is the procedure that estimates or measures the intensity, frequency and duration of exposure to a chemical compound. A complete exposure assessment should include description of the sources, pathways, routes, magnitude, duration and pattern of exposure, the characteristics of the exposed population and uncertainties in the assessment (International Programme on Chemical Safety, 2001).

The dietary intake of the studied compounds are defined by the amount the compounds are present in foods as well as factors concerning the consumption of the later. This step is often considered as a source of uncertainty which is related to the low levels of these compound are present in foods along with the sensitivity of the analytical methods applied for their determination (Kroes et al., 2002). Uncertainty related to the analytical methods applied is associated with the number of samples detected in levels below the limit of detection (LOD) or the limit of quantification (LOQ) of the method and the way these "non-detects" are

treated (O'Brien et al., 2006). In literature, data below the LOD are replaced by the corresponding LOD (upper bound scenario), by ½ LOD (medium bound scenario) or by zero (lower bound scenario) (Tressou, Leblanc, Feinberg, & Bertail, 2004; Vinci et al., 2012).

An estimate of exposure to a compound can be obtained after combining concentration data in foods with data related to the consumption of these foods by a given population. The three most common applied methodologies are: a) point estimates or deterministic approach, b) probabilistic analysis and c) simple distributions.

The deterministic analysis is an approach for estimation of exposure that in principle involves fixed values of food intake and food contamination data. It is simply obtained by multiplication of the mean, the 95th or the 97.5th percentile of food consumption data with the corresponding mean 95th or 97.5th percentile of contamination data (Lambe, 2002). This approach is usually applied to obtain an insight in exposure assessments due to its simplicity. However, deterministic analysis is based on the assumptions that all individuals consume the involved foods at the same level and that the compound of interest is always present in the foods in an average concentration. Therefore, the conclusions made from this approach are not always accurate due to possible over- or underestimation of the exposure levels (Lambe, 2002).

In probabilistic analysis, the variables are not described in terms of point estimates but as distributions instead. The best fit distributions for consumption and contamination data are determined and first order Monte Carlo simulations are performed. Monte Carlo simulations are based on random sampling of each probability distribution within a model. One value is obtained for each distribution, is entered in the model and the model is solved. This procedure is repeated for a given number of times (iterations) and instead of obtaining a discrete number for model outputs (as in a deterministic simulation) a set of output samples is obtained. The important advantage in probabilistic analysis is that a more complete exposure assessment is allowed because the whole distribution is taken under consideration with respect to the consumption and contamination data (Vose, 1996).

1.2.4 Risk characterization

Risk characterization is the fourth step in risk assessment. According to its definition it is the quantitative or semi-quantitative estimate, including attendant uncertainties, of the probability of occurrence and severity of adverse effects in a given population under defined exposure conditions based on hazard identification, hazard characterization and exposure assessment (European Commission, 2000). In other words, is the stage of risk assessment that integrates information from exposure assessment and hazard characterization into advice suitable for use in decision-making (Renwick et al., 2003). When threshold level is available the risk can be estimated by direct comparison of the outcome of exposure assessment with ADI or TDI (Benford, 2001). When threshold is not available or applicable the following approaches can be applied: low-dose extrapolation of data from rodent carcinogenicity bioassays, the threshold of toxicological concern (TTC) and the margin of exposure (MOE).

Extrapolation of animal dose-response data or use of the median toxic dose (TD50), the carcinogenic potency index T25 or the benchmark dose lower confidence limit ($BMDL_{10}$) as the point of departure for simple linear extrapolation, can be used to estimate the risk associated with human exposure to a compound (O'Brien et al., 2006). However, this approach can introduce a high level of uncertainty related to the mathematical models applied which do not always reflect the complexity of the biological phenomena involved. Thus, this approach is not the most recommended.

The TTC is a concept that allows establishment of a level of exposure that should raise concerns about human health for chemicals which no specific toxicity data are available but there is knowledge of their chemical structure. According to the TTC concept, a "safe" level of exposure can be identified for many chemicals based on their chemical structure and the known toxicity of chemicals that share similar structural characteristics. The TTC approach is exclusively designed as a substitute for substance-specific information in situations where there is limited or no information on the toxicity of the compound and information on exposure indicates that human exposure is very low. All risk assessment approaches have some degree of uncertainty. When the TTC approach is applied, it should be considered that it is a probability-based screening tool and may have additional uncertainty. The derivation of the various TTC values is based on frequency distributions. (Kroes, Kleiner, & Renwick, 2005; Koster et al., 2011; Kroes et al., 2004). Chemicals are classified into three classes

according to Cramer decision tree (Cramer, Ford, & Hall, 1978) and therefore the TTC for chemicals are considered 1800, 540, 90 µg person d-1 for those belonging to Cramer class I, II and II respectively. These TTC values that have been proposed for use are not based on the lowest value in each of the distributions but on a point close to the lowest value. (Hennes, 2012; Kroes & Kozianowski, 2002). It should be mentioned however, that genotoxic and high potency carcinogens are not included in this approach.

The MOE is an approach which considers the ratio between a dose leading to tumors in experimental animals and the human dietary intake. In the MOE approach the higher the MOE the lower the risk due to exposure to the studied compound. However, the possible magnitude of risk is not defined. In order to calculate the MOE, definition of the point on the dose-response curve and knowledge of the human exposure are required. The TD50, T25 or BMD are often taken as comparative estimates of the potency of genotoxic carcinogens (Sanner, Dybing, Willems, & Kroese, 2001). The European Food Safety Authority (EFSA) Scientific Committee has stated: "In general a margin of exposure of 10,000 or higher, if it is based on the $BMDL_{10}$ from an animal carcinogenicity study, and taking into account overall uncertainties in the interpretation, would be of low concern from a public health point of view and might be reasonably considered as a low priority for risk management actions. However, such a judgment is ultimately a matter for the risk managers. Moreover an MOE of that magnitude should not preclude the application of risk management measures to reduce human exposure" (EFSA, 2012). When animal based dose response data are available the MOE approach is preferred to characterize risks without a threshold.

In the upcoming chapters, the evaluation of existing analytical methods for determination of the targeted aldehydes and development and validation of new methods will be discussed. Furthermore, studies on the formation of these aldehydes under various conditions are included. Finally, exposure assessment and characterization of the potential risk in the Belgian population in relation to consumption of specific food categories will be discussed.

CHAPTER 2

EVALUATION OF ANALYTICAL METHODS FOR DETERMINATION OF MALONDIALDEHYDE IN VARIOUS FOOD MATRICES

Redrafted from:

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CHAPTER 2. EVALUATION OF ANALYTICAL METHODS FOR DETERMINATION OF MDA IN VARIOUS FOOD MATRICES

SUMMARY

The ability of the spectrophotometric thiobarbituric acid reactive substances (TBARS) test to determine malondialdehyde (MDA) in various food matrices was evaluated. MDA was extracted from the foods, the extract reacted with thiobarbituric acid (TBA) and the formed TBA-MDA adduct was measured spectrophotometricaly at 532nm. In parallel, the TBA-MDA adduct was analyzed with High Performance Liquid Chromatography (HPLC) coupled with fluorescence detection. Oils, unprocessed and uncooked meat and fish products, did not exhibit any significant difference in the amount of MDA measured by the two methods, indicating that the major substance reacting with TBA and forming an adduct that absorbs at 532nm was malondialdehyde. However, in products such as dry nuts, pork sausages, cooked fish and gouda cheese an overestimation of malondialdehyde was observed, indicating that the TBARS test is unsuitable for accurate determination of MDA. Furthermore, the results in the present work suggest that overestimation of MDA by the TBARS test as it was applied, is related to the interference of other than secondary lipid oxidation products.

KEYWORDS: Lipid oxidation, Malondialdehyde, Thiobarbituric Acid Reactive Substances

2.1 INTRODUCTION

As already described in detail in chapter 1, TBARS is widely used for quantification of MDA in foods and is preferred for routine analysis of large amount of samples due to its simplicity and low cost (Raharjo & Sofos, 1993). It is based on the spectrophotometric measurement of the pink colored adduct of MDA with 2-thiobarbituric acid (TBA) which gives a maximum absorbance at 532-535nm (Botsoglou et al., 1994). In the literature there was no available study evaluating the efficiency of TBARS test in measuring MDA in fatty foods as well as different type of oils. Thus, an evaluation of the ability of TBARS test to measure MDA within a broad range of foods was considered necessary prior to analysis of the foods and model systems presented in the following chapters. The aim of the work presented in this chapter was to evaluate the potential of the spectrophotometric TBARS test to measure MDA in a broad variety of foods and oils. The amount of MDA measured with the TBARS test was compared with the results obtained from the HPLC method. Furthermore, a novel enzyme based extraction technique for MDA was developed for food samples that have a high starch content.

2.2 MATERIALS AND METHODS

2.2.1 Chemicals and materials

2- thiobarbituric acid (TBA), alpha amylase, 1,1,3,3- tetraethoxypropane (TEP), were purchased from Sigma Aldrich (St. Louis, MO, USA). KH_2PO_4, glacial acetic acid of analytical grade was purchased from Chem-Lab (Zedelgem, Belgium), trichloroacetic acid (TCA) was purchased from Acros Organics (Geel, Belgium), methanol HPLC grade was provided by Fisher Scientific (Leicestershire, UK).

2.2.2 Food samples

Various food samples ***Table 2.1***, of limited knowledge of storage history, with developed rancid odor at collection time were analyzed in the lab with both spectrophotometric (TBARS test) and HPLC method. Furthermore, a storage experiment with controlled conditions was conducted with products supplied from the local supermarket in order to further investigate the ability of TBARS test to measure the MDA formation during storage. This group included peanuts, almonds in sliced and powder form, walnuts, two different brand names of

potato crisps (crisps A and B), tortilla crisps, sweet cookies with cinnamon, rainbow trout and herring. Beheaded frozen vacuum packed herring was provided by the industry and was unpacked and stored at -27 ^{o}C. Rainbow trout was bought fresh from the local supermarket, was filleted and stored at -27 ^{o}C. All the rest of the samples (peanuts, almond slices and powder, walnuts, potato crisps and tortilla crisps) were packed in transparent PE bags, sealed and stored under light with an average illuminance of 1577 Lux. The temperature measured throughout storage varied between 20-26 ^{o}C. In order to further evaluate the ability of TBARS test to measure MDA in oil samples, different types of oils and mixtures of them were stored at 75 ^{o}C in the dark for six days to induce oxidation.

Table 2.1 Short description of the oxidized products used in the experiment

Product	Description
pork meat sausages	stored at 4 ^{o}C
peanut paste	stored at room temperature, bought in bulk from Uganda
peanuts	opened packaging for unknown period
walnuts	opened packaging for unknown period
salmon	cooked and stored in the freezer
pork	uncooked minced meat stored in the freezer
herring	stored in the freezer
potato crisps	opened packaging for unknown period
vegetable oil A	disposed frying oil from restaurant
vegetable oil B	stored at room temperature exposed to air
beef	uncooked, stored for several days at 4^{o}C
Soybeans	stored in bulk, provided by the industry
soy-corn mixture A	fresh preparation before extrusion
soy-corn mixture B	fresh preparation after extrusion
gouda cheese	stored at 4^{o}C for unknown period

2.2.3 MDA extraction

Depending on the nature of the product, different extraction techniques were applied as described below.

Meat products, cheese and dry nuts

Approximately 7 g of sample were weighted in a 50 mL falcon tube, 15 mL of 7.5% TCA added, the mixture was homogenised with an Ultraturax (Janke & Kunkel, IKA-Werk, Staufen, Germany) for 1min at 18000 rpm and the volume was adjusted to 30 mL by addition of TCA. The homogenate was filtered through 150 mm filter paper and a specific volume reacted with TBA reagent as described later (Mendes et al., 2009).

Oil samples

A total of 1 g of oil was weighted in a 50 mL falcon tube, 5 mL of water was added, and the mixture was vortexed (VWR, Leuven, Belgium) for 2 min and centrifuged (Sigma 4K15, Sartorius, Goettingen, Germany) at 5000 g for 5 min. The aqueous layer was collected and the procedure was repeated two times. The collected extract reacted with TBA reagent as described later.

Products rich in starch

Food products having a high starch content (potato crisps) were analysed using an enzyme based analytical technique. Approximately 7 g of sample were weighted in a 50 mL falcon tube and homogenised with 15 mL of water. 1 mL of 3000 U alpha-amylase preparation was added and the mixture incubated in a water bath at 30 ^{o}C for 40 min. 1 U corresponds to the amount of enzyme which liberates 1 μmol maltose per minute at pH 6.0 and 25 °C. The enzymatic reaction was stopped by the addition of TCA to a fixed volume followed by centrifugation at 5000 g for 10 min. The supernatant was filtered through a 150 mm filter paper and a specific volume reacted with TBA. All the aforementioned extractions were performed in triplicate.

2.2.4 MDA determination

The reaction of MDA with TBA and the determination of the formed adduct with the two different methods was set as follows: For the HPLC determination of MDA the procedure described by Mendes et al., (Mendes et al., 2009) was followed with slight modifications. A total of 1 mL of extract and 3 mL of TBA reagent (40 mM dissolved in 2 M acetate buffer pH 2.0) were mixed in a test tube and heated in a boiling water bath for 35 min. The reaction mixture was chilled prior to addition of 1 mL of methanol and 20 μL of the sample were injected into a Varian C18 HPLC column (5 μm, 150 x 4.6 mm) hold at 30 °C. The mobile phase consisting of 50 mM KH_2PO_4 buffer solution, methanol and acetonitrile (72:17:11 v/v) was pumped isocratically at 1 mL min^{-1}. Fluorometric detector excitation and emission wavelengths were set at 525 and 560 nm respectively. For the spectrophotometric determination of MDA (Vyncke, 1975), 2.5 mL of extract and 2.5 mL of TBA reagent (46 mM dissolved in acetic acid) were mixed in a test tube and heated in a boiling water bath for 35 min. The reaction mixture was chilled and the absorbance was measured at 532 nm using

a Cary 50 UV–Vis Spectrophotometer from Varian (Sint-Katelijne-Waver, Belgium). For quantification with both methods, standard solutions of MDA in 7.5% TCA were prepared from 1,1,3,3- tetraethoxypropane (TEP) and calibration curves were prepared at a concentration ranged from 0.6 to 10 μM.

2.2.5 Fat content determination

The fat content of the samples was determined according to the Weibull method (Egan, Kirk, & Sawer, 1981). This technique involves acid dissolution of the sample followed by continuous extraction of the fat with petroleum ether using a Soxhlet apparatus.

2.2.6 Fatty acid composition

The fatty acid profile was determined after preparation of fatty acid methyl esters (FAME) and analysis with gas chromatography according to AOCS official method (AOCS, 1989). A total of 50 mg of fat was placed in a tube containing a known amount of nonadecanoic acid (Internal standard). A total of 2 ml 0.5 N NaOH-solution was added, the tube was vortexed and placed in a boiling water bath for 7 min. The tube was cooled at room temperature, a total of 2 ml BF_3/MeOH-reagent was added and the mixture was placed in a boiling water bath for 5 min. The prepared FAMEs were extracted with a total of 6 mL of Hexane. Chromatographic analysis of the prepared FAMEs was performed as follows: A total of 0.1 μL of the hexane solution containing the FAMEs was injected cold on column into a CP-Sil 88 capillary column 60 m x 0.25 mm ID (Agilent Technologies, Palo Alto, CA), with a film thickness of 0.2 mm, installed in an Agilent 6890N series GC. (Agilent Technologies, Palo Alto, CA) The temperature program applied was the following: initial temperature 100 ^{o}C for 1 min, then 5 ^{o}C min^{-1} to 150 ^{o} C, followed by a ramp of 10 ^{o}C m^{-1} to 220 ^{o}C held for 10 min.

Extraction of the oil was carried out according to the method described by Bligh & Dyer (Bligh & Dyer, 1959) with some modifications. In brief, approximately 20 g of sample was weighted in a centrifuge tube, 20 and 40 mL of dichloromethane and methanol were added respectively and the mixture was homogenized with the Ultraturax at 18000 rpm for 2 min. Subsequently 20 mL of dichloromethane were added followed by homogenization for 30 s. Finally, 20 mL of water were added and mixed for 30 seconds. The mixture was centrifuged at 2800 g for 10 min, the lower layer was collected and the oil was recovered after

evaporation of the solvent using a rotary evaporator (Heidolph Instruments GmbH & Co, Schwabach, Germany) operating at low temperature.

2.2.7 Statistical analyses

One way Analysis of Variance (ANOVA) was applied to detect differences between samples and to evaluate the significance of the correlation. Whether differences were detected multiple comparisons were done by the Student Newman–Keuls (S-N-K) test. All the analysis was carried out with SPSS 18 statistics package.

2.3 RESULTS AND DISCUSSION

In order to evaluate the efficiency of the spectrophotometric TBARS test to determine MDA compared to the HPLC separation method of the TBA-MDA complex, fifteen food samples with developed rancidity were collected and the results are shown in ***Table 2.2***.

TBA test resulted in higher or similar amounts of MDA compared to the results obtained by the HPLC method .Oils, unprocessed and uncooked meat and fish products, did not exhibit any significant difference in the amount of MDA measured by the two methods, indicating that the major substance reacting with TBA was malondialdehyde. Mendes et al., (Mendes et al., 2009) were able to detect the same amounts of MDA with both methods in stored sardines, while others (Tsaknis et al., 1998) have reported a slight overestimation of MDA with the TBARS test in oxidized vegetable oil.

Hirayama *et al.*, (Hirayama, Yamada, Nohara, & Fukui, 1983) however, detected significantly higher MDA levels with the TBARS test compared to HPLC analysis of MDA-dansyl hydrazine derivatives in vegetable oils. In cooked fish, processed meat, potato crisps and dried nuts, spectrophotometric measurements demonstrated an overestimation of MDA. In the case of the soy-corn mixture, extrusion resulted in a considerable decrease in MDA content that was not detected by the TBA test method. The observed overestimation of MDA by the classic TBARS test in meat products has also been reported by others (de las Heras, Schoch, Gibis, & Fischer, 2003).

Table 2.2 MDA values measured with the TBARS test and the HPLC method and total lipid content. Values are the average of three and two replications for MDA and total lipids determination respectively ± the standard deviation. Different letters within the lines indicate statistically significant differences ($p<0.05$) in MDA content

Sample	MDA (μg g^{-1} sample)		Total lipids %
	TBARS test	HPLC	
pork meat sausages	0.62±0.02 [a]	0.40±0.03 [b]	41.15±1,70
peanut paste	5.00±0.08 [a]	0.36±0.06 [b]	48.83±1.76
peanuts	5.50±0,83 [a]	0.86±0.10 [b]	51.87±0.03
walnuts	11.59±1.21[a]	3.24±0.03 [b]	67.12±0.04
Salmon	2.69±0.26 [a]	1.53±0.04 [b]	9.96±0.49
pork	1.02±0.11 [a]	0.87±0.08 [b]	18.96±2.44
herring	2.49±0.71 [a]	2.33±0.70 [a]	8.76±0.38
potato crisps	7.27±1.38 [a]	0.51±0.03 [b]	37.87±1.77
vegetable oil A	0.79±0.09 [a]	0.71±0.11 [a]	-
vegetable oil B	1.07±0.11 [a]	0.94±0.02 [a]	-
beef	3.56±0.13 [a]	3.64±0.01 [a]	N/A
soybeans	18.22±0.58 [a]	2.18±0.33 [b]	17.88±0.55
soy-corn mixture A*	7.08±0.28 [a]	1.06±0.10 [b]	6.89±0.16
soy-corn mixture B*	6.09±0.06 [a]	0.51±0.01 [b]	8.07±0.07
gouda cheese	0.25±0.01 [a]	0.11±0.02 [b]	30.26±1.27

*Results expressed on dry matter basis

In one of the first systematic works related to quantification of MDA in foods (Shamberger, Shamberger, & Willis, 1977) where MDA was determined based on the distillation method, the levels MDA was found in cooked meat were within the range of 1.3-27 μg g^{-1} of sample. These high values are a clear indication of an overestimation provided by the TBARS method, in this study possibly related to formation of MDA during the distillation.

Specificity of the TBARS method to measure accurately MDA has always been contested. It is well accepted that components present in food matrices such as browning reaction products, protein and sugar degradation products participate in the formation of the TBA colour complex (Frankel, 2005) resulting in false estimation of the MDA content. Reduction of the reaction temperature along with the usage of higher concentrations of TBA reagent has been suggested as potential solutions in order to increase the specificity of TBA for MDA (Wang, Pace, Dessai, Bovell-Benjamin, & Phillips, 2002). However, even though the results suggest that in some matrices MDA can be accurately measured by the TBARS test, presence or absence of interfering compounds cannot be assumed prior to the analysis. Furthermore,

statistical analysis did not reveal any significant correlation between the MDA content ($p>0.05$ $r^2= 0.123$) measured by the two methods when all the samples were taken into account.

In order to further investigate the ability of TBARS test to measure the MDA formation during storage a controlled storage experiment was carried out and the results are shown in ***Table 2.3*** and the regression coefficients along with regression equations are shown in ***Table 2.4*****.** For peanuts and both types of almonds no significant correlation between MDA measured with the two methods ($p>0.05$) was observed. The results suggest that MDA is not the major compound that reacts with TBA and forms a complex that absorbs at 532 nm. Thus TBARS test cannot be considered reliable for MDA determination in the aforementioned samples.

Table 2.3 Changes in MDA content (μg g^{-1} of sample) as measured by the two methods. Values are the average of three replications ± the standard deviation Different letters within the columns indicate statistical significant differences ($p<0.05$) in MDA content

Storage Time (Days)	***Peanuts***		***Almond slices***		***Almond powder***		***Walnuts***		*Storage Time (Days)*	***Herring***	
	TBARS test	*HPLC*	*TBARS test*	*HPLC*	*TBARS test*	*HPLC*	*TBARS test*	*HPLC*		*TBARS test*	*HPLC*
0	5.01±0.43a	0.44±0.12a	3.16±0.08a	0.32±0.02a	5.21±0.29a,b	0.44±0.04a	7.21±0.41a	0.72±0.07a	0	0.46±0.15a	0.30±0.04a
14	5.45±1.86a	0.52±0.06a	4.15±0.42b	0.33±0.02a	5.38±0.77a,b	0.32±0.01b	9.08±0.67b	1.52±0.11b	21	2.65±1.34a	2.72±1.13a
37	7.52±0.16b	0.81±0.05b	6.37±0.54c	0.48±0.06b	6.06±0.35b,c	0.52±0.01c	14.15±0.65c	3.23±0.17c	103	12.64±1.71b	12.87±2.51b
69	8.75±0.66c	0.38±0.03a	4.99±0.34d	0.38±0.06a	6.90±0.72c	0.33±0.08b	15.95±0.82c,d	4.32±0.24d	159	15.03±1.43b	14.42±1.46b
92	6.16±0.50a	1.00±0.13c	4.45±0.09b	0.48±0.04b	4.46±0.38a	0.38±0.02a,b	14.84±0.60d	4.46±0.38d			
Storage Time (Days)	**Cookies**		**Crisps A**		**Crisps B**		**Tortilla Crisps**		Storage Time (Days)	**Rainbow trout**	
	TBARS test	*HPLC*	*TBARS test*	*HPLC*	*TBARS test*	*HPLC*	*TBARS test*	*HPLC*		*TBARS test*	*HPLC*
0	3.13±0.00	1.60±0.01a	2.48±0.28a	0.33±0.07a	3.99±0.31a	0.62±0.24a	3.56±0.51a	0.52±0.04a	0	0.19±0.04a	0.09±0.02a
14	28.18±0.65b	1.06±0.13b,c	2.03±0.48a	0.57±0.11a	3.58±0.17a	0.84±0.04a	1.66±0.07b	0.28±0.02b	15	0.31±0.07a	0.15±0.06a
37	28.98±2.26b	1.41±0.15a	6.40±0.00	1.46±0.00b	4.06±0.00	1.34±0.35b	2.44±0.21c	0.41±0.01c	36	0.27±0.01a	0.15±0.02a
69	27.16±1.47b	1.15±0.03b	5.87±0.70b	0.99±0.08c	15.04±3.06b	3.07±0.23c	3.93±0.45a	0.41±0.03c	118	0.43±0.03a	0.33±0.01b
92	24.07±4.38b	0.87±0.11c	3.59±0.16c	1.21±0.11c	8.49±1.13c	2.94±0.10c	2.70±0.21c	0.60±0.05d	176	2.48±0.21b	1.53±0.05c

It should be also mentioned that the applied extraction technique mainly focuses on the free MDA. Aldehydes and especially MDA have been found to react with ε-amino and sulfhydryl groups of proteins resulting in alterations of their functionality (Draper & Hadley, 1990; Wu, Zhang, & Hua, 2009). In peanut samples the observed decrease in MDA content as measured with the HPLC method at the 69th day of storage could be possibly explained by MDA-protein interactions. The further increase could be related to a possible difference between the rates of formation of MDA and the rates of interaction of the compound with the proteins.

Table 2.4 Regression significance and regression equations between MDA values measured with the two methods in a control storage experiment involving various matrices (X=HPLC measured value, Y=TBARS measured value)

Product	Regression significance	Regression equation	Regression coefficient
peanuts	p= 0.830	-	-
almond slices	p=0.185	-	-
almond powder	p=0.767	-	-
walnuts	p<0.05	Y=2.23X+5.89	0.893
cookies	p=0.365	-	-
crisps A	p=0.014	Y=2.94X+1.33	0.812
crisps B	p=0.001	Y=3.64X+0.80	0.773
tortilla crisps	p=0.088	-	-
brined herring	p<0.05	Y=1.00X+0.12	0.997
rainbow trout	p<0.05	Y=1.59X+0.02	0.995

Results from walnut samples indicate a significant correlation between the two methods ($p<0.05$). This correlation could be explained by the high levels of MDA present in the samples. Analysis of walnut oil found 11.5% to be PUFAs with more than two double bonds, the peroxides of which are known to be the main precursors of MDA. However, 10 fold overestimation of MDA at day 0 indicates that also in this case TBARS test primarily measures other compounds. Similar were the results for two types of potato crisps (A and B). Statistical analysis indicated significant correlation between the two methods ($p<0.05$) for both samples. In contrast, no correlation was found in tortilla crisps. Regarding the results obtained from two different types of frozen fish, both demonstrated significant correlation between the two methods of analysis ($p<0.05$). Similar strong correlation was found by others working with fresh and oxidized fatty fish species (Tsaknis, Lalas, & Evmorfopoulos, 1999). In case of herring samples the results suggest that the TBARS test can give a precise estimation of MDA, while in rainbow trout MDA is overestimated when measured with the TBARS test. Alghazeer et al.,(Alghazeer, Saeed, & Howell, 2008) reported similar results

from frozen Atlantic mackerel and LC-MS analysis indicated the presence of a gluteraldehyde-TBA adduct that can be considered interference in the TBARS test. The overestimation of MDA in trout samples could also be attributed to the pigments present in the flesh of the fish consisting of compounds such as astaxanthin (Sheehan, O'Connor, Sheehy, Buckley, & FitzGerald, 1998). In order to further evaluate the ability of TBARS test to measure MDA in oil samples, different types of oils with diverse fatty acid composition and mixtures of them were stored at 75^{o}C in the dark for six days to induce oxidation. The levels of MDA measured with both methods were similar (***Table 2.5***) and furthermore, a strong correlation was observed (***Figure 2.1***) ($p<0.05$, $R^2= 0.999$).

Table 2.5 Fatty acid composition (%) and changes in MDA content (μg g^{-1} of sample) as measured by the two methods. Values are the average of three replications ± the standard deviation. Different letters indicate statistically significant differences ($p<0.05$) in MDA content

Oil Samples	Saturated fatty acids	Mono-unsaturated fatty acids	Poly-unsaturated fatty acids	ω-6	ω-3	MDA (μg g^{-1})	
						TBARS test	HPLC
corn oil	13.7	28.8	56.7	55.8	0.9	1.28±0.17a	1.17±0.14a
sunflower oil	10.6	34.7	53.8	53.6	0.2	0.88±0.15b	0.76±0.11b
arachid oil	15.7	64.3	19.0	18.9	0.1	1.05±0.10a	0.92±0.08a
colza oil	7.1	63.1	29.0	20.0	9.0	11.75±0.44c	11.70±0.30c
olive oil	15.4	73.5	10.1	9.5	0.7	1.58±0.59a	1.50±0.54a
mix 1 (Colza oil/olive oil 50:50)	11.3	68.3	19.6	14.8	4.9	4.51±1.17d	4.28±1.47d
mix 2 (Colza oil/Arachid oil 50:50)	11.4	63.7	24.0	19.4	4.6	4.99±2.49d	4.77±2.52d
mix 3 (mix1/mix2 80:20)	11.1	63.8	24.2	19.3	4.9	5.65±2.94d	5.66±3.023d

The main secondary oxidation products of mono-unsaturated fatty acids subjected to autoxidation, for example C18:1, include volatiles such as decanal, 2-undecanal, nonanal, octanal (Frankel, 2005). Autoxidation of linoleate (C18:2 ω-6) leads to the formation of 2,4-decadienal, 3-nonenal, hexanal and pentanal. Furthermore formation of HNE from oxidised linoleate has attracted a lot of interest in the recent years (Han & Csallany, 2009). As with oleate and linolate, decomposition of linolenate (C18:3 ω-3) hydroperoxides derived from autoxidation lead to the formation of numerous volatiles. Furthermore, it is well accepted that oxidized PUFAs with more than two double bonds are the major source of MDA due to

degradation of hydroperoxides (Esterbauer et al., 1991; Esterbauer & Cheeseman, 1990). It has also been proposed that linoleic acid is a weak precursor of MDA (Esterbauer & Cheeseman, 1990). All the above mentioned secondary oxidation products can potentially react with TBA.

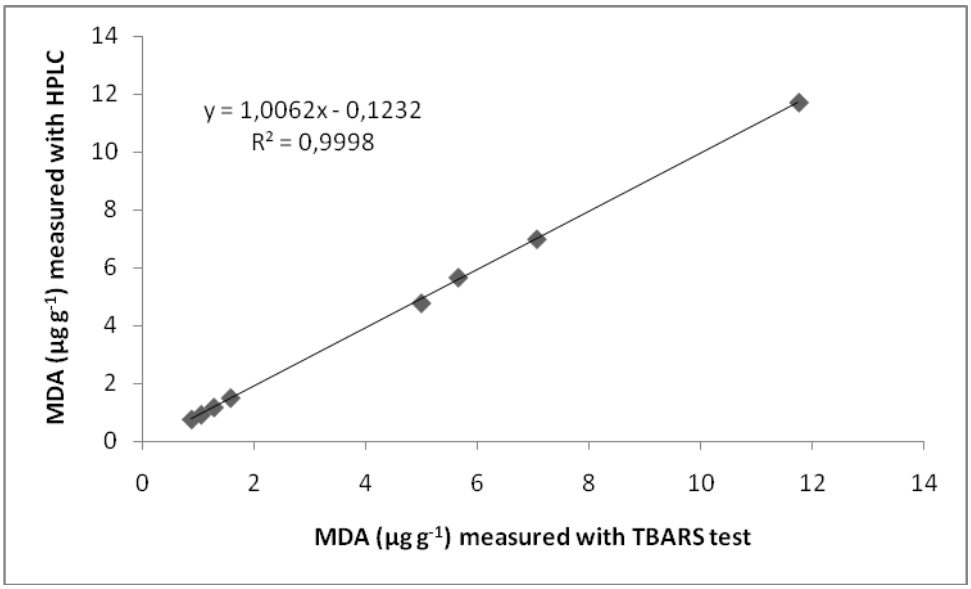

Figure 2.1 Linear correlation between the MDA amounts determined with both methods in oxidized oil samples

Regarding the fatty acid composition of the oil samples, the presence of other aldehydes other than MDA can be expected. However, these TBA-aldehyde derivatives did not interfere with the TBA test indicating that in complex food matrices it is more likely that other than secondary lipid oxidation products are measured along with MDA. The results suggest that the spectrophotometric method we applied can precisely determine the amount of MDA present in oil samples. On the other hand, regarding the diversity of the fatty acids in various vegetable oils and taking into account the variety of secondary lipid oxidation products formed during oxidative deterioration, the importance of MDA formation compared to other compounds should be further evaluated.

2.4 CONCLUSIONS

The results suggest that the TBARS test is reliable when applied for the determination of MDA in vegetable oils and in unprocessed meat and fish products. In processed beef, pork and fish, dry nuts, cheese and potato crisps TBARS test is overestimating the content of MDA due to interferences of other compounds with TBA. Furthermore the results also indicate that other than secondary lipid oxidation products interfere with the TBARS test.

In the following chapter the development of an analytical method suitable for the determination of HNE and HHE in various foods is presented. Furthermore, a short survey related to the levels of these compounds in foods available in the Belgian market is incorporated.

CHAPTER 3

DEVELOPMENT AND VALIDATION OF A STABLE ISOTOPE DILUTION ASSAY FOR SIMULTANEOUS DETERMINATION OF 4-HYDROXY-2-(E)-NONENAL AND 4-HYDROXY-2-(E)-HEXENAL IN VARIOUS FOOD MATRICES

Redrafted from:

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CHAPTER 3. DEVELOPMENT AND VALIDATION OF A STABLE ISOTOPE DILUTION ASSAY FOR SIMULTANEOUS DETERMINATION OF 4-HYDROXY-2-(E)-NONENAL AND 4-HYDROXY-2-(E)-HEXENAL IN VARIOUS FOOD MATRICES

SUMARY

As already mentioned in chapter 1 there is a lack of a suitable and reliable analytical method for determination of 4-Hydroxy-2-(E)-Nonenal (HNE) and 4-Hydroxy-2-(E)-Hexenal (HHE) in matrices such as dairy products, meat and meat products as well as in oils and fried foods. The aim of the work presented in this chapter was the development of an analytical method suitable for the determination of HNE and HHE in various food matrices. The presented method involves the use of deuterated HNE and HHE as internal standards, extraction of the analytes from the matrices followed by derivatization and detection with gas chromatography - mass spectrometry (GC-MS). Four different food matrices were chosen as model systems including vegetable oils, unprocessed meat, fried potato crisps and infant formula and three different extraction techniques suitable for the different matrices were applied including the QuEchERS method. The simplicity of the extraction techniques allows the method to be applied for routine analysis of a large amount of samples. The results verify the accuracy and reproducibility of the analytical technique and its ability to provide reliable quantification of both analytes at concentrations as low as 12 ng g^{-1} of sample. Furthermore, a short overview of the levels of HNE and HHE in several products available in the Belgian market is presented.

Keywords: Lipid oxidation, 4-Hydroxy-2-(E)-Nonenal, Hydroxy-2-(E)-Hexenal, QuEchERS

3.1 INTRODUCTION

As described in chapter 1 after an extensive review of the available literature it was figured out that the analytical methods were not suitable for providing reliable results on quantification of HNE and HHE in foods. Thus, in the study presented in this chapter a method for simultaneous determination of HNE and HHE in various food matrices was developed and validated. The method is based on: incorporation in the sample of a deuterated isotopes as IS, extraction of the analyte with an appropriate technique and derivatization of the extracted compound. Analysis of the derivatives was performed in GC-MS operating in SIM. Derivatization of the extracted compound aimed in improvement of the selectivity and sensitivity of the analytical technique. It is the first time, that a method combining HNE and HHE determination in a broad range of foods has been reported. In addition, a brief overview on the levels of HNE and HHE in several fatty foods available in the Belgian market is provided.

3.2 MATERIALS AND METHODS

3.2.1 Supplies and reagents

HNE and HHE standards were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). HNE *d11* and HHE *d5* dimethyl acetals were purchased from C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada). *O*-(2,3,4,5,6-Pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA) was supplied by Sigma-Aldrich (Bornem, Belgium), N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) was obtained by Acros Organics (Geel, Belgium). Deionised water (Milli-Q, Millipore Corp.) of 18.0 MΩ cm^{-1} resistivity was used throughout the experiments and all the solvents and reagents used were of analytical grade. All the foods used in the experiments were purchased from the local market and were analyzed as soon as they were unpacked. The dry nuts were packed under modified atmosphere. To simulate conditions occurring during frying, 5 g of ω-3 enriched and regular frying oils were placed in glass test tubes without a cap and were heated in an oven at 170 ^{o}C for 5 hours.

3.2.2 Extraction Techniques

The foods chosen for development and validation of the analytical method were vegetable oil (palm kernel oil), infant formula, potato crisps and unprocessed beef meat. These matrices were chosen as representatives of foods with different properties in order to evaluate the performance of the developed technique. Three different extraction procedures for HNE and HHE were applied depending on the food matrix. In order to evaluate the efficiency of the extraction techniques to recover the analytes from the matrices and evaluate any matrix effect, addition curves were prepared. Samples were spiked with 0.36, 0.28, 0.2, 0.12, 0.04 µg of HNE and HHE and 0.2 µg of HNE *d11* and HHE *d5* were added as IS. HNE and HHE standards were dissolved in methanol, HNE *d11* and HHE *d5* dimethyl acetals were deprotected prior to use by dissolving in 0.5 mL of 20 mM HCl and kept at 4 ^{o}C for 1 h.

For oils, 500 mg sample were weighed in a 15 mL polypropylene tube, spiked with the analytes and the internal standards, 0.5 mL of hexane was added and the mixture was vortexed (VWR, Leuven, Belgium) for 1 min in order to achieve complete incorporation of the analytes and the IS. 2 mL of water / methanol (60/40) were added and the sample was vortexed for 2 min. The tubes were centrifuged at 2000 g for 2 min (Sigma 4K15, Sartorius, Goettingen, Germany) and 1 mL of the aqueous portion was collected and subjected to derivatization.

For infant formulas, a modified procedure as described by Payá et al. (Payà et al., 2007) known as Quick Easy Cheap Effective Rugged Safe (QuEChERS) was applied. A total amount of 500 mg sample were weighed in a 15 mL polypropylene tube, spiked with the analytes and the IS, 2 mL of water was added and the mixture was vortexed for 1 min. A total of 3 mL of acetonitrile was added followed by vortexing for 1 min. A total of 0.3 g of NaCl and 1 g of MgSO4 were added and the tubes were mixed manually for one more minute. The tubes were centrifuged at 3600 g for 5 min and 2 mL of the supernatant was collected and evaporated under a gentle flow of nitrogen prior to derivatization.

Concerning potato crisps, a total of 500 mg of sample previously ground were weighed in a 15 mL polypropylene tube, spiked with the analytes and the IS. A total of 5 mL of 1.66 M H2SO4 / methanol (60/40) was added and the mixture was vortexed for 2 min. The tubes were centrifuged at 3600 g for 5 min and the aqueous phase was filtered through a wet 150 mm filter paper. 2.5 mL of the filtrate were collected and subjected to derivatization. The

same procedure was followed for the beef samples, with the difference that, 1 g of sample was homogenized with an Ultraturax (Janke & Kunkel, IKA-Werk, Staufen, Germany) for 1 min at 18000 rpm after spiking.

External calibration curves were prepared by applying the corresponding extraction technique for each matrix excluding the sample. All the extractions were performed in duplicate. The exact amount of HHE and HNE standards used in the experiments was determined by measuring the absorbance at 220 and 223 nm respectively (Cary 50 UV–Vis, Varian, Sint-Katelijne-Waver, Belgium). The mass extinction coefficient provided by the supplier is 16000 M^{-1} cm^{-1} for HHE and 13750 M^{-1} cm^{-1} for HNE.

3.2.3 Derivatization procedure

The extracts were mixed with 1 mL of methanolic solution of 4 mg mL^{-1} PFBHA reagent in a screw caped glass tube and the samples were incubated for 1 h at 40 ^{o}C. The formed oximes were extracted two times with 2 mL of pentane and dried over sodium sulphate prior to collection in a 25 mL pear shaped evaporation flask. The solvent was evaporated in a rotary evaporator (Heidolph Instruments GmbH & Co, Schwabach, Germany) at 30 ^{o}C and the remaining oximes were quantitatively transferred to a glass vial with 200 µL of pentane. Subsequently, the solvent was evaporated under a gentle stream of nitrogen. For silylation, 20 µL of 10% TMCS in BSTFA and 80 µL of pyridine were added. The mixture was vortexed for 1 min and 1 µL was injected in the GC-MS.

3.2.4 Instrumental analysis

Chromatographic analysis was performed in an Agilent 7890A GC equipped with a 5975C Mass Spectrometer (Agilent Technologies, Palo Alto, CA). The derivatised sample (1 µL) was introduced into the injector operating in the splitless mode at 200^{o}C and the separation was carried out in an Agilent HP-5 MS 30 m, 0.25 mm, 0.1 µm capillary column. The carrier gas was helium at a constant flow of 0.8 mL min^{-1} and the oven temperature was programmed from 50 (held for 1 min) to 150 ^{o}C at a rate of 10 ^{o}C min^{-1}, from 150 to 200 ^{o}C at a rate of 3 ^{o}C $min^{-1}$and finally up to 250 ^{o}C at a rate of 40 ^{o}C min^{-1}. The MSD conditions were the following: capillary direct interface temperature, 250 ^{o}C; ionization energy, 70 eV; operating in selective ion mode (SIM); selected ions monitored, *m/z* 200, *m/z* 205, *m/z* 242, *m/z* 253 and *m/z* 352; scan rate 3.64 cycles per second. In order to obtain the full mass spectra

of the HNE, HHE and their deuterated isotopic oximes, a full scan analysis was performed between *m/z* 50 and *m/z* 400.

3.2.5 Statistical analysis

One-way analysis of variance (ANOVA) was applied to detect differences between the slopes of the addition curves. All the analysis was carried out with SPSS 18 statistics package (IBM, SPSS, Inc.).

3.3 RESULTS AND DISCUSSION

Initial experiments were carried out in order to obtain the mass spectra and the retention times of the HNE, HHE and the deuterated isotopic oximes. The corresponding mass spectra are shown in ***Figure 3.1*** The mass spectra of HNE are in agreement with those published from LaFond et al.(LaFond et al., 2011) All the oximes share the following ions m/z 73, m/z 181 and m/z 352, along with the characteristic ions m/z 242 for HNE, m/z 200 for HHE, m/z 253 for HNE d11 and m/z 205 for HHE d5. From the shared ions, m/z 352 was chosen for monitoring the compounds in SIM along with the characteristic ion of each analyte. The ratios of the common (m/z 352) and the characteristic ion in combination with the retention times were used for positive identification of the analytes. HNE, HHE and their corresponding isotopes eluted as two peaks, since two stereoisomers were formed during the derivatization. ***Figure 3.2*** illustrates typical chromatograms of each analyte and IS obtained at SIM. Quantification was based on the sum of the two peak areas of the corresponding characteristic ion of each compound. Calibration and addition curves were constructed by plotting the ratio of the area of the analyte to the area of the isotope against the ratio of the mass of the analyte to the mass of the isotope. Knowing the exact mass of IS added in the sample prior to extraction the mass of the analyte can be calculated.

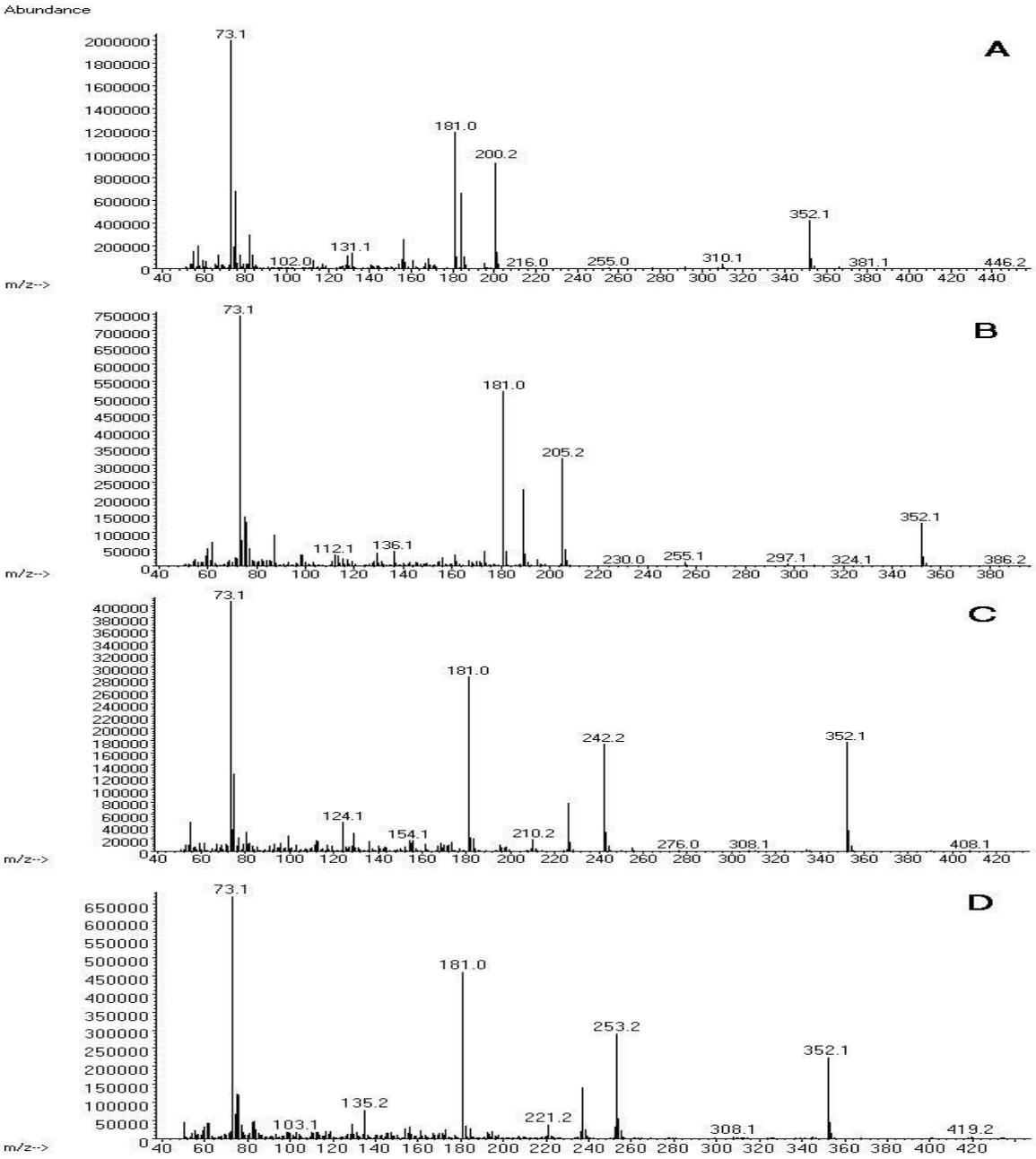

Figure 3.1 Mass spectra of A) HHE, B) HHE-*d5*, C) HNE, D) HNE-*d11*. Mass spectra were obtained after injection of derivatised standards and deuterated analogues

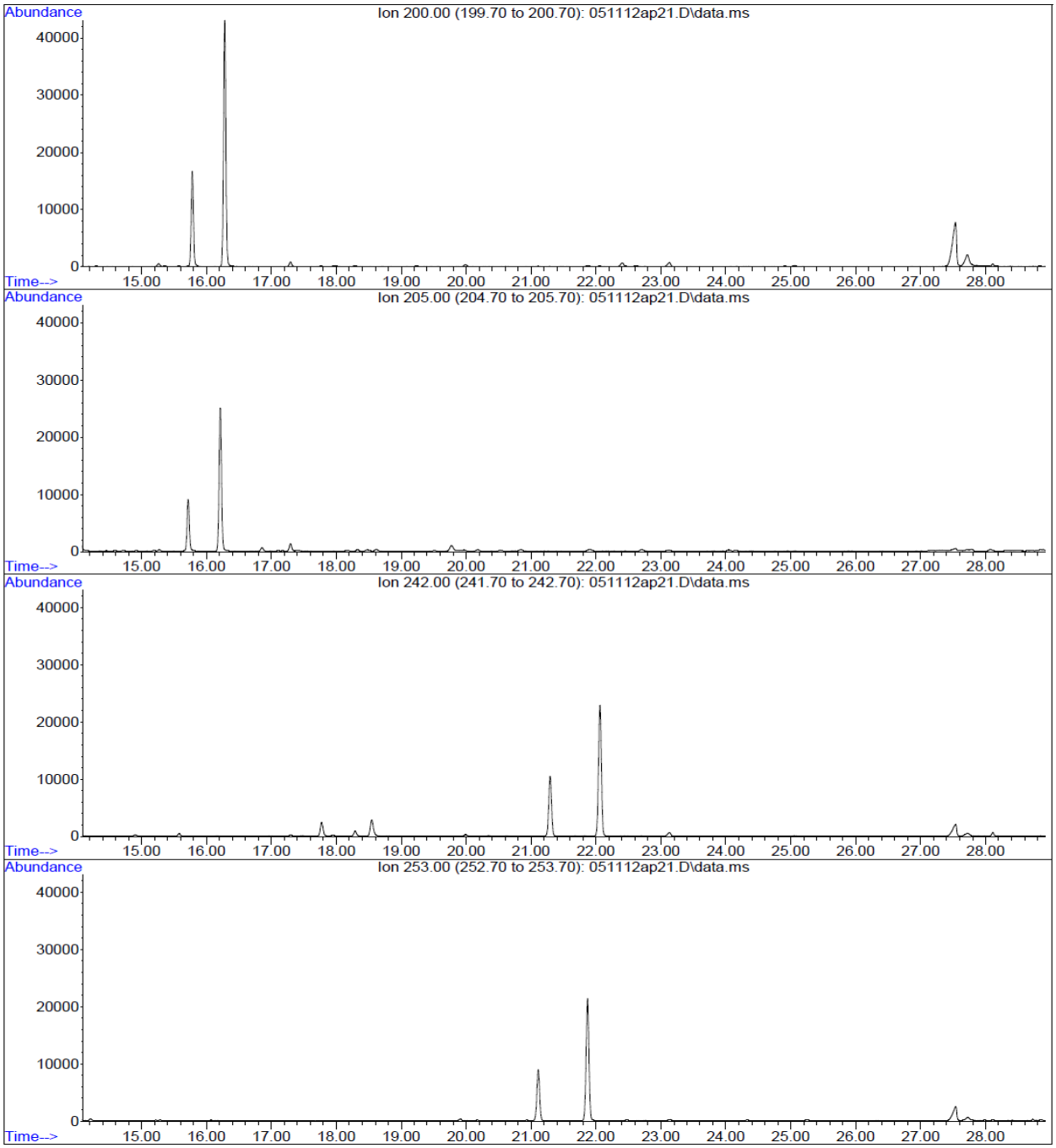

Figure 3.2 Typical chromatograms of HHE *m/z* 200 retention time 15.81, 16.33 min, HHE-*d5*, *m/z* 205 retention time 15.74, 16.26 min, HNE, *m/z* 242 retention time 21.32, 22.10 min, HNE-*d1*, *m/z* 253 retention time 21.13, 21.93 min. The chromatograms were obtained from HNE and HHE standard preparations

In preliminary experiments we have tried to derivatize HNE and HHE with PFBHA prior to the extraction as it has been reported in the literature (Michalski et al., 2008) in various food matrices. In the later study, the authors have reported the use of relatively high amount of

PFBHA per analyzed sample. Due to the high cost of the reagent, we have tried to reduce the amount of reagent to the level of 20 mg per sample for direct derivatization in the matrix. However, this approach was proven unsuccessful since no aldehyde-PFBHA adducts could be detected. Thus, extraction of the analytes was considered necessary

As mentioned previously, recovery of the analytes during the extraction can be highly influenced by the nature of the sample. Addition of HNE *d11* and HHE *d5* can eliminate underestimation of the analytes and minimize the variation between samples, but at the same time high actual recoveries of the analyte in order to increase the sensitivity of the method is also desired. Extraction of the analytes from oils with a mixture of water and methanol at proportions of 60/40 (v/v) and vortexing for 2 minutes was found to be efficient for actual recovery of HNE and HHE 75 and 85% respectively at spiking levels between 0.1 and 1 μg g^{-1}. Thus, the same proportion of methanol was chosen as extraction mixture for samples such as meat and potato crisps. When this extraction technique was applied on infant formula and other dairy products, the formed oximes could not be further extracted with pentane due to the incomplete separation of the aqueous and organic solvent phase which most probably occurred due to the presence of dairy proteins. In order to overcome this limitation, the QuEChERS method (Anastassiades, Lehotay, Stajnbaher, & Schenck, 2003) for determination of pesticide residues in fruits and vegetables was selected for the extraction of the analytes. In preliminary experiments, HNE along with HHE and their deuterated analogs were added in aqueous solutions of beef and infant formula and incubated at room temperature overnight. The analytes were extracted from the meat with a mixture of water and methanol 60/40 (v/v) and from the infant formula using the QuEChERS method. The amount of the analytes recovered was equal to the blank samples and notable low recovery of the IS was observed. α, β-unsaturated aldehydes are known to form Schiff bases with the ε-NH_2 groups of lysine and Michael adducts between the electrophilic double bond and the nucleophilic groups of cysteine, histidine and lysine (Uchida & Stadtman, 1992; Rauniyar & Prokai, 2009; Naveena et al., 2010).

In order to avoid interactions of the proteins present in the food samples with the IS and to ensure the recovery of the free aldehydes present, the extraction from meat samples was carried out with 60/40 (v/v) 1.66 M H_2SO_4 / methanol. The acid present in the extraction mixture is expected to minimize the reactivity of the nucleophilic groups of the amino acids towards the analytes and the IS. For the extraction of the analytes from infant formula using

the QuEChERS method addition of acid was not found to be necessary as far as the extraction was carried out as soon as the IS was added. This could be attributed to alterations of the proteins and thus reduced reactivity with the aldehydes.

Table 3.1 Statistical comparison of the addition curves with the calibration curves. p values below 0.05 indicate statistically significant difference between the two slopes

		Addition curve		**Calibration curve**		**p value**
		Slope	**R^2**	**Slope**	**R^2**	
HNE	potato crisps	0.7431	0.9986	0.7696	0.9989	0.4257
	beef	0.7696	0.9989	0.7418	0.9940	0.2809
	Oil	0.7643	0.9929	0.6940	0.9995	0.0153
	infant formula	0.6928	0.9909	0.5873	0.9625	0.0818
HHE	potato crisps	1.0691	0.9984	1.1277	0.9994	0.0011
	Beef	1.1963	0.9977	1.1504	0.9979	0.0842
	Oil	1.1277	0.9994	1.1640	0.9970	0.1694
	infant formula	1.0932	0.9966	1.2053	0.9984	0.0003

The matrix effect on the recovery of the analytes was evaluated by comparing the slopes of the calibration curves with those of the addition curves. A slight difference in the polarity of HNE compared to HNE *d11* due to the high level of deuterium atoms can be expected. Thus, calibration curves were prepared by applying the corresponding extraction method for each matrix excluding the sample. However, apart from the difference in polarity between HNE and the deuterated analogue, chemical interactions with the matrix due to the aldehyde group, double bond and hydroxyl group are expected to be identical. The results of the statistical evaluation of the slopes of the calibration and addition curves are shown in ***Table 3.1***. Concerning HNE, only the oil samples appear to have a slight but statistically significant matrix effect (p= 0.0153). In the case of HHE, a matrix effect is evident in potato crisps and infant formula (p=0.0011, p=0.0003). The trueness of the measurement was assessed through the recovery of the analytes added at different levels (addition curves) as suggested by the European Union Commission decision 2002/657/EC (EC., 2002) and the results are illustrated in ***Table 3.2.***

Table 3.2 HNE and HHE recoveries from the spiked matrices.

Matrix	HNE HHE Added µg g^{-1}	HNE detected µg g^{-1}	StDev	% CV	% Recovery	HHE detected µg g^{-1}	StDev	% CV	% Recovery
potato	0.72	0.71	0.06	9.1	99.0	0.66	0.01	1.7	93.2
crisps	0.57	0.57	0.08	14.0	99.9	0.52	0.02	4.8	92.5
	0.39	0.38	0.02	4.9	95.6	0.36	0.01	3.7	91.2
	0.24	0.22	0.01	2.2	92.8	0.21	0.00	3.4	89.2
	0.08	0.09	0.01	10.5	113.5	0.08	0.00	1.5	107.2
beef	0.37	0.37	0.04	11.1	98.0	0.37	0.06	15.3	98.1
	0.28	0.26	0.03	11.0	91.9	0.26	0.05	17.5	94.1
	0.20	0.19	0.01	5.1	90.4	0.19	0.02	11.2	94.7
	0.12	0.11	0.01	3.9	89.5	0.11	0.01	10.7	92.4
	0.04	0.04	0.01	13.0	89.6	0.03	0.00	11.3	90.6
oil	0.71	0.74	0.04	5.9	104.3	0.76	0.01	1.8	99.0
	0.54	0.59	0.06	10.4	108.0	0.55	0.03	5.1	101.1
	0.37	0.36	0.02	7.0	97.7	0.38	0.01	3,1	100.6
	0.23	0.22	0.02	10.1	95.5	0.21	0.01	4.9	90.6
	0.08	0.07	0.02	22.4	90.0	0.07	0.01	19.6	85.5
infant	0.72	0.62	0.05	12.9	87.0	0.66	0.03	5.0	92.7
formula	0.53	0.46	0.01	14.2	87.1	0.49	0.02	5.0	93.4
	0.40	0.36	0.06	16.3	89.8	0.39	0.03	8.0	96.5
	0.23	0.20	0.03	14.2	86.7	0.22	0.02	7.5	95.2
	0.08	0.07	0.01	18.3	87.2	0.08	0.02	23.7	107.6

The values are the mean of four replications of analysis performed in two different days. The results indicate that in all the matrices the error from the actual amount added in the sample is always, with the exception of the recovery of HNE from potato crisps at the lower spiked level, between -20 to +10%. This is within the limits of the directive for concentrations above 10µg g^{-1}. The data suggest that, the matrix effect, indicated by the comparison of the slopes of the addition and calibration curves, did not have a significant impact on the accuracy of the method. In order to examine the repeatability, different samples of each group were analysed in duplicate in two different days and the coefficient of variation (CV) was found to be below 20% for both analytes in four different matrices with the exception of HNE in oil samples at concentration of 80 ng g^{-1} (CV 22.4%) and HHE in infant formula samples at concentration of 79 ng g^{-1} (CV 23.7%). Furthermore, reproducibility was evaluated by analysing oil samples using two different analysts in different days and the results are shown in ***Table 3.3***. Regarding HNE, the CV varied between 5.9 and 20 % while for HHE between 2.9 and 15.5 %.

Table 3.3 HNE and HHE recoveries from the spiked oil samples

Matrix	HNE HHE Added µg g^{-1}	HNE detected µg g^{-1}	StDev	% CV	% Recovery	HHE detected µg g^{-1}	StDev	% CV	% Recovery
oil	0.71	0.72	0.04	5.9	101.6	0.69	0.03	3.8	96.5
	0.55	0.57	0.06	9.8	104.9	0.54	0.03	5.9	99.0
	0.38	0.37	0.02	6.3	98.1	0.37	0.01	2.9	97.9
	0.24	0.23	0.03	11.6	97.1	0.22	0.01	4.5	91.5
	0.08	0.07	0.02	20.2	93.9	0.07	0.01	15.5	87.8

Limits of Detection (LOD) and Limits of Quantification (LOQ) were calculated based on the standard deviation (StDev) of the residuals of the response and the slope of the addition curve (Mocak, Bond, Mitchell, & Scollary, 1997). LOD equals to 3.3 x (StDev / Slope), while LOQ equals to 10 x (StDev/Slope). LODs and LOQs were determined for each matrix and the results are shown in ***Table 3.4***. Statistical determination of the LODs and LOQs is indicating higher values compared to empirical methods i.e. methods based on the calculation of the signal-to-noise ratio. Based on the available results in the literature (Surh, Lee, & Kwon, 2007; Surh & Kwon, 2005; Seppanen & Csallany, 2002) together with the results from the samples analyzed with the present method the LODs and LOQs are below the expected concentrations.

Table 3.4 LOD and LOQ (ng g^{-1} of sample) for the different matrices

		Potato crisps	Beef	Oil	Infant formula
HNE	LOD	20.1	4.2	17.2	32.1
	LOQ	61.2	12.8	52.1	97.3
HHE	LOD	7.3	4.2	10.4	9.4
	LOQ	21.2	12.7	31.1	28.4

Table 3.5 illustrates the levels of HNE and HHE detected in selected samples available in the Belgian market. As already mentioned HNE is expected to be formed during oxidation in foods containing ω-6 fatty acids, while formation of HHE is related to the presence of ω-3 fatty acids. In fact in samples such as peanuts where the dominating PUFA is linoleic and ω-3

PUFAs are not present, only HNE could be detected. In walnuts where linolenic acid was found to be on average 11 % of the fatty acids compared to 60% of linoleic, HHE was detected but in significantly lower levels than HNE. Extra virgin olive oils with an average fatty acid composition of 78 % oleic, 4.5 % linoleic and 0.7 % linolenic acid demonstrated higher amounts of HHE as compared to HNE, indicating that linolenic acid was more prone to oxidation (Frankel, 2005). Heated frying oil contained 40.6 and 2.6% ω-6 and ω-3 fatty acids respectively (Detailed information concerning the fatty acid profile of the ω-3 enriched oil samples can be found in table 4.2 in Chapter 4). Heating of the oils at temperatures applied in frying resulted in formation of significant amounts of HNE and HHE indicating that, consumption of fried foods can contribute to high intake of these aldehydes. The levels of HNE and HHE in the frying oils before heating were below the LODs. In the available literature, levels of HNE in thermally oxidized oils have been reported between 5.6 μg g^{-1} (LaFond et al., 2011) and as high as 40 μg g^{-1} when oils rich in PUFAs were used (Seppanen & Csallany, 2004). Concerning infant formulas the levels of HNE and HHE were comparable to was has been reported by others (Surh et al., 2007).

Table 3.5 HNE and HNE levels in analyzed food samples. (n=4) whereas ± standard deviation is mentioned. The rest are obtained from a single analysis of each sample

Sample description	**HNE ng g^{-1} sample**	**HHE ng g^{-1} sample**
heated frying oil ω-3 enriched	3750 ± 24	520 ± 10
heated frying oil	3760 ± 20	181 ± 5
infant formula	209 ± 10	21 ± 3
sardines canned	Not detected	25 ± 4
colza oil	111	149
extra virgin olive oil A	69	155
extra virgin olive oil B	117	141
extra virgin olive oil C	57	99
peanuts A	598	Not detected
peanuts B	124	Not detected
cookies A	132	25
cookies B	103	23
cookies C	179	21
walnuts A	136	24
walnuts B	406	33

3.4 CONCLUSIONS

We have designed, implemented and validated an accurate and reproducible analytical technique for determination of free HNE and HHE that can be applied in a broad range of food samples. The results are not affected by the analyzed matrix and are not dependent on the recovery of the analyte during the extraction. We suggest that milk and dairy products or generally samples containing surface active compounds can be analyzed with the QuEChERS method. The method in which the analytes are extracted with acidified methanolic solution developed and validated on raw beef and crisps could also be applied on cooked meat and meat products, dry nuts and cookies. Oils, butter, margarines and mayonnaise could be analyzed by the same method.

The next chapter is related to the development of an analytical method suitable for the determination of 2-butenal in foods. In addition factors influencing the formation of this compound during frying are investigated.

CHAPTER 4

DEVELOPMENT AND VALIDATION OF A STABLE ISOTOPE DILUTION ASSAY FOR DETERMINATION OF 2-BUTENAL AND INVESTIGATION OF ITS FORMATION IN OILS AND FOODS DURING FRYING

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CHAPTER 4. DEVELOPMENT AND VALIDATION OF A STABLE ISOTOPE DILUTION ASSAY FOR DETERMINATION OF 2-BUTENAL AND INVESTIGATION OF ITS FORMATION IN OILS AND FOODS DURING FRYING

SUMARY

The formation of 2-butenal in oils subjected to temperatures applied in frying as well as in fried foods was evaluated. For this purpose, a sensitive and accurate stable isotope dilution essay was developed and validated using as an internal standard 2-butenal d6 synthesized in the lab. Heating of corn oil, sunflower oil, extra virgin and refined olive oil, ω-3 frying oil and palm oil at 170 °C for a total of 8 hours resulted in the formation of 2-butenal at levels between 0.05 to 1 μg g-1and the highest amounts were already detected after two hours of heating. The highest 2-butenal formation was observed in the oil containing ω-3 unsaturated fatty acids indicating that 2-butenal content is mainly affected by the fatty acid composition. No significant amount of 2-butenal was detected in fried foods purchased from fast food shops with the exception of one chicken sample and a pork brochette. Further experiments including several foods did not indicate any increase of 2-butenal content due to frying. It can be suggested that fried foods may not be a significant dietary source of 2-butenal for humans. However, contamination of the indoors environment where frying is taking place could be of potential concern.

Keywords: Lipid oxidation, 2-butenal, Stable Isotope Dilution Assay, Frying oils

4.1 INTRODUCTION

2-Butenal, also referred as crotonaldehyde, is a four carbon α,β-unsaturated aldehyde which is an ubiquitous environmental pollutant and can be also produced from the decomposition of hydroperoxides resulting from oxidation of ω-3 polyunsaturated fatty acids (PUFAs) (Frankel, 2005). Oxidation of PUFAs can occur during storage and processing of oils. Heating of oils at high temperatures during cooking or deep frying can result in formation of undesired compounds which can have an impact on sensorial properties or can also be of toxicological concern. Modern analytical techniques such as Head Space and or Solid Phase Micro Extraction combined with Gas Chromatography and Mass spectrometry (HS-SPME-GC-MS) have been employed the last years for the determination of these volatile compounds. Acrolein has been recently detected and quantified in heated oils at frying temperatures with a HS-GC-MS based technique (Ewert et al., 2011). Furthermore, α,β-unsaturated aldehydes along with other compounds have been detected in vegetable and fish oils during storage (Guillen & Goicoechea, 2008a; Guillen et al., 2009) and during heating at frying temperatures (Guillen & Uriarte, 2012). The later study also revealed that the profile of aldehydes produced, depended upon the temperature at which oxidation took place. However, to the best of our knowledge no data have been published, reporting the actual quantities of 2-butenal formed during heating of oils at frying temperature. Furthermore, the levels of 2-butenal in fried products and how they are affected by frying process remain unknown.

Therefore, the aim of the study presented in this chapter was to develop a sensitive and accurate stable isotope dilution assay for determination of 2-butenal in oils and fried products. For this purpose deuterated 2-butenal was synthesized in order to be used as an internal standard (IS). In addition, the 2-butenal content of frying oils and fried products was determined and in addition a short survey on its levels in fried products purchased from fast food restaurants in Belgium was conducted.

4.2 MATERIALS AND METHODS

4.2.1 Chemicals and samples

Six commercial oils including corn, sunflower, palm, extra virgin olive oil, refined olive oil and an ω-3 rich frying oil were purchased from the local market. Pre fried French fries, chicken nuggets, beef burger and Alaska Pollock fish sticks covered by breadcrumbs was purchased frozen from the local market. Already fried snacks were purchased from local fast food shops. 2-butenal, O-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride (PFBHA), deuterium oxide and sodium deuteroxide were supplied by Sigma-Aldrich (Bornem, Belgium). Acetaldehyde *d4* was purchased from C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada). Deionised water (Milli-Q, Millipore Corp.) of 18.0 MΩ cm^{-1} resistivity was used throughout the experiments and all the solvents used were of analytical grade.

4.2.2 Oil sample preparation

Heat treatment of the oils was applied as follows: five grams of the oil were placed in an open glass test tube of 16 mm diameter. The oil was heated at 170 ^{o}C in an oven for 2, 4, 6 and 8 hours. Frying experiments were performed in a household fryer of 4 liters capacity, the oil was heated for two hours at 170 °C prior to frying and the products were fried for 4 minutes. All samples were analyzed after cooling at ambient temperature and the oil temperature was measured throughout the experiments with a thermocouple (Testo, Lenzkirch, Germany).

4.2.3 Fatty acid composition, polar fraction and p-Anisidine value

Fatty acid profile of the oils was determined after preparation of fatty acid methyl esters (FAMEs) and analysis with gas chromatography according to the American Oil Chemists' Society (AOCS) official method Ce 1b-89 (AOCS, 1989). p-Anisidine value was determined in duplicate for each of the samples based on AOCS Official Method Cd 18-90 (AOCS, 1992). Polar fraction was determined with column chromatography according to the IUPAC standard method 2.507(IUPAC, 1987).

4.2.4 Preparation, characterization and quantification of 2-butenal *d6*

A total of 1.63 moles of acetaldehyde *d4* were added to 2 mL of 0.2 N sodium deuteroxide solution prepared in deuterium oxide and the mixture was reacted for 10 min at room temperature. The formed 2-butenal *d6* was extracted five times with 2 mL of pentane and was subjected to mass spectrometric characterization to confirm its identity. For this reason 10 µL of the pentane extract was placed in a 20 mL headspace glass vial and pentane was evaporated under a gentle stream of nitrogen. The vial was sealed with a PTFE septum cap, kept at 40 ^{o}C for 10 min and a CAR-PDMS fiber was inserted in the headspace of the vial for 10 min at 60 ^{o}C. Chromatographic analysis was performed in an Agilent 7890A GC (Agilent, Palo Alto, CA) equipped with a CTC PAL auto sampler and a 5975C Mass Spectrometer. The analyte was desorbed from the fiber in a PTV inlet at 250 ^{o}C in splitless mode for 2 min and chromatographic separation was carried out in a DB 624 capillary column 60 m x 0.25 mm ID, with 1.4 µm film thickness. The temperature program was set as follows: initial temperature 35 ^{o}C for 5 min, then 10 ^{o}C min^{-1} to 100 ^{o}C and kept at this temperature for 5 min, followed by a ramp of 50 ^{o}C min^{-1} to 230 ^{o}C held for 10 min. The MSD conditions were the following: capillary direct interface temperature, 250 ^{o}C; ionization energy, 70 eV; operating mode scan from *m/z* 30 to *m/z* 100; scan rate 3.64 cycles/second.

The 2-butenal *d6* concentration in the pentane extract was quantified prior to use as an internal standard by GC combined with flame ionization detection. A total of 0.1 µL of the pentane solution was injected cold on column into a CP-Sil 88 capillary column 60 m x 0.25 mm ID, with a film thickness of 0.2 mm, installed in an Agilent 6890N series GC. The temperature program applied was the following: initial temperature 40 ^{o}C for 5 min, then 10 ^{o}C min^{-1} to 120 ^{o} C, followed by a ramp of 20 ^{o}C m^{-1} to 220 ^{o}C held for 2 min. Quantification was carried out by preparing an external calibration curve using 2-butenal at a range of 0.06 to 1 mM.

4.2 5 Extraction and derivatization of 2-butenal

For 2-butenal extraction from oils a total amount of 1 g of sample was weighted in a 15 mL polypropylene tube and spiked with a known amount of IS (2.04 µg). A total of 3 mL of water was added and the sample was vortexed for 1min followed by centrifugation at 3,000 x g for 5 min (Sigma 4K15, Sartorius, Goettingen, Germany). A total of 1 mL of the aqueous phase was mixed with 1 mL of aqueous solution of 2 mg mL^{-1} PFBHA in a 20 mL headspace

vial. The vial was sealed with a PTFE septum cup and the samples were incubated for 30 minutes at 40 ^{o}C in order to form the PFBHA-2-butenal oximes.

Concerning meat samples and French fries, a total of 2 g of sample was weighted in a 50 mL falcon tube and spiked with a known amount of IS, 10 mL of extraction buffer was added and the contents were homogenized with an Ultraturax (Janke & Kunkel, IKA-Werk, Staufen, Germany) at 12,000 rpm for 1 min. The homogenate was centrifuged at 3,000 x g for 5 min and the aqueous phase was subjected to derivatization as mentioned previously. Extraction buffer consisted of 0.1 M of Na_2HPO_4 and KH_2PO_4 and the pH was adjusted to 2.00 with H_2SO_4.

4.2.6 Analysis of the PFBHA- 2-butenal- oximes

The vials containing the oximes were kept at 70 ^{o}C for 10 minutes and 0.5 mL of the headspace was extracted with a gastight syringe. Chromatographic analysis was performed on the same GC used for the characterization of butenal *d6*. The temperature program was the following: initial temperature 50 ^{o}C for 1 min, then 30 ^{o}C min^{-1} to 200 ^{o}C, followed by a ramp of 5 ^{o}C min^{-1} to 230 ^{o}C held for 8 min. The MSD conditions were the following: capillary direct interface temperature, 250 ^{o}C; ionization energy, 70 eV; operating mode selective ion (SIM); selected ions monitored, *m/z* 181, *m/z* 250, *m/z* 265, *m/z* 253 and *m/z* 271; scan rate 3.64 cycles/second. In order to obtain the full mass spectra of 2-butenal oxime and its deuterated isotopic oxime, a scan mode analysis was performed between *m/z* 50 and *m/z* 400.

4.2.7 Statistical analysis

One-way analysis of variance (ANOVA) was applied in order to detect differences between the slopes of the addition and calibration curves in the validation of the analytical technique. Multiple-way ANOVA was performed on the measured variables to find significant differences between treatments. For the experiment related to heating of the oils, 2-butenal formation was studied based on two experimental factors (two-way ANOVA): (a) oil type (6 levels) and (b) heating time (5 levels). For the experiment related to fried products, one-way ANOVA was applied (1factor, 10 levels). Whereas significant effects were detected ($p < 0.05$), Student-Newman-Keuls test of mean comparisons was applied. All the analysis was carried out with SPSS 18 statistics package (IBM, SPSS, Inc.).

4..3 RESULTS AND DISCUSSION

4.3.1 Development and validation of the analytical method for the determination of 2-butenal

Successful synthesis of labeled 2-butenal was verified by mass spectrometry. ***Figure 4.1*** illustrates the mass spectra obtained from 2-butenal and its deuterated analogue 2-butenal *d6.* The mass spectra of 2-butenal contained the molecular ion *m/z* 70 as well as the following fragments: *m/z* 69, *m/z* 41, *m/z* 39 and *m/z* 29. The mass spectra of the labeled isotope (**Figure 1B**) contained the molecular ion *m/z* 76 as well as *m/z* 74, *m/z* 46, *m/z* 42 and *m/z* 30. All these ions are expected to be present after the ionization of 2-butenal *d6*. Mass spectrometric analysis confirmed that self-condensation of acetaldehyde *d4* under the controlled conditions applied resulted only in the formation of 2-butenal *d6*. In detail, α-cleavage of the deuterium of the aldehyde group is responsible for the presence of *m/z* 74. Absence of any significant signal from *m/z* 69, *m/z* 70, *m/z* 71, *m/z* 72 and *m/z* 73 indicated that heterogeneous labeling in the carbons 2, 3 and 4 did not take place. Furthermore, α-cleavage between the first and second carbon is responsible for the formation of the fragments with *m/z* 46 and *m/z* 30. Therefore, absence of *m/z* 29 (typical fragment of aldehydes) confirmed that no hydrogen was present in the aldehyde group. Derivatization with PFBHA after the extraction of the analyte and prior to GC-MS analysis was chosen in order to improve the performance of the analytical technique. It is well accepted that derivatization of small molecular weight volatile compounds prior to analysis is improving the chromatographic separation. Moreover the formed adducts have easily distinguishable characteristic ions in mass spectrometry, thus derivatization is resulting in more sensitive analytical techniques (Shibamoto, 2006; Saison, De Schutter, Delvaux, & Delvaux, 2009). The formation of 2-butenal and 2-butenal *d6* oximes with PFBHA was studied in a similar pattern. The ratios *m/z* 250 and *m/z* 265 and *m/z* 253 and *m/z* 271 for 2-butenal and 2-butenal *d6* oximes respectively together with the retention times were used for positive identification of the compounds. *m/z* 250 and *m/z* 253 were chosen for quantification of 2-butenal and 2-butenal *d6* oxime respectively.

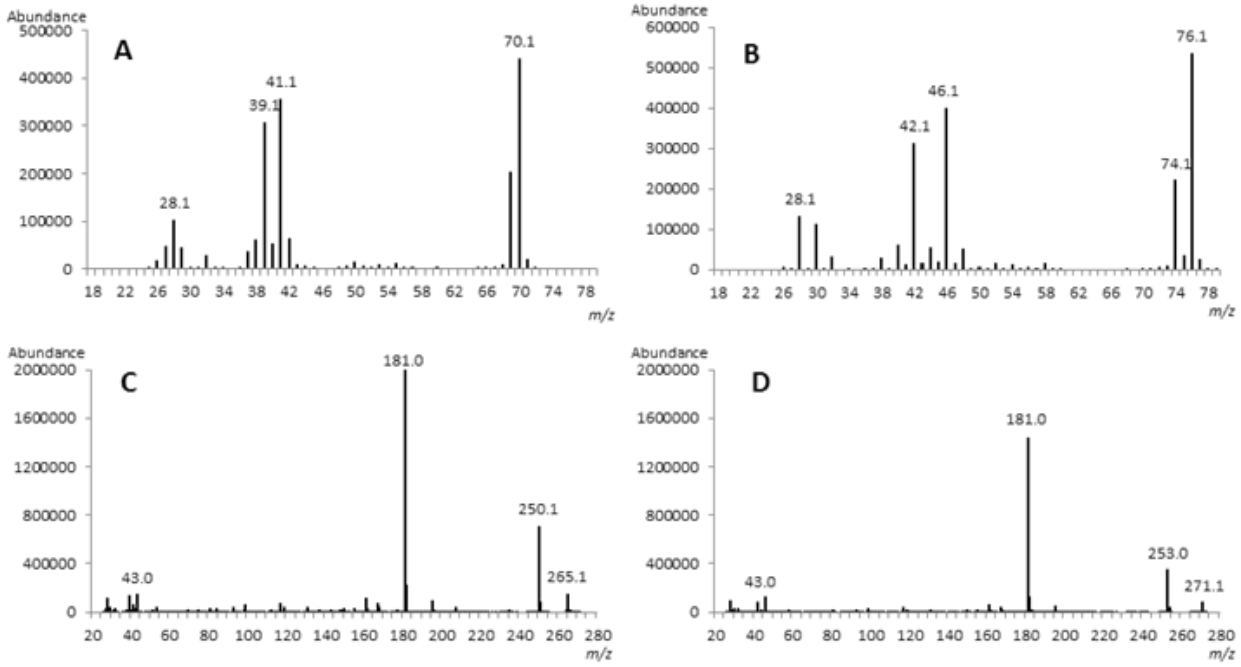

Figure 4.1 Mass spectra of A) 2-butenal, B) 2-butenal *d-6*, C) PFBHA-2-butenal derivative, D) PFBHA-2-butenal *d-6* derivative

Characteristic chromatograms obtained from analysis of oil samples in selective ion mode are illustrated in ***Figure 4.2***. 2-butenal and its corresponding isotope eluted as two peaks, since two stereoisomers were formed during the derivatization with PFBHA, thus for quantification the sum of both peaks was taken into account.

In order to evaluate the efficiency of the extraction technique to recover 2-butenal from the oil and evaluate any matrix effect, an addition curve was prepared by spiking the samples with 0.03, 0.04, 0.09, 0.3, 0.45, 0.9, 3, 4.5 µg $g^{-1}$of 2-butenal and 1.2 µg $g^{-1}$of deuterated analogue as IS. For validation of the extraction technique from meat products, fresh meat was spiked with 0.03, 0.05, 0.10, 0.5, 1 µg $g^{-1}$of 2-butenal and 2.4 µg g^{-1} of IS. The matrix effect on the recovery of 2-butenal was evaluated by comparing the slope of the calibration curve with the slope of the addition curve. No statistical significant difference between the calibration and addition curve was detected ($p>0.05$) indicating no matrix effect.

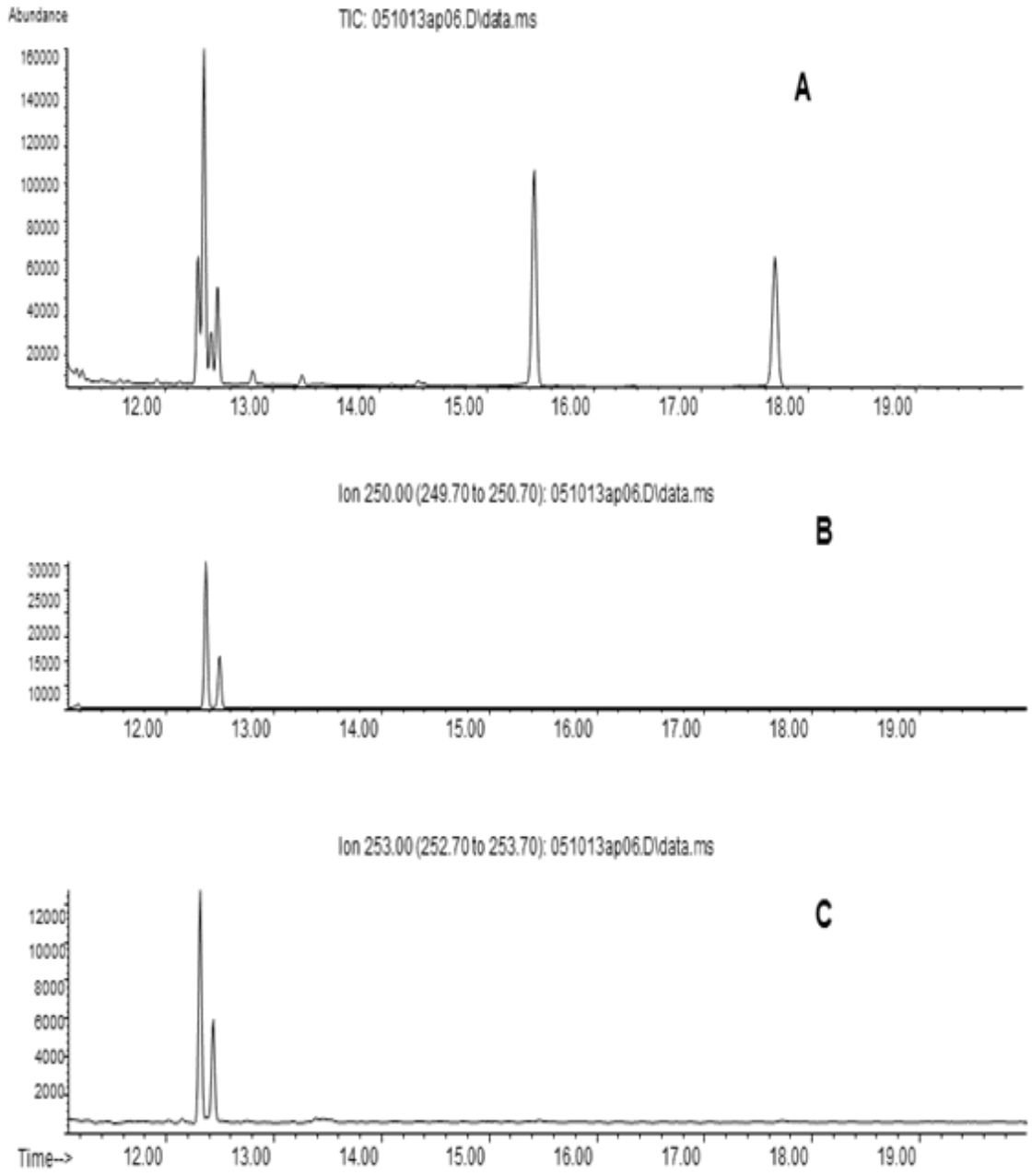

Figure 4.2 Characteristic chromatograms of 2-butenal and 2-butenal *d-6* oximes. A) Total ion chromatogram in SIM of oil sample, B) 2-butenal oxime, extracted ion m/z 250, retention time 12.36, 12.493, C) 2-butenal *d-6* oxime, extracted ion m/z 253, retention time 12.31, 12.43

The trueness of the measurement was evaluated through the recovery of the analytes added at different levels (addition curves) as suggested by the European Union Commission decision 2002/657/EC (EC., 2002) and the results are shown in ***Table 4.1.*** According to the results the assay was proven to be sensitive and reproducible.

Table 4.1 2-butenal recoveries from the spiked oils and meat samples (n=5)

Matrix	**2-butenal added µg g^{-1}**	**2-butenal recovery %**	**CV % of the recovery**
oil	0.03	97.8	12.1
	0.04	92.7	8.8
	0.09	71.8	17.1
	0.30	89.8	6.6
	0.45	84.2	9.5
	0.90	92.0	10.2
	3.00	98.0	2.7
	4.50	101.0	3.3
pork meat	0.03	81.2	5.8
	0.05	95.0	12.7
	0.10	109.3	7.6
	0.50	120.9	5.3
	1.00	113.6	3.4

LOD and LOQ were calculated based on the standard deviation (StDev) of the residuals of the response and the slope of the addition curve (Mocak et al., 1997) from 0.03 to 0.3 µg g^{-1}. LOD equals to 3.3 x (StDev / Slope), while LOQ equals to 10 x (StDev/Slope). For the oil samples LOD and LOQ were 0.013 and 0.02 µg g^{-1} respectively, while for the meat samples LOD and LOQ were 0.014 and 0.028 µg g^{-1} respectively.

4.3.2 2-butenal formation during heating of the oils

The fatty acid profile of the samples subjected to the heating experiment are shown in ***Table 4.2***. The ω-3 rich frying oil was found to contain 3.6% of 18:3 and lower amounts of 18:2 compared to corn and sunflower oil. For the heating experiment, the six types of vegetable oils were subjected to 170 ^{o}C up to 8 hours and 2-butenal levels as well as p-anisidine values were monitored. The total heating time applied reasonably resembled the operating timeframe of a fryer in a restaurant and the experiment was performed in triplicate and the polar fraction of the oils was measured after 8 hours of heating.

Table 4.2 Fatty acid composition of the oils used in the heating experiments. Values represent g of fatty acid per 100 g of fatty acids

Fatty acid	Corn oil	ω-3 frying oil	Olive oil extra virgin	Olive oil refined	Palm oil	Sunflower oil
16:0	10.4	9.6	11.8	12.2	46.5	6.0
16:1	0	0	0.9	0.9	0.4	0
18:0	1.9	2.6	3.6	3.6	5.1	3.5
18:1	30.3	58.0	78.1	77.8	39.1	25.1
18:2	55.8	25.0	4.5	4.5	8.4	65.4
18:3	0.9	4.1	0.6	0.7	0.1	0
20:0	0.4	0.4	0.3	0.3	0.4	0.3

Polar fraction was 8.7, 7.6, 6.5, 5.8, 9.6 and 9.8% for corn, ω-3 frying oil, extra virgin olive oil, refined olive oil, palm and sunflower oil respectively. These percentages were far below 27%, which are considered the limit for edible oils (Nawar, 1996). ***Figure 4.3*** illustrates the evolution of 2-butenal formation throughout heating in the different oils. The results indicate the highest levels of 2-butenal were formed after two hours of heating and remained stable throughout the experiment for all the oils. Exception was the ω-3 containing oil, in which the maximum 2-butenal content was observed after four hours of heating. The stabilization of 2-butenal levels could be attributed to evaporation of 2-butenal from the oils at a rate equal to the rate of formation of the compound. The fact that 2-butenal is reaching its maximum level in oils after short period of heating has been also reported by others (Guillen & Uriarte, 2012). 2-butenal has been found in the indoor environment at the level of 6.55 μg m^{-3} when soybean oil was subjected to frying temperatures and the emission rate was 3.06 μg g^{-1} of oil h^{-1} (Seaman, Bennett, & Cahill, 2009).

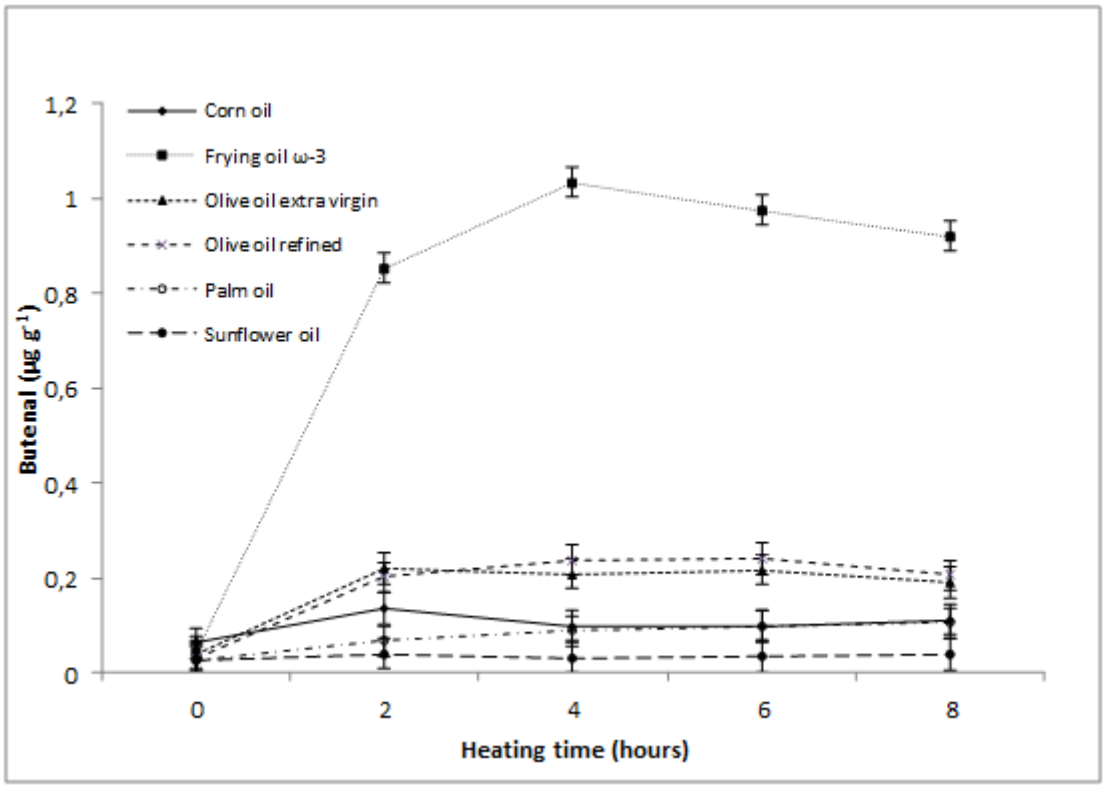

Figure 4.3 Changes in 2-butenal content (μg g^{-1}) in the oils during heating at 170 °C. Points represent the mean of 3 replications ± 95% CIs

Formation of 2-butenal was the lowest in sunflower oil, reaching a maximum of 0.031 μg g^{-1} after two hours of heating. Extra virgin and refined olive oil reached the levels of 0.242 and 0.217 μg g^{-1} after six hours respectively and no significant difference ($p>0.05$) was observed between them. Corn and palm oil contained 0.097 μg g^{-1} after 6 hours of heating. The highest levels of 2-butenal were observed in the ω-3 rich frying oil (1.034 μg g^{-1}) and it is related to the relatively high levels (3.6%) of linolenic acid present. This is supported from the observation that the levels of 2-butenal of the heated oils and the ω-3 fatty acid content were found to correlate (R^2= 0.95). On the other hand ω-6 fatty acid content was not found to correlate with the levels of 2-butenal. The 2-butenal levels detected were lower than what has been reported for other α,β-unsaturated aldehydes. Acrolein has been found at levels of 5.4 and 8.5 μg g^{-1} in coconut and olive oils and up to 101 μg g^{-1} in linseed oils subjected to 180 °C for six hours (Ewert et al., 2011). 4-hydroxynonenal has been detected at levels between 6 to 40 μg g^{-1} in thermally oxidized soybean oils (Seppanen & Csallany, 2002; LaFond et al., 2011; Papastergiadis, Mubiru, Van Langenhove, & De Meulenaer, 2013). In thermally oxidized sunflower oils of which the polar fraction amounted 25 %, epoxy unsaturated

aldehydes, such as (E)-4,5-epoxy-(E)-2-decanal were detected at a level of 30 μg g^{-1} (Guillen & Uriarte, 2012).

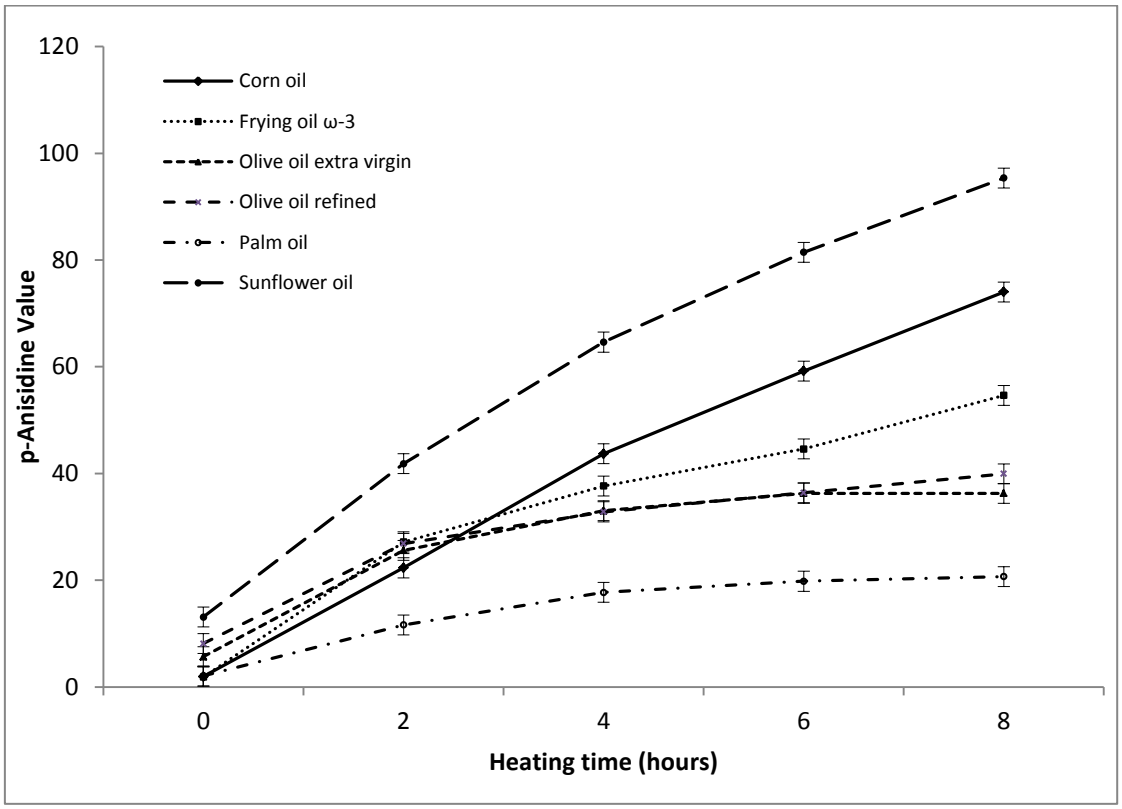

Figure 4.4 Changes in p-Anisidine value in the oils during heating at 170 °C. Points represent the mean of 3 replications ± 95% CIs

The total level of unsaturated aldehydes formed in the oils, as measured with the p-anisidine value test (***Figure 4.4***), was not in agreement with the amounts of 2-butenal detected. The p-anisidine value method measures principally the content of unsaturated aldehydes (2-alkenals and 2,4-alkadienals) in oils (Shahidi & Zhong, 2005). The p-anisidine value was continuously increasing up to the end of the experiment for all the oils Interestingly, corn and sunflower oil which demonstrated the lowest levels of 2-butenal, reached the highest levels of p-anisidine value. This could be explained by the high amounts of 18:2 present, the hydroperoxides of which are considered precursors of unsaturated aldehydes. Moreover, this discrepancy shows that 2-butenal is not the principle unsaturated aldehyde produced as a result of oil oxidation.

4.3.3 Presence of 2-butenal in fried products

A small survey on the presence of 2-butenal in fried products was conducted on twelve fried snacks purchased from three individual local fast food shops. Detailed description of the samples and 2-butenal contents are listed in ***Table 4.3***. It is remarkable the low levels 2-butenal was detected which was below the LOD in several samples.

Table 4.3 2-butenal levels detected in fried samples from fast food shops. Values were obtained by a single analysis

Sample	2-butenal (μg g^{-1})
pork brochette (1)	0.049
pork brochette (2)	<LOD
pork brochette with vegetable slices (1)	0.015
pork brochette with vegetable slices (2)	0.014
fish brochette (1)	0.014
fish brochette (2)	<LOD
beef burger	0.014
chicken sausage	0.018
chicken legs	0.022
hot dog (Frikadel)	<LOD
French fries (1)	0.014
French fries (2)	<LOD

The highest levels were detected in pork brochette from one shop (0.049 μg g^{-1}). As a common practice, fats with low amount of PUFA are usually used for frying operations in restaurants and industry. Thus it was not surprising that only very low 2-butenal levels were found in the analyzed samples. Therefore another research strategy was followed in order to evaluate the potential presence of 2-butenal in fried foods by frying different types of samples (French fries, beef burgers, chicken nuggets and fish stick) in the ω-3 rich frying oils which demonstrated earlier to produce higher 2-butenal levels upon frying. The frying oil was heated at 170 °C for two hours prior to frying and 2-butenal levels were measured in the food products and in the oil prior and after the actual frying experiments. Frying was performed in triplicate and the results are presented in ***Figure 4.5.*** 2-butenal was detected at 0.044 μg g^{-1} in the frying oil prior to heating and 0.156 μg g^{-1} after two hours at 170 °C.

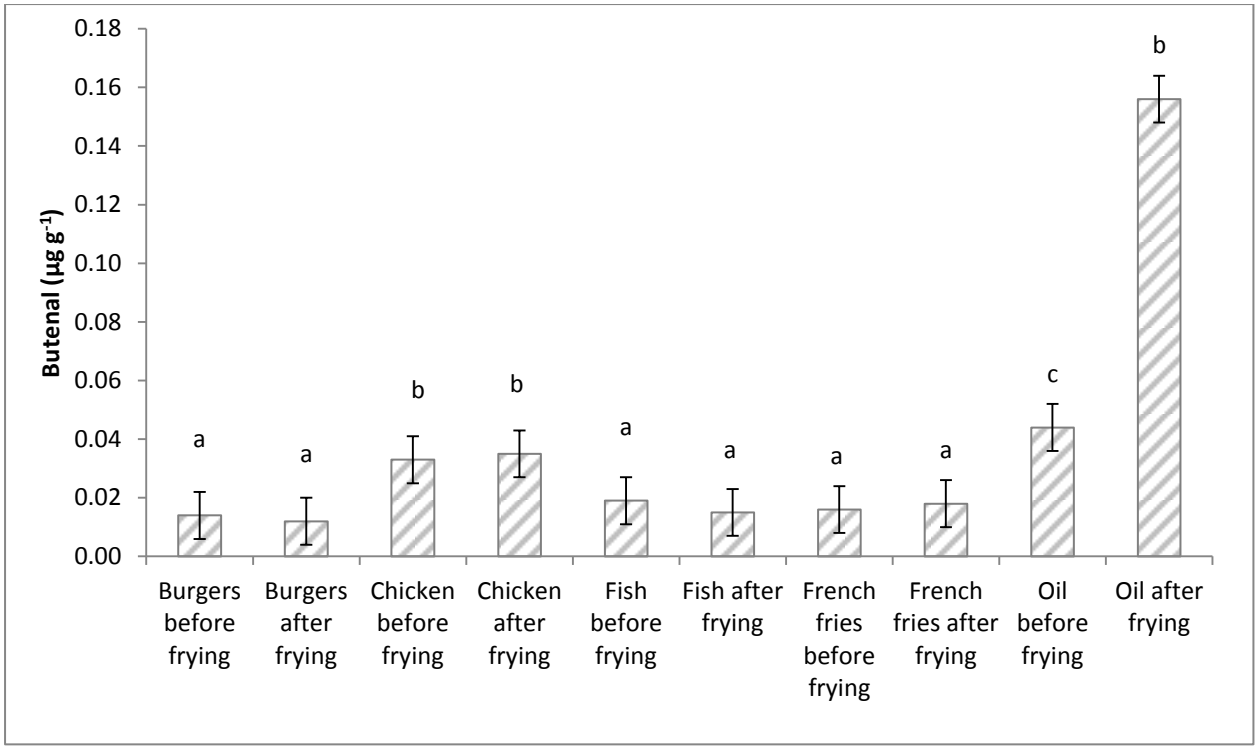

Figure 4.5 Levels of 2-butenal (μg g^{-1}) in foods and frying oil before and after frying at 170 °C. Data are the mean of 3 replications ± 95% CIs

This was ten times lower than what was found earlier in the oils heated in the test tubes and could be attributed to the bigger surface to volume proportion in the fryer compared to the test tubes, facilitating 2-butenal evaporation from the oil. Beef burgers, fish sticks and French fries had initial 2-butenal contents of 0.014, 0.019 and 0.016 μg g^{-1} respectively which was between the LOD and LOQ of the method. 2-butenal was detected at levels of 0.033 μg g^{-1} prior to frying in the chicken nuggets, which was higher compared to the other products. For all samples, no significant changes in the 2-butenal content could be observed as a result of the frying, despite the fact in the frying oil a clear increase in the 2-butenal content was observed. At this point it should be mentioned that any potential increase in 2-butenal levels should be expected to be a result of carry-over through absorbance of oil in the matrix, rather than formation due to oxidation of the fat naturally present in the food. This indicates that either 2-butenal was not absorbed by the food matrices subjected to frying in sufficient amounts or it entered in the matrix and reacted immediately with the food components. The latter hypothesis seems more plausible since it has been reported that α, β-unsaturated aldehydes are known to form Schiff bases with the ε-NH_2 groups of lysine and Michael adducts between the electrophilic double bond and the nucleophilic groups of cysteine,

histidine and lysine (Uchida & Stadtman, 1992; Rauniyar & Prokai, 2009; Naveena et al., 2010). In comparison of the levels of 2-butenal found in this study and the levels of MDA, HNE and HHE reported in the previous chapters, can be concluded that 2-butenal is found in lower amounts. However, based on these results conclusions only on the amounts of 2-butenal present in the oils and foods in a free form can be drawn and thus, no robust information of possible adduct formation and their importance can be evaluated.

4.4 CONCLUSIONS

We have developed, implemented and validated a sensitive and reproducible stable isotope dilution essay for the determination of 2-butenal in oils and food matrices. This is the first time, to the best of our knowledge, that 2-butenal is precisely quantified in oils and food matrices. The results indicate that 2-butenal is formed during heating of the oil at frying temperatures at levels between 0.05 to 1 μg g^{-1} of oil. Oils containing ω-3 PUFAs demonstrated the highest levels of 2-butenal formation. Furthermore, it was shown that at the studied temperature the highest level of 2-butenal determined in oils was already formed after two hours of heating. It can be suggested that 2-butenal content is mainly affected by the fatty acid composition than by the extent of lipid oxidation. Fried foods purchased from fast food shops did not contain significant amounts of 2-butenal and frying of several foods did not result in any increase of 2-butenal content. It can be suggested that fried foods may not be a significant dietary source of 2-butenal for humans. Contamination of the indoors environment with 2-butenal if inappropriate ventilation is applied could be of potential concern.

In the upcoming chapter, a study on the factors influencing the formation of MDA, HNE, HHE and 2-butenal in oil in water emulsions is presented. The factors incorporated in the study include the fatty acid composition, storage time as well as photosensitized oxidation.

CHAPTER 5

FACTORS INFLUENCING THE FORMATION OF MALONDIALDEHYDE, 4-HYDORXY-2-NONENAL, 4-HYDROXY-2-HEXENAL AND 2-BUTENAL IN OIL-IN-WATER EMULSION SYSTEMS

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CHAPTER 5. FACTORS INFLUENCING THE FORMATION OF MALONDIALDEHYDE, 4-HYDORXY-2-NONENAL, 4-HYDORXY-2-HEXENAL AND 2-BUTENAL IN OIL-IN-WATER EMULSION SYSTEMS

SUMARY

The formation of malondialdehyde (MDA), 4-Hydroxy-2-(E)-Nonenal (HNE) and 4-Hydroxy-2-(E)-Hexenal and 2-butenal was investigated in oil-in-water emulsions prepared with different fatty acid profiles and stored at 4 ^{o}C for a total of thirty days with and without illumination in the presence of riboflavin. The results suggest that the amounts of MDA, HNE, HHE and 2-butenal in the studied system is strongly affected by the type and extent of lipid oxidation along with the fatty acid profile. In illuminated samples, MDA was found to be the major aldehyde formed in emulsions prepared with fish oil, where eicosapentaenoic (EPA) and docosahexaenoic (DHA) were the major ω-3 poly unsaturated fatty acids (PUFAs). HHE was found to be the second most important, while 2-butenal was produced in only a small portion. HNE was found to be the major aldehyde formed when linoleic acid was the dominant PUFA. When lipid oxidation proceeded in the dark , EPA and DHA did not produce any significant amount of MDA, HHE and 2-butenal, indicating that there are other secondary oxidation products formed in more significant amounts than the studied aldehydes. In contrast, samples containing linoleic and linolenic acid produced considerably higher amounts of MDA, HNE, HHE and 2-butenal when stored under dark.

Keywords: Lipid oxidation, emulsions, malondialdehyde, 4-Hydroxy-2-(E)-Nonenal (HNE), 4-Hydroxy-2-(E)-Hexenal, 2-butenal

5.1 INTRODUCTION

As already mentioned previously, it has been suggested that MDA can originate from the decomposition of hydroperoxides of fatty acids with more than two double bonds, including bothω-3 and ω-6 PUFAs (Esterbauer et al., 1991; Esterbauer & Cheeseman, 1990). Regarding hydroxylated α,β- unsaturated aldehydes, it is well accepted that HNE is formed due to decomposition of ω-6 PUFA hydroperoxides, while HHE is mainly related to the oxidation of ω-3 PUFA (Long & Picklo, 2010; Surh et al., 2010; Han & Csallany, 2009; Pryor & Porter, 1990), as is 2-butenal (Frankel, 2005). Thus, it is easily understood that foods rich in PUFA can be a potential major dietary intake source of these aldehydes. However, it is not well understood which of these aldehydes is the most abundantly produced in matrices containing oils with different PUFA profiles.

In the work presented in this chapter the effect of fatty acid composition on the formation of MDA, HNE, HHE and 2-butenal in oil in water emulsions upon autoxidation and photooxidation was investigated. Oil in water emulsions were chosen as a model system representing foods containing 3% of fat, excluding all the other components (such as proteins) and thus their impact on the presence of free aldehydes. The results clearly indicated which of the studied aldehydes are of major importance under different oxidation conditions and fatty acid composition during storage. It is the first time a systematic investigation on the factors affecting the formation of the studied aldehydes in emulsions is reported.

5.2 MATERIALS AND METHODS

5.2.1 Supplies and reagents

HNE and HHE standards were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). HNE *d11*, HHE *d5* dimethyl acetals and acetaldehyde *d4* were purchased from C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada). 2-butenal, TWEEN 20®, silica gel 60 for column chromatography (particle size 63-100 μm), Aluminum oxide activated, basic, Brockmann I, *O*-(2,3,4,5,6-Pentafluorobenzyl) hydroxylamine hydrochloride (PFBHA), 1,1,3,3- tetraethoxypropane (TEP), riboflavin, peptone and 2- thiobarbituric acid (TBA) were supplied by Sigma-Aldrich (Bornem, Belgium). N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA), trimethylchlorosilane (TMCS) and trichloroacetic acid (TCA) were obtained by

Acros Organics (Geel, Belgium). Methanol HPLC grade was obtained from Fisher Scientific (Leicestershire, UK). Deionized water (Milli-Q, Millipore Corp.) of 18.0 MΩ cm^{-1} resistivity was used throughout the experiments. K_2HPO_4, KH_2PO_4, petroleum ether and hexane of analytical grade and were purchased from Chem-Lab (Zedelgem, Belgium). Olive oil was purchased from the local market, linseed and fish oil was kindly donated by Cargill Refined Oils Europe (Izegem, Belgium).

5.2.2 Oil and emulsion preparation

Triacylglycerols (TAGs) were isolated from olive, linseed and fish oil with a modified method described by Mariod et al (Mariod, Matthaeus, & Hussein, 2011). Initially a total of 25 g of oil were dissolved in 30 mL of petroleum ether and loaded in a glass chromatographic column (diameter 2.5 cm) containing 25 g of silica gel. The oil was eluted with 150 mL of petroleum ether, the solvent was evaporated in a rotating evaporator (Heidolph Instruments GmbH & Co, Schwabach, Germany) operating at 30 ^oC and the oil was subjected to the next purification step as described further. A total of 100 g of activated aluminum oxide was packed in a glass chromatographic column, the oil obtained from the previous step was dissolved in 60 mL of petroleum ether, loaded into the column and eluted with a total of 100 mL of hexane. The solvent was evaporated in a rotating evaporator operating at 30 ^oC and the oils were further used for the preparation of the emulsions. Throughout the oil purification procedure the samples were prevented from direct exposure to light in order to avoid oxidation. Tocopherol content of the oils and TAGs and the peroxide values were determined as described by Shrestha et al (Shrestha, Stevens, & De Meulenaer, 2012).

The emulsions were made with 10 mM phosphate buffer (K_2HPO_4, $KH_2PO_{4)}$ pH 7.2, using Tween 20® as emulsifier at a ratio 1:10 emulsifier : oil as described by Waraho et al (Waraho, Cardenia, Rodriguez-Estrada, McClements, & Decker, 2009). A total of 200 mL of emulsion were prepared by adding 3% striped oil, emulsifier and 1mg riboflavin. A coarse emulsion was prepared by mixing with an Ultraturax (Janke & Kunkel, IKA-Werk, Staufeb, Germany) for 1min at 18000 rpm and the emulsion was further homogenised with a Microfluidiser 110S (Microfluidics Corporation, Newton, Massachusetts, USA), at 560 bar for three times. A total of 5 g of emulsion was placed in transparent SPME glass vials of 20 mL volume, closed with a PTFE septum cap and heated at 70 ^oC for 7 min to ensure microbiological stability throughout storage. The particle size distribution of the emulsions was determined before and

after pasteurization with a Mastersizer S long bed (version 2.15, Malvern Instruments Ltd) equipped with MSX-17 wet sample dispersion unit. Sample was added drop-wise until an obscuration between 10 and 15 % was obtained. For the results calculation, the presentation code incorporated a continuous phase refractive index of 1.33 and a real and imaginary dispersed phase refractive index of 1.4600 and 0.0100, respectively. The 300RF lens was used in the wet laser diffraction analysis and for the particle size distribution determination, the polydisperse model was chosen in the Malvern Mastersizer software.

For the microbial enumeration of the emulsions a representative sample of 1 mL was aseptically transferred in a tube containing 9 mL of peptone physiological solution (PPS) 0,85 % w/v NaCl and 0,1 % w/v peptone in distilled water in order to prepare the primary decimal dilution. Subsequently, decimal dilution series were prepared from the primary using PPS and aliquots were pour plated in Plate Count Agar (PCA, Biorad) aiming at the determination of the total aerobic count (International Standard ISO: 4833:2003, 2003). All plating was performed in triplicate and incubation was carried out at 30^{o}C for 3 days and analysis was performed day 15 and 30 of storage. During the storage experiment the samples were kept in a cold room at 4 ^{o}C in the dark or under illumination at an average of 4600 lux throughout the storage period. Each emulsion preparation was prepared in duplicate.

5.2.3 Aldehyde determination

For determination of HNE and HHE, a total of 500 mg of emulsion was spiked with a known amount of internal standard and quantification was performed as described by Papastergiadis et al. for dairy products (Papastergiadis et al., 2013). A total amount of 500 mg sample were weighed in a 15 mL polypropylene tube, spiked with the analytes and the IS, 2 mL of water was added and the mixture was vortexed for 1 min. In brief a total of 3 mL of acetonitrile was added followed by vortexing for 1 min. A total of 0.3 g of NaCl and 1 g of $MgSO_4$ were subsequently added and the tubes were mixed manually for one more minute. The tubes were centrifuged at 3600 g for 5 min and 2 mL of the supernatant was collected and evaporated under a gentle flow of nitrogen prior to derivatization. Chromatographic analysis was performed using an Agilent 7890A GC equipped with a 5975C Mass Spectrometer (Agilent Technologies, Palo Alto, CA). The derivatised sample (1 μL) was introduced into the injector operating in the splitless mode at 200 ^{o}C and the separation was carried out using an Agilent HP-5 MS 30 m, 0.25 mm, 0.1 μm capillary column. The carrier gas was helium at a constant

flow of 0.8 mL min^{-1} and the oven temperature was programmed from 50 (held for 1 min) to 150 ^{o}C at a rate of 10 ^{o}C min^{-1}, from 150 to 200 ^{o}C at a rate of 3 ^{o}C $min^{-1}$and finally up to 250 ^{o}C at a rate of 40 ^{o}C min^{-1}. The MSD conditions were the following: capillary direct interface temperature, 250 ^{o}C; ionization energy, 70 eV; operating in selective ion mode (SIM); selected ions monitored, *m/z* 200, *m/z* 205, *m/z* 242, *m/z* 253 and *m/z* 352; scan rate 3.64 cycles per second. Concerning HNE the limit of detection (LOD) and the limit of quantification (LOQ) were 0.20 and 0.61 µmol $kg^{-1}$of sample respectively. In regards to HHE LOD and LOQ were 0.08 and 0.24 µmol $kg^{-1}$of sample respectively.

Concerning quantification of MDA, a modified method involving HPLC determination of MDA-TBA adduct (Papastergiadis, Mubiru, Van Langenhove, & De Meulenaer, 2012) was applied. Briefly, two grams of emulsion were subjected to centrifugation at 20000 g for 10 min (Sigma 4K15, Sartorius, Goettingen, Germany) and 0.5 mL of the aqueous portion (lower layer) was collected and subjected to derivatization with TBA. A total of 20 µL of the solution containing the MDA-TBA derivatives were injected into a Varian C18 HPLC column (5 µm, 150 x 4, 6 mm) hold at 30 °C. The mobile phase, consisting of 50 mM KH2PO4 pH 5.3 buffer solution, methanol and acetonitrile (72:17:11 v/v) was pumped isocratically at 1 mL min-1. Fluorometric detector excitation and emission wavelengths were set at 525 and 560 nm respectively. The LOQ and LOD of the method were 0.05 and 0.10 µmol kg-1of sample respectively.

Quantification of 2-butenal was performed with HS-GC-MS as described in chapter 4 after derivatization of the above mentioned aqueous phase with PFBHA. Briefly, 2-butenal *d-6* as internal standard was added in the emulsion and centrifugation at 20000 g for 10 min was applied. A total of 0.5 mL of the aqueous phase was mixed with 1 mL of aqueous solution of 2 mg mL^{-1} PFBHA in a 20 mL headspace vial. The vial was sealed with a PTFE septum cup and the samples were incubated for 30 minutes at 40 ^{o}C in order to form the PFBHA-2-butenal oximes. The vials containing the oximes were kept at 70 ^{o}C for 10 minutes and 0.5 mL of the headspace was extracted with a gastight syringe. Chromatographic analysis was performed using an Agilent 7890A GC equipped with equipped with a CTC PAL auto sampler and a 5975C Mass Spectrometer. The derivatised sample (1 µL) was introduced into the PTV inlet operating in the splitless mode at 250 ^{o}C and the separation was carried out using an Agilent DB 624 capillary column 60 m x 0.25 mm ID, with 1.4 µm film thickness. The temperature program was the following: initial temperature 50 ^{o}C for 1 min, then 30 ^{o}C

min^{-1} to 200 ^{o}C, followed by a ramp of 5 ^{o}C min^{-1} to 230 ^{o}C held for 8 min. The MSD conditions were the following: capillary direct interface temperature, 250 ^{o}C; ionization energy, 70 eV; operating mode selective ion (SIM); selected ions monitored, *m/z* 181, *m/z* 250, *m/z* 265, *m/z* 253 and *m/z* 271; scan rate 3.64 cycles per second. The LOD and LOQ were 0.20 and 0.40 μmol $kg^{-1}$of sample respectively.

5.2.4 Statistical analyses

Multiple-way ANOVA was performed on the measured variables to find significant differences between treatments. Changes in the formation of the studied aldehydes was studied based on two experimental factors (two-way ANOVA): (a) fatty acid composition (5 levels) and (b) storage time (7 levels). When deviations from normal distribution and homogeneity of variation were observed suitable transformations were applied in the data. Whereas significant effects were detected ($p < 0.05$), Student-Newman-Keuls test of mean comparisons was applied.

5.3 RESULTS

The striped oils were tested for any contamination with the aldehydes of interest prior to preparation of the emulsions and all the samples were found aldehyde free. Furthermore, the PV of the fish, linseed and olive oil were found to be 6.2, 5.8, and 4.5 respectively prior to preparation of the emulsions. In order to investigate the effect of the fatty acid composition and oxidation conditions on the formation of HNE, HHE, MDA and 2-butenal in oil-in-water emulsions during storage, five different fatty acid profiles were chosen. The emulsions containing 3% of oil were prepared from blends of stripped olive, fish and linseed oil and the concentration of each fatty acid (mmol kg-1 of emulsion) is illustrated in **Table 5.1**. The content of 18:2 and 18:3 of linseed oil was 43.5 and 15.5% respectively.

Table 5.1PUFA composition of the prepared emulsions (mmol fatty acids kg^{-1} emulsion)

	mmol fatty acids kg^{-1} emulsion				
	3%Olive	2%Olive 1%Fish	1%Olive 2%Fish	2%Olive 1%Linseed	1%Olive 2%Linseed
C18:2ω-6	4.63	5.12	5.62	17.68	30.73
C18:3ω-3	0.68	0.90	1.13	5.66	10.64
C20:5ω-3	0.00	6.58	13.16	0.00	0.00
C22:6ω-3	0.00	2.82	5.64	0.00	0.00
Total ω-3	0.68	10.30	19.93	5.66	10.64

Mixing of olive oil with fish oil aimed in preparation of emulsions with high levels of ω-3 fatty acids, mainly due to eicosapentaenoic (EPA) and docosahexaenoic (DHA) fatty acids while the levels of ω-6 (mainly linoleic) remained low. On the other hand, mixing of olive oil with linseed oil resulted in an increase of both ω-3 and ω-6 due to α-linolenic and linoleic acid respectively. HPLC analysis of the tocopherol content of the oils indicated that no tocopherols remained in the striped oils. No difference was observed in the droplet size distribution of the emulsions prepared with different oils, before and after pasteurization. An average droplet diameter of 0.62 μm was determined. During storage of the emulsions no creaming was observed in all experiments. HNE, HHE, MDA and 2-butenal were detected bellow the LOD of the analytical methods in any of the samples after heating at 70 ^{o}C for 7 min, thus it can be concluded that the initial pasteurization procedure did not have any impact on the formation of the studied aldehydes. Right after pasteurization, the samples were kept at 4 °C illuminated or under dark for a total of thirty days and every five days HNE, HHE, MDA and 2-butenal content was determined.

Concerning HNE in illuminated samples stored at 4 ^{o}C **(*Figure 5.1 A*)** statistically significant differences ($p<0.05$) were detected between all the treatments. The highest amount of the aldehyde produced after five days of storage was 40 μmol kg^{-1} of emulsion in the samples containing 1% linseed oil. The levels of HNE increased throughout storage in all the samples reaching 180 μmol kg^{-1} of emulsion in those containing 2% linseed oil. Both samples prepared with olive and fish oil in different portions contained 10 μmol kg^{-1} of emulsion of HNE at the first day of measurement and HNE increased up to 60 μmol kg^{-1} of emulsion in the end of storage for the samples containing one and two percent of fish oil.

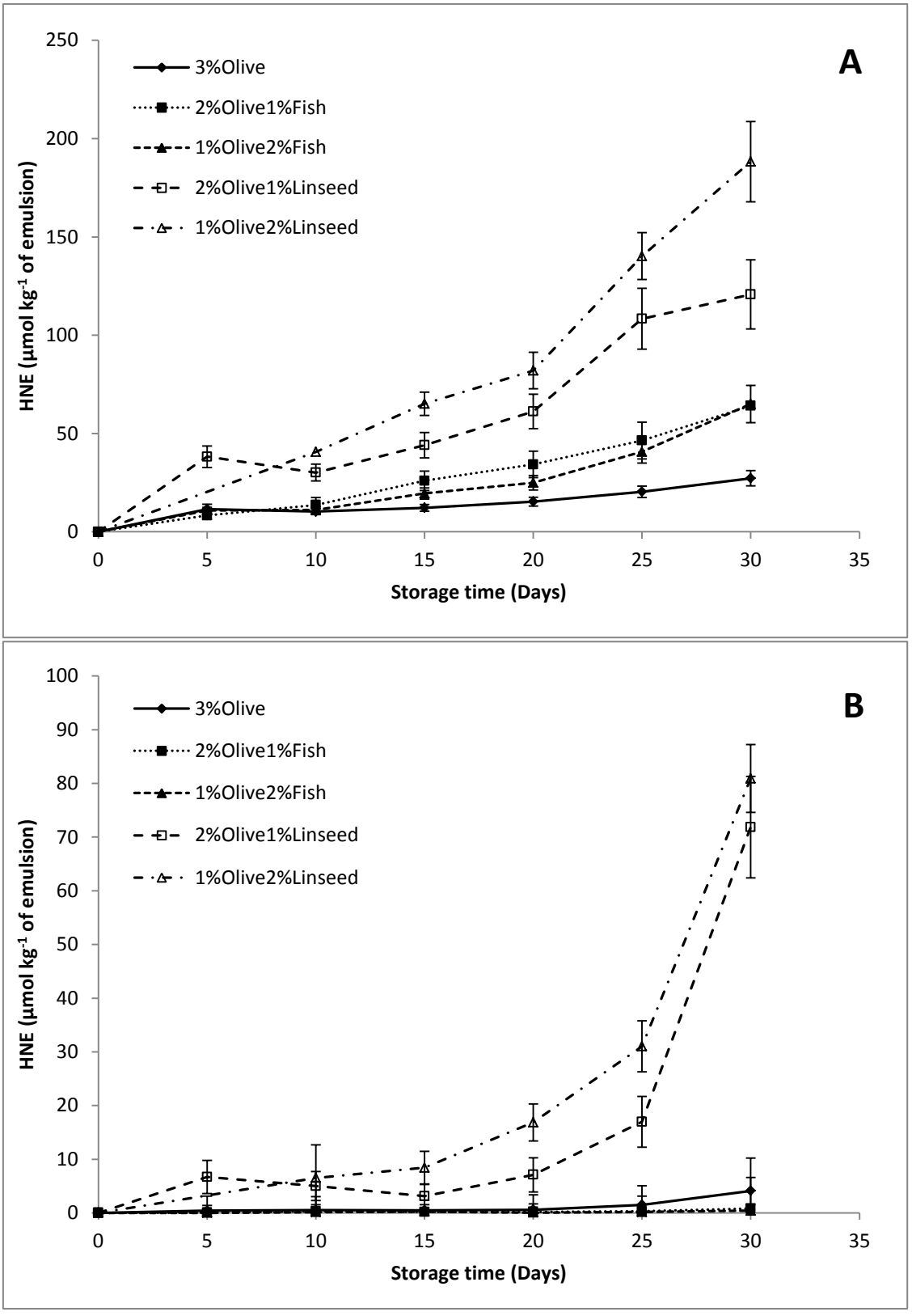

Figure 5.1 Formation of HNE (µmol kg^{-1} of emulsion) during storage at 4 ^{o}C A) Under illumination, B) Under dark. Values are the mean of two replications ± 95% CIs

The highest HNE content in the emulsions prepared from 3% olive oil was 22 μmol kg^{-1} of emulsion in the last day of storage.. In the case of samples kept in the dark (***Figure 5.1* B)**, statistical analysis did not indicate any significant difference ($p>0.05$) in HNE between the samples prepared with fish oil. HNE in both fish oil containing emulsions was detected below 0.8 μmol kg^{-1} of emulsion throughout storage. The level of HNE during the first five days of storage in both samples prepared with linseed oil was below 10 μmol kg^{-1} of emulsion and reached 72 μmol kg^{-1} of emulsion (2%Olive 1%Linseed) and 80 μmol kg^{-1} of emulsion (1%Olive 2%Linseed) in the end of the experiment Statistical analysis indicted the levels of HNE in the samples prepared with 2% linseed oil higher ($p<0.05$).

The levels of HHE detected in the emulsions under illumination and in the dark are illustrated in ***Figure 5.2***. Among illuminated samples, statistically significant differences ($p<0.05$) were detected between all treatments (as indicated by the main effects of two-way ANOVA, results not shown). Emulsions prepared with 3% olive oil developed the lowest amount of HHE reaching 5 μmol kg^{-1} of emulsion on the thirtieth day of storage. Emulsions containing 2% fish oil demonstrated the highest values of HHE, determined as 123 and 254 μmol kg^{-1} of emulsion after five and thirty days of storage respectively. The corresponding HHE levels of emulsions prepared with 1% fish oil were 49 and 143 μmol kg^{-1} of emulsion on the first and last day of measurement respectively. Emulsions prepared with 1 and 2% of linseed oil reached 64 and 113 μmol kg^{-1} of emulsion of HHE after thirty days of storage. In regards to samples kept in the dark, emulsions prepared with fish oil did not show any significant formation of HHE throughout storage. Indeed, HHE levels were not found statistically different ($p>0.05$) from those determined in emulsions made only with olive oil. Samples prepared with linseed oil contained less than 10 μmol kg^{-1} of emulsion of HHE after five days of storage and no further increase was observed up to the fifteenth day. However, for both samples, a gradual increase was observed from twenty days on and HHE was found in both slightly above 100 μmol kg^{-1} of emulsion the last day of storage. Statistical analysis indicated a difference ($p<0.05$) in HHE levels between these two treatments.

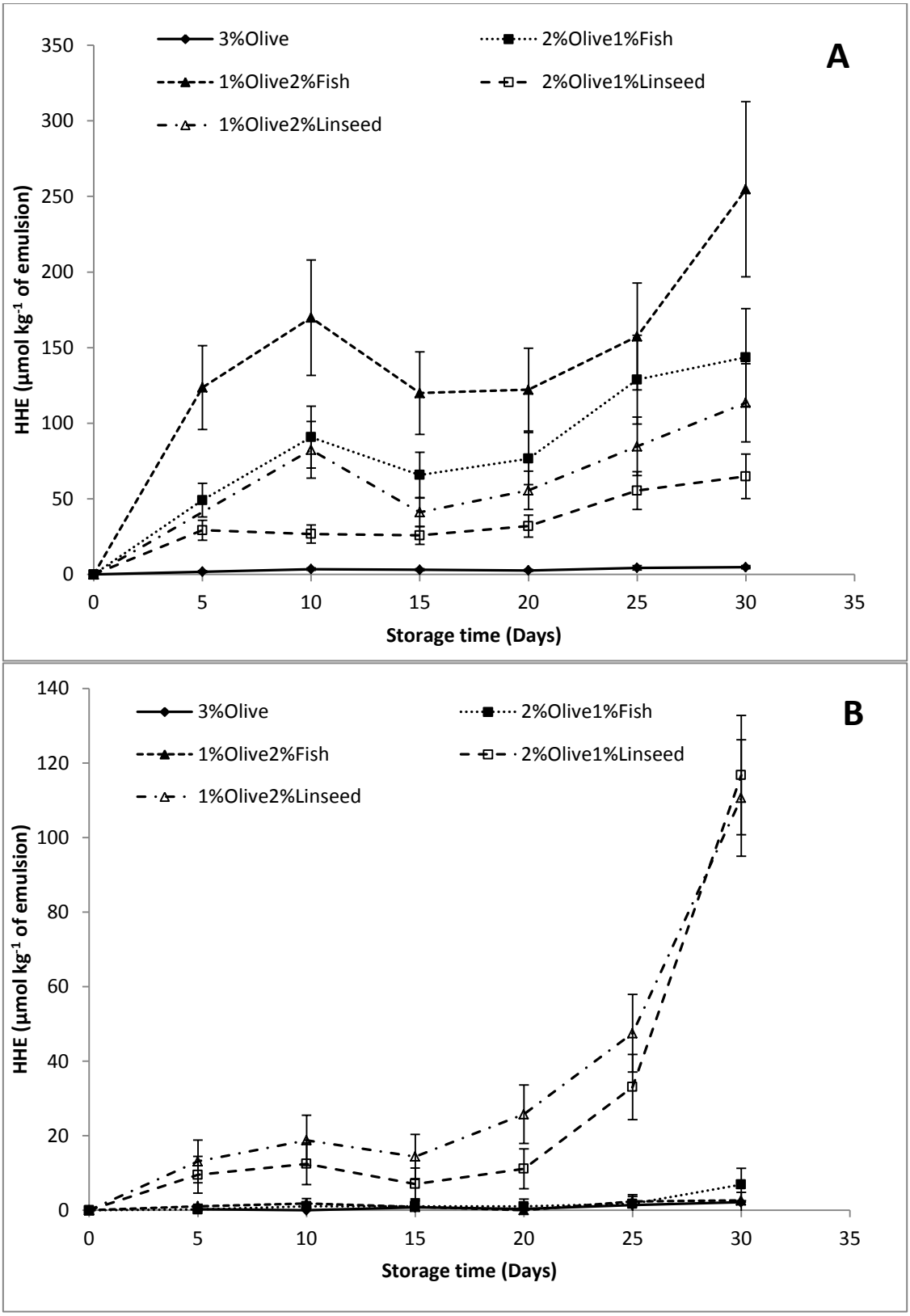

Figure 5.2 Formation of HHE (µmol kg^{-1} of emulsion) during storage at 4 ^{o}C A) Under illumination, B) Under dark. Values are the mean of two replications ± 95% CIs

Regarding MDA formation in illuminated samples (***Figure 5.3 A***), statistical analysis indicated no changes in the concentrations of the aldehyde between five and thirty days of storage ($p>0.05$) for all the different emulsion preparations. Furthermore, differences were detected between all the treatments except for emulsions prepared with 2%linseed oil and those prepared with 1% fish oil ($p>0.05$), Samples prepared with 2% fish oil demonstrated the highest MDA levels (200 μmol kg^{-1} of emulsion), while no difference was indicated between emulsions prepared with 1% fish and 2% linseed oil ($p>0.05$). In emulsions prepared with 1% linseed oil up to 90 μmol kg^{-1} of emulsion of MDA was detected. In contrast, when samples were stored in the dark (***Figure 5.3 B***), an increase in MDA content was observed up to ten days of storage. Samples prepared with 1 and 2% of linseed oil demonstrated the highest levels of MDA reaching up to 47 and 67 μmol kg^{-1} of emulsion respectively in the end of storage. The lowest levels of MDA were detected in the samples containing 3% olive oil.

The concentrations of 2-butenal detected in the emulsions are presented in ***Figures 5.4 A*** and ***B***. Regarding illuminated samples, statistical analysis indicated an increase in 2-butenal content up to the fifteenth day of storage. During the last fifteen days of the experiment 2-butenal levels remained stable for all emulsions studied. The highest levels of 2-butenal were detected for the illuminated samples prepared with 2% fish and linseed oil. Similar was the behavior of the emulsions prepared with 1% fish and linseed oil, however the 2-butenal was detected in lower amounts and no difference between these two treatment was indicated ($p>0.05$). Emulsions prepared with 3% olive oil did not demonstrate any statistically significant ($p>0.05$) increase in 2-butenal content throughout the storage period. Concerning samples stored in dark, only emulsions prepared with 1 and 2% linseed oil demonstrated formation of 2-butenal during storage. Statistical analysis indicated that the highest levels were detected in the last day and were 4 and 9 μmol kg^{-1} of emulsion for emulsions containing 1 and 2% of linseed oil respectively. Samples prepared with 3% olive oil and those prepared with 1 and 2% of fish oil did not demonstrate any increase in 2-butenal content and no difference was observed between them ($p>0.05$).

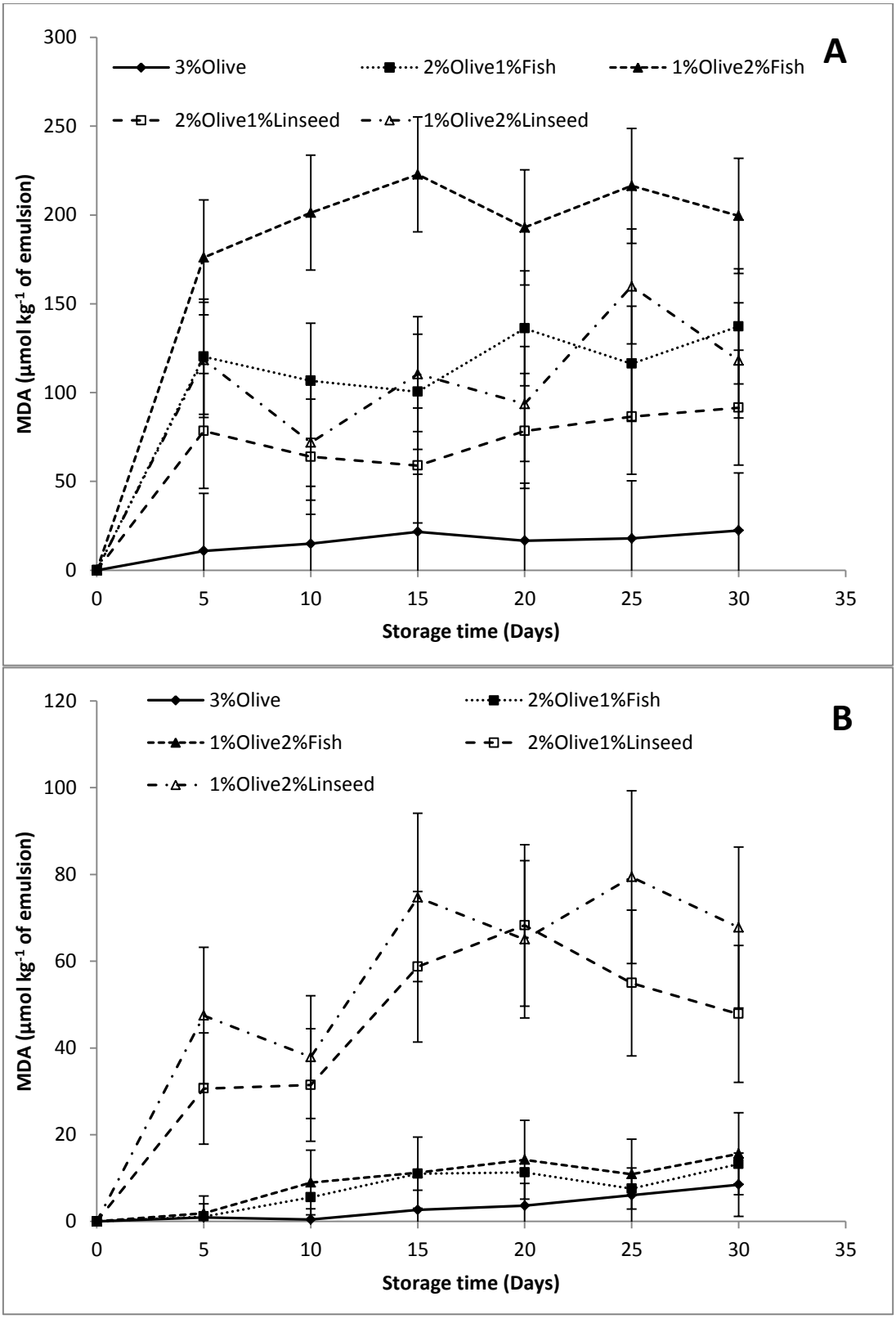

Figure 5.3 Formation of MDA (µmol kg^{-1} of emulsion) during storage at 4 ^{o}C A) Under illumination, B) Under dark. Values are the mean of two replications ± 95% CIs

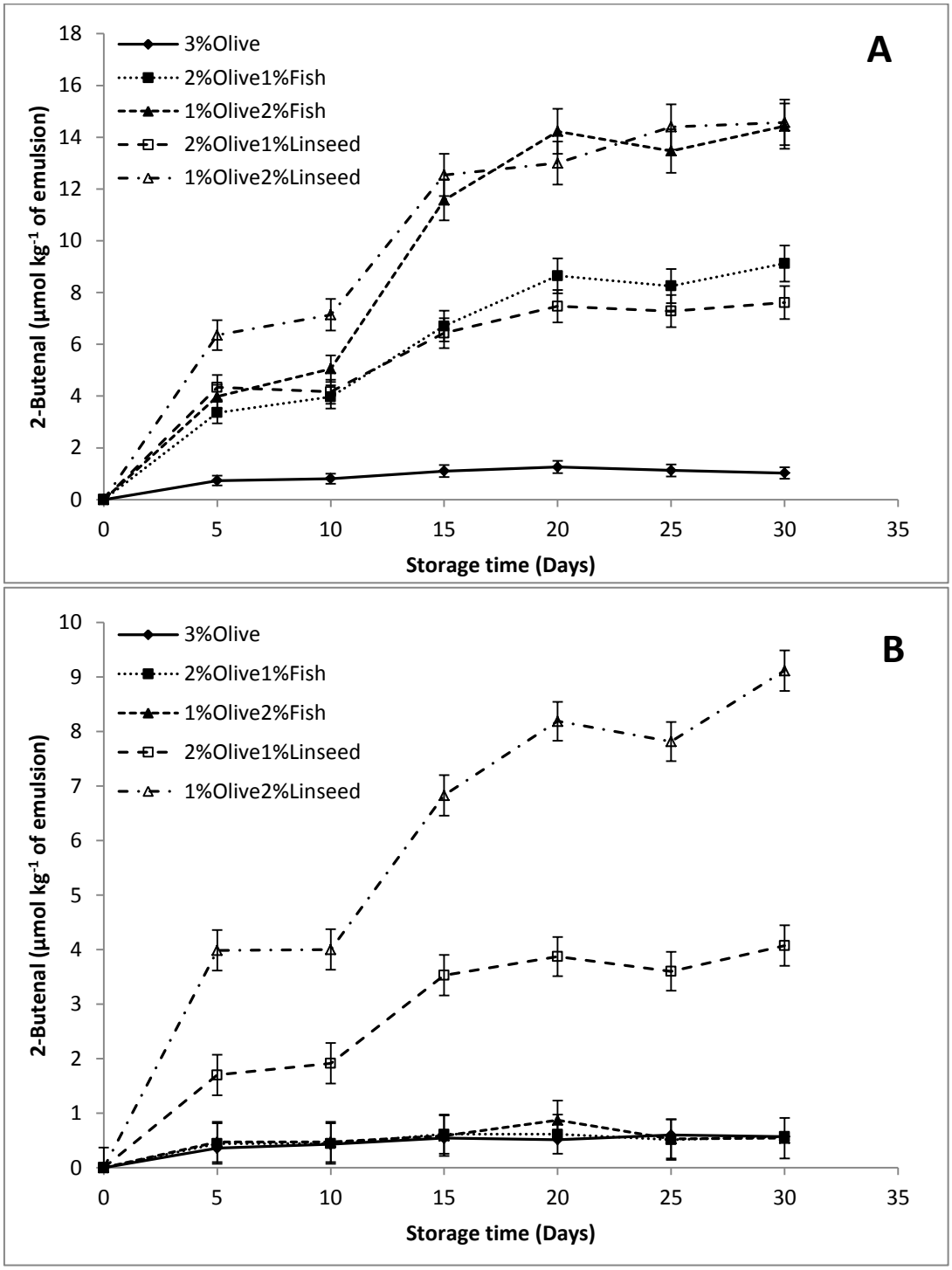

Figure 5.4 Formation of 2-butenal (µmol kg^{-1} of emulsion) during storage at 4 ^{o}C A) Under illumination, B) Under dark. Values are the mean of two replications ± 95% CIs

5.4 DISCUSSION

Riboflavin, which has been added in all the emulsions, is classified as Type I and II sensitizer and can act as a photochemically activated free radical initiator (Type I) or it can react with oxygen by energy transfer to produce non-radical singlet oxygen (1O2) (Type II) (Huang, Choe, & Min, 2004; Min & Boff, 2002). Recent studies on the photooxidation occurring in oil-in-water emulsions in the presence of riboflavin, indicated that riboflavin is capable of participating in both photooxidation mechanisms in such model systems. However, type I mechanism was found to be more important compared to type II and this was attributed to the short shelf life of singlet oxygen and superoxide anion in the aqueous phase of the emulsion (Lee & Decker, 2011). Interestingly in this work it was also demonstrated that photooxidation involving singlet oxygen was responsible for the formation of specific secondary lipid oxidation products such as 2-heptenal. This supports the opinion that the formation of secondary lipid oxidation products is strongly dependent upon the lipid hydroperoxide isomers formed. In the present work, the higher amounts of the studied aldehydes in the illuminated samples can be attributed to the acceleration of lipid hydroperoxide formation due to photooxidation.

Regarding the samples containing portions of linseed oil, HNE was detected in higher levels compared to MDA after the twentieth day of storage under light. HNE is known to be formed during the oxidation of ω-6 PUFA (Surh et al., 2010; Han & Csallany, 2009) and in the present study the main ω-6 PUFA in the emulsions is linoleic acid at concentrations varying between 4.6 and 30.7 mmol kg-1 of emulsion. Autoxidation of linoleic acid is resulting in the formation of hydroperoxides on the 9 and 13 (9-OOH and 13-OOH) carbon at almost equal ratios at different oxidation temperatures (Frankel, Neff, & Weisleder, 1990). Two different mechanisms have been proposed for the formation of HNE from linoleic acid, one involving the 13-OOH and the other the 9-OOH (Schneider et al., 2001). In both cases 4-hydroperoxy-2-nonenal is formed which is further reduced to 4-hydroxy-nonenal. Thus, every isomer of linoleic acid hydroperoxides can be reasonably considered as a potential precursor of HNE. In both samples containing fish oil stored in the dark, HNE never reached levels above 4 μmol kg-1 of emulsion. This could be attributed to a delay in the formation of linoleic acid hydroperoxides in combination with the relatively low levels of this fatty acid in these particular emulsions. On the other hand, both emulsions prepared with linseed oil

demonstrated a rapid increase in HNE, most likely related to the high levels of linoleic acid present.

HHE is known to be produced from the hydroperoxides of ω-3 PUFAs. Even though the mechanism of HHE is not studied extensively it has been proposed that it is formed in a similar manner as HNE from ω-6 PUFAs (Long & Picklo, 2010). From that perspective, formation of HHE from linolenic acid requires the formation of hydroperoxides on the 12 and 16 carbon atoms, from EPA formation of hydroperoxides on the 14 and 18 carbon atoms and from DHA formation of hydroperoxides on the 16 and 20 carbon atoms. Concerning linolenic acid, it has been found that the proportions of the formed 9, 12,13 and 16 position hydroperoxides are unevenly distributed (Frankel et al., 1977). This was attributed to the tendency of the 12 and 13 peroxyl radicals to form five membered hydroperoxy epidioxides. As already mentioned these are precursors of MDA and this can explain the reason HHE was lower than MDA up to the twentieth day of storage in the emulsions prepared with linseed oil and stored in the dark. The importance of DHA as a precursor of HHE remains controversial. HHE has been reported as a minor secondary oxidation product of DHA by some authors (Beckman, Howard, & Greene, 1990) while others have reported HHE as the major secondary oxidation product of DHA (Long, Smoliakova, Honzatko, & Picklo, 2008). In the case of EPA, autoxidation is leading to the formation of eight regioisomeric hydroperoxides. As already mentioned, the 14 and 18- OOH can be considered as precursors of HHE. However, it has been demonstrated that 14 and 18-OOH can be further oxidized to five membered mono-, bi-, tricyclic peroxides and bicyclic endoperoxides (Yin, Brooks, Gao, Porter, & Morrow, 2007). These cyclic peroxides, as already mentioned, are important precursors of MDA.

MDA was found to be the most abundant aldehyde detected in the emulsions prepared with 1 and 2% fish oil up to the twenty fifth day of storage under light. The same observation is valid also for both samples prepared with linseed oil, where MDA was the major aldehyde detected up to twenty days of storage. MDA is known to be produced from both ω-3 and ω-6 PUFAs. The most important precursors of MDA have been found to be the hydroperoxy epidioxides of linoleic acid followed by the hydroperoxy bis-epidioxides and bicycloendoperoxides of linolenic acid (Frankel, 2005). Interestingly, the reported high rate of hydroperoxy epidioxides formation of linoleic acid requires participation of singlet oxygen, which is produced during type II photooxidation promoted by riboflavin. MDA can

also be produced, but in lower amounts, from the monohydroperoxides produced due to autoxidation of linoleic and linolenic acid. The high levels of MDA generated can possibly be linked to the high rate of formation of the aforementioned hydroperoxides during the first twenty days of storage. Concerning samples stored in the dark, the levels of MDA in emulsions prepared with fish oil remained lower than 10 μM throughout storage, more than five times lower than the samples prepared with linseed oil. This could be an indication that singlet oxygen plays an important role in the formation of MDA during oxidation of EPA and DHA.

Formation of 2-butenal occurs due to decomposition of hydroperoxides of ω-3 PUFAs. A proposed mechanism suggests the formation of 2-butenal after homolytic β-scission of the 15-OOH of linolenic acid (Frankel, Neff, & Selke, 1981). However, these hydroperoxides are formed during photooxidation where singlet oxygen is involved, thus this mechanism cannot support the observation of 2-butenal formation even in the dark. The results indicate that 2-butenal is a minor unsaturated aldehyde formed during autoxidation of ω-3 PUFAs and also suggest that 2-butenal is not formed in the early stages of EPA and DHA oxidation.

The results indicated low levels of formation for all the studied aldehydes in emulsions prepared with portions of fish oil and stored under dark. An explanation for this observation could be that in initial stages of lipid oxidation when photooxidation is not involved, mainly other than the studied aldehydes are formed from the oxidation of EPA and DHA. Alkenals, alkadienals and alkatrienals have been reported within the volatile compounds present in fish enriched milk (Venkateshwarlu, Let, Meyer, & Jacobsen, 2004) and more specifically hexanal, heptenal, heptadienal, nonanal and nonadienal have been reported as main aldehydes associated with lipid oxidation of fish oils (Aidos et al., 2002) It has been also demonstrated that the major aldehydes formed during oxidation of mayonnaise samples enriched with fish oil were hexanal, trans-2-hexenal, trans, cis-2,4-heptadienal and trans, trans-2,4-heptadienal (Hartvigsen, Lund, Hansen, & Holmer, 2000). MDA and HHE was detected in higher levels in the illuminated samples prepared with fish oil compared to samples prepared with linseed oil. However, the opposite effect was observed in samples stored in the dark which could be an indication that different mechanisms of lipid oxidation are involved. These results are a first insight on the formation of the studied aldehydes in a system where light and a photosensitizer are present and the two mechanisms of photooxidation are not studied separately. In order to have a more detailed view on the effect of singlet oxygen in the

formation of the studied aldehydes, experiments including a physical singlet quencher such as sodium azide should be performed.

5.5 CONCLUSIONS

It can be concluded that during photooxidation in the presence of riboflavin, where EPA and DHA were the major ω-3 PUFAs, MDA was the most abundant aldehyde formed of those studied. HHE was found to be the second most important, while 2-butenal represented only a small portion. HNE was found to be the major aldehyde formed when linoleic acid was the dominant PUFA. When lipid oxidation proceeded in a slower rate in the dark, where only autoxidation is expected to occur, EPA and DHA did not produce any significant amount of MDA, HHE and 2-butenal. This implies that there are other secondary oxidation products formed in more significant amounts than the studied aldehydes. However, samples containing linoleic and linolenic acid (prepared with linseed oil) produced considerable amounts of MDA, HNE, HHE and 2-butenal when stored under dark.

In the upcoming chapter, results on the exposure and risk assessment related to the dietary intake of MDA, HNE and HHE from the Belgian population are introduced and discussed.

CHAPTER 6

RISK ASSESSMENT OF MALONDIALDEHYDE, 4-HYDROXY-2(E)-NONENAL AND 4-HYDROXY-2(E)-HEXENAL THROUGH CONSUMPTION OF SPECIFIC FOODS AVAILABLE IN BELGIUM

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CHAPTER 6. RISK ASSESSMENT OF MALONDIALDEHYDE, 4-HYDROXY-2-NONENAL AND 4-HYDROXY-2-HEXENAL THROUGH CONSUMPTION OF SPECIFIC FOODS AVAILABLE IN BELGIUM

SUMARY

Malondialdehyde (MDA), 4-Hydroxy-2-(E)-Nonenal (HNE) and 4-Hydroxy-2-(E)-Hexenal (HHE) are reactive aldehydes found in foods and are formed due to decomposition of polyunsaturated fatty acid hydroperoxides. In the present study, a total of sixteen food categories were analyzed for the aforementioned aldehydes and in combination with consumption data obtained from a national representative sample of the Belgian population, a quantitative exposure assessment was performed. MDA was detected above the detection limit in 84% of the analyzed samples while HNE and HHE in 63% and 16% of the samples respectively. Consumption of dry nuts, fried snacks, French fries and cured minced meat products were found to contribute the most to the intake of MDA and HNE. Intake of HHE from the foods analyzed was found not to be significant. An evaluation of any potential risk related to the intake of the studied aldehydes for the consumers of the specific food categories analyzed was made by applying the Threshold of Toxicological Concern concept. No risk to human health could be identified related to the consumption of these foods for the vast majority of the consumers, with the only exception of a small proportion (1.4 %) of those who consume cured and minced raw meat, that could be at risk.

Keywords: Lipid oxidation, Malondialdehyde, 4-Hydroxy-2-(E)-Nonenal, Hydroxy-2-(E)-Hexenal, Exposure assessment, Probabilistic analysis, Threshold of Toxicological Concern

6.1 INTRODUCTION

MDA, HNE and HHE have attracted the attention in biological systems due to their potential toxicity to humans which is attributed to their high reactivity with proteins and DNA, consequently leading to structural damage and alteration of their functionality (Esterbauer, 1982; Esterbauer & Cheeseman, 1990; Esterbauer et al., 1991; StAngelo, 1996; Uchida, 2003; Guillen & Goicoechea, 2008b; Voulgaridou et al., 2011). Even though phenomena related to the endogenously formed MDA and α,β-unsaturated aldehydes due to lipid peroxidation are well studied, the role and importance of these aldehydes when absorbed through the diet is not extensively investigated to date apart of a few studies (Goicoechea et al., 2008; Grootveld et al., 1998; Kanazawa & Ashida, 1998). Furthermore, there is a limited knowledge concerning the dietary exposure of humans to these aldehydes (Surh & Kwon, 2005) and to the best of our knowledge there are no available toxicological data of these compounds. Due to lack of specific toxicological data, the concept of Threshold of Toxicological Concern (TTC) can be applied. TTC is a useful tool for preliminary risk characterization of chemicals of known structure to which humans are exposed at low levels (Rennen, Koster, Krul, & Houben, 2011).

The objective of the study presented in this chapter was the determination of free MDA, HNE and HHE contents in specific food categories available in the Belgian market, followed by a quantitative exposure assessment of dietary intake of the studied aldehydes for the Belgian population. Evaluation of the potential risk was performed comparing the estimated intakes with the corresponding TTC values of each aldehyde.

6.2 MATERIALS AND METHODS

6.2.1 Supplies and reagents

2- thiobarbituric acid (TBA) and O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA) were purchased from Sigma Aldrich (St. Louis, MO, USA). KH2PO4, glacial acetic acid of analytical grade was purchased from Chem-Lab (Zedelgem, Belgium), trichloroacetic acid (TCA), N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) were obtained by Acros Organics (Geel, Belgium). Methanol HPLC grade was provided by Fisher Scientific (Leicestershire, UK). HNE and HHE standards were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). HNE

d11 and HHE d5 dimethyl acetals were purchased from C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada). Deionized water (Milli-Q, Millipore Corp.) of 18.0 MΩ cm-1 resistivity was used throughout the experiments and all the solvents used were of analytical grade.

6.2.2 Sampling plan for MDA and hydroxylated alkenals determination in foods

Specific foods were included in the sampling plan according to their potential to be contaminated with the secondary oxidation products. Fat content as well as the degree of unsaturation of the fatty acids and consequently their sensitivity to oxidation were taken into account. Foods were divided into three groups including foods of plant origin, foods of animal origin and chilled cooked meals (containing multiple ingredients) respectively. Each group consisted of several food categories as shown in ***Table 6.1.*** A total of 390 samples were purchased from five supermarket chains located in Ghent, Belgium, except of French fries and fried snacks that were purchased from local fast food shops. The samples included brand named and private label products. Foods were purchased between September 2012 and May 2013 and were analyzed immediately upon delivery in the lab. Analysis near the end of the shelf life was performed for samples stored under the recommended conditions from the producer. In the case of vegetable oils, the bottles were opened and stored at room temperature, avoiding direct exposure to light and analyzed after two months. Milk samples were analyzed soon after purchasing, the opened bottles were kept at 4 oC for three days and analysis was carried out again. French fries and fried snacks were analyzed upon arrival in the lab. In the case of cooked chilled foods all the components of the meals were homogenized prior to the analysis.

Table 6.1 Description of the food groups and categories analyzed.

Food group	Food category	Food description
Foods of plant origin	plant oils	refined corn, sunflower, colza, arachid, soya oils and commercial mixtures of those, extra virgin olive oils
	dry nuts	roasted and peeled salted and not salted peanuts, peeled walnuts, almonds and also mixtures of the aforementioned
	potato crisps	salted and not salted
	French fries	with mayonnaise, tartar sauce, andaluse sauce, purchased from fast food shops
	cookies	speculoos, petit beurre
	soya based products	Quorn based products
Foods of animal origin	fried snacks	chicken, fish, beef, pork meat, purchased from fast food shops
	frozen and fresh salmon	filleted and stored at -20 ^{o}C
	smoked salmon	filleted and stored at 4 ^{o}C
	full fat milk	pasteurized and sterilized
	cheese	Gouda, Parmesan, Brugge, brie
	cured and cooked meat products	Knacki, Paris, frankfurter
	bacon	smoked and non-smoked
	cured raw ham	prosciutto, Ganda ham
	cured minced raw meat products	dry sausage, salami, chorizo
Cooked chilled foods	ready to eat meals	stewed beef, lasagna, pork meat balls, burgers and chicken accompanied with potatoes, rice, pasta including the sauces accompanying the meals

6.2.3 Determination of MDA and hydroxylated alkenals in foods

The method described by (Papastergiadis et al., 2012) was adopted for determination of MDA. Briefly, MDA was extracted from the samples after homogenization with 7.5% trichloroacetic acid (TCA) and a specific volume of the extract reacted with 2-thiobarbituric acid (TBA). The formed chromogen was injected in a C18 reversed phase HPLC column and was detected fluorometrically. The LOD and LOQ of the method were determined as 4 and 8 μg kg-1of sample respectively.

HNE and HHE content was determined based on a GC-MS method involving deuterated HNE and HHE analogues as internal standards (Papastergiadis et al., 2013). Three different extraction techniques were applied depending on the nature of the sample and therefore three different LODs and LOQs were considered, which can be found in detail in the aforementioned study. Briefly, the analytes along with the internal standard were extracted, derivatised with PFBHA, silylated with TMCS-BSTFA and the derivatives were analyzed with GC-MS. The derivatised sample (1 μL) was introduced into the injector operating in the

splitless mode at 200 ^{o}C and the separation was carried out in an Agilent HP-5 MS 30 m, 0.25 mm, 0.1 µm capillary column. The mass spectrometric analysis was carried out in SIM monitoring the selected ions *m/z* 200, *m/z* 205, *m/z* 242, *m/z* 253 and *m/z* 352 at scan rate 3.64 cycles per second.

6.2.4 Consumption data

Food consumption data were obtained from the Belgian National Food Consumption Survey (BNFCS) conducted in 2004 (De Vriese et al., 2005), where information on dietary intake was collected from 3083 individuals of fifteen years old or older, residing in Belgium. The survey was based on two non-consecutive 24 h recalls combined with a self- administered food frequency questionnaire. Consumption information of the specific foods were extracted from the BNFCS database based on their description. The usual food intake was determined from the total data set including the zero intakes, when a food is not consumed by an individual, with the Multiple Source Method (MSM) program (Harttig, Haubrock, Knueppel, Boeing, & Consortium, 2011; Haubrock et al., 2011; German Institute of Human Nutrition Postdam-Rehbrücke (DLfE), 2013). All the consumers where considered habitual and food intake was expressed in kg of food (kg bw day)-1. BNFCS does not contain information about cooked chilled meals (bought as ready-to-eat products), therefore data related to the consumption of cooked chilled foods were obtained from a consumer survey conducted by Daelman et al. (Daelman et al., 2013) A total of 681 individuals of fifteen years or older were interviewed on their consumption behavior of commercially available and industrial prepared ready-to-eat meals in spring of 2011. This survey was based on frequency of consumption, respondents had to answer the question 'how often do you eat a ready-to-eat meal?'. Seven possible answers were posed: 5-7 times a week, 2-4 times a week, once a week, 3-5 times a month, once a month, once a year and never. These responses were first converted to a daily consumption by a conversion factor (i.e. 5-7 times a week corresponded to 1/day; once a week corresponded to 1/7 days; 3-5 times a month corresponded to 4/30 days, once a month corresponded to 1/30 and once per year corresponded to 1/365), followed by a multiplication of the average weight of all analyzed products with the average body weight from Belgian consumers. By application of these calculations, the consumption dataset (kg ready-to-eat meals (kg bw day)-1) was obtained.

6.2.5 Exposure assessment

Exposure assessment was performed with the consumption data and the contamination data collected from the analyzed samples. Concerning the non-detects (<LOD), three scenarios were applied. Non-detects were considered as zero, ½ LOD and LOD for lower, medium and upper bound scenarios respectively. For the exposure assessment, foods were divided into sixteen categories including plant oils, dry nuts, potato crisps, French fries, cookies, soya based products, fried snacks, frozen and fresh salmon, smoked salmon, full fat milk, cheese, cured and cooked meat products, bacon, cured raw ham, cured minced raw meat products and ready to eat meals.

Deterministic exposure assessment

Dietary exposure of the consumers to the targeted aldehydes was initially estimated on basis of the deterministic approach. Estimated intakes were calculated by multiplication of the mean, maximum or P 99.5 percentile of the contamination levels with the corresponding mean, maximum or P 99.5 percentile of the consumption data of each food category. Deterministic analysis could not be applied for the cooked chilled meals.

Probabilistic exposure assessment

Best fit distributions for the consumption and contamination data of each food category were determined for all three scenarios (lower, medium, upper). The type of distribution best fitted for the upper bound scenario of the contamination data was applied to the lower and medium scenarios. Monte Carlo simulations with 50,000 iterations were performed for each food category and the estimated daily intake was expressed in µg aldehyde (kg bw day)$^{-1}$. Simulations were performed with the software package @Risk for Microsoft Excel (Palisade Corporation, USA) Best fit distributions for the consumption and contamination data of each food category were determined for all three scenarios (lower, medium, upper). The type of distribution best fitted for the upper bound scenario of the contamination data was applied to the lower and medium scenarios. Monte Carlo simulations with 50,000 iterations were performed for each food category and the estimated daily intake was expressed in µg aldehyde (kg bw day)$^{-1}$. Simulations were performed with the software package @Risk for Microsoft Excel (Palisade Corporation, USA).

6.3 RESULTS AND DISCUSSION

6.3.1 Occurrence of MDA, HNE and HHE in foods available in the Belgian market

Concentrations of MDA, HNE and HHE in the samples for the upper bound scenario are illustrated in ***Table 6.2.*** A total of 390 food samples were analyzed and MDA was detected above the LOD in 84% of the samples. HNE and HHE were detected above the LOD in 63% and 16% of the samples, respectively. At this point, it should be mentioned that the analytical methods applied in the present study are targeting towards the aldehydes that can be found in a free form in the foods. Thus these results cannot indicate with confidence which aldehyde is actually produced in higher amounts. MDA, which was found to be the predominant aldehyde, was detected in all the dry nut samples, potato crisps, French fries and fried snacks, cookies, frozen and fresh salmon, cured raw ham, cured minced meat products and cooked chilled meals. More specific, dry nuts and cookies were found to contain more than 1,300 μg kg^{-1} which was statistically significant higher than the other food categories. Statistical analysis further indicated that potato crisps, French fries and fried snacks, soya based products, frozen salmon and chilled cooked meals had similar levels of MDA. MDA levels were relatively low in vegetable oils and milk, cheese and bacon as compared to other samples. The vast majority of vegetable oils used in this study were refined and their initial peroxide values (data not shown) were low, possibly as a result of the refining process. Furthermore, no significant increase in MDA levels during storage could be detected indicating the stability of these oils. In milk samples the low MDA concentrations detected can be attributed to the low level of unsaturated fatty acids typically observed in dairy products. MDA formation in foods is well studied and is associated with the decomposition of both ω-3 and ω-6 fatty acid hydroperoxides that are formed during processing and storage of foods (Frankel, 2005; Belitz et al., 2009). Traditionally, MDA is used as a marker of lipid peroxidation in foods due to the diversity of the fatty acid of origin (Shahidi & Hong, 1991).

Table 6.2 Concentrations of MDA, HNE and HHE (µg kg^{-1}) in the different food categories analyzed (Upper bound scenario).

	MDA µg kg^{-1}					HNE µg kg^{-1}				HHE µg g^{-1}			
Food categories	**N**	**N<LOD**	**LOD<N<LOQ**	**Mean**	**Max**	**N<LOD**	**LOD<N<LOQ**	**Mean**	**Max**	**N<LOD**	**LOD<N<LOQ**	**Mean**	**Max**
plant oils	76	27	9	85^{a}	1662	19	39	$42^{c,d,e}$	185	59	2	20	158
dry nuts	13	0	0	1581^{d}	8090	0	0	308^{f}	1264	11	0	8	30
potato crisps	14	0	0	$312^{b,c,d}$	760	0	0	$115^{e,f}$	184	4	6	8	14
French fries	8	0	0	$547^{c,d}$	1220	2	0	$65^{c,d,e,f}$	158	9	0	4	4
cookies	13	0	0	1344^{d}	4175	8	1	$88^{d,e,f}$	367	1	1	4	14
soya based products	4	1	0	$370^{c,d}$	534	1	0	$58^{c,d,e}$	99	4	0	2	2
fried snacks	23	0	0	$615^{c,d}$	1981	4	0	$58^{c,d,e}$	202	24	0	2	2
frozen and fresh salmon	13	0	0	$313^{b,c,d}$	1029	12	0	7^{a}	45	7	0	34	60
Smoked salmon	13	1	1	$695^{c,d}$	1596	7	5	$15^{a,b}$	79	11	1	2	8
full fat milk	17	5	0	40^{a}	164	18	0	$32^{c,d,e}$	32	18	0	5	5
cheese	17	5	0	$197^{a,b,c}$	665	10	0	$32^{c,d,e}$	32	10	0	5	5
cured and cooked meat products	19	8	2	106^{a}	882	9	5	$110^{a,b,c}$	2047	18	3	5	49
bacon	17	3	1	$143^{a,b}$	669	11	6	$8^{a,b}$	45	20	0	2	2
cured raw ham	15	2	0	$1235^{c,d}$	4145	3	12	$82^{c,d,e}$	357	15	0	2	2
cured minced raw meat products	19	1	0	$1633^{c,d}$	9265	5	1	$554^{d,e,f}$	3701	14	0	48	475
chilled cooked meals	109	10	0	$579^{b,c,d}$	7399	35	4	$95^{b,c,d}$	5074	102	0	6	234

Different superscripts within the columns indicate statistically significant differences ($p<0.05$).

The highest levels of HNE, reaching 553 µg kg^{-1}, were found in cured minced raw meat products, followed by 308 µg kg-1 detected in dry nuts. These high levels could be attributed to a combination of the processing and the fatty acid composition of these foods. The lowest levels were detected in bacon and, fresh, frozen and smoked salmon samples. In the case of bacon, the low concentrations of HNE detected can be explained by the low levels of PUFAs present in the samples. HNE was lower in comparison to the high levels reported for cured minced raw meat products. This could be explained by the fact that processing of the latter can have an impact on lipid oxidation resulting in higher formation of secondary lipid oxidation products. In milk and cheese samples, HNE was not detected above the LOD and thus, the values illustrated in

Table 6.2 are corresponding to the LOD values. Regarding salmon samples, the majority of PUFAs present are ω-3 which are not precursors of HNE (Seppanen & Csallany, 2001). In cooked chilled meals, levels of HNE were found to be six times lower than MDA and this could be due to either the actual lower amount of HNE formed or the reactivity of HNE with components present in the food matrix such as proteins. Indeed, it has been demonstrated that, HNE can form Michael addition adducts with amino acids such as lysine, glutamine, histidine (Uchida & Stadtman, 1992; Zhao, Chen, Zhu, & Xiong, 2012). These type of adducts involve stable C-C bond formation, thus, once they are formed they can be considered irreversible. On the other hand, MDA is mainly forming Schiff bases with amino acids that have an available amine group and this adduct can easily be broken down (e.g. under acidic conditions, as applied during the analytical protocol). A statistically significant correlation was found ($p=0.002$, Pearson correlation 0.714) between MDA and HNE content when all the food categories were taken into account. This suggests that the majority of MDA formed in the analyzed samples originated from ω-6 fatty acids and more specifically from linoleic (18:2), which is known to be the most common PUFA in the analyzed samples.

HHE was the aldehyde detected at the lowest levels in the analyzed samples. It was found at a level of 34 µg kg-1 in fresh and frozen salmon. Plant oils had an average of 20 µg kg-1 and it was also found in cured minced raw meat products. In all the other samples HHE was detected below the detection limit. As already mentioned HHE is produced after the decomposition of ω-3 fatty acid hydroperoxides thus it is expected to be present in high levels in oxidized samples containing fatty acids such as α-linolenic acid (18:3), eicosapentanoic acid (20:5) and docosahexaenoic acid (22:6) (Long & Picklo, 2010).

However, HHE was not detected above the detection limit in smoked salmon samples, while MDA was found at 695 μg kg^{-1}. This shows that, despite the samples were oxidized up to a particular degree, either HHE was not the major aldehyde formed, or the formed HHE readily reacted with other components of the food (i.e. proteins). At this point it should be mentioned that statistical analysis did not reveal any significant difference in the levels of aldehydes in the beginning and end of shelf life. This does not necessarily mean that lipid oxidation did not occur, since as already mentioned, the studied compounds exhibit high reactivity towards various components of the food matrix.

6.3.2 Consumption data

The total sample of 3083 individuals provided useful information on the consumption of the analyzed samples from the Belgian population. The food categories were classified into three groups according to the relative number of individuals consuming them. The first group includes foods that are consumed from more than 20% of the participants in the survey and it consists of plant oils, French fries and cheese which are consumed by 37.4, 26.1 and 48.2% of the individuals respectively. The second group includes foods that have been consumed by 10 to 20% of the individuals, including potato crisps, cookies, milk, cooked and processed meat products, cured unprocessed meat products and cured processed meat products, been consumed by 10.1, 12.7, 15.8, 10.6, 11.4 and 14.0% respectively. The last group consists of foods that have been consumed by less than 10% of the individuals. It includes dry nuts, soya based products, fried snacks, frozen and fresh salmon, smoked salmon and bacon, that have been consumed by 4.9, 0.5, 3.1, 2.8, 3.5 and 9.8% respectively. In case of cooked chilled meals, a survey was conducted of which 77.7% of the participants were considered consumers.

6.3.3 Deterministic exposure assessment

An initial attempt to estimate the exposure of the consumers to MDA, HNE and HHE through consumption of each defined food category individually was based on a deterministic approach. This approach was applied for the lower, medium and upper bound scenarios for each food category and the results for the upper bound scenario (worst case scenario) are illustrated in ***Table 6.3.*** Results or the lower and medium bound scenario indicated that the non-detects did not have any significant impact in the conclusions thus are not shown.

Deterministic analysis for the chilled cooked meals could not be applied due to the nature of the available consumption data.

MDA was detected in highest levels in samples compared to HNE and HHE. Therefore it can be suggested that consumers are exposed the most to MDA. Food categories that have the lowest contribution to the mean intake of MDA were plant oils, followed by full fat milk, bacon, cooked and processed meat products and cheese at levels of 0.008, 0.023, 0.033, 0.051 and 0.073 μg kg bw day^{-1} respectively. Low exposure to MDA through consumption of plant oils can be attributed to the combination of low contamination levels in the samples and the low average daily food intake (9 x 10-5 kg of food (kg bw day)$^{-1}$). Exposure due to the consumption of milk appears higher due to the fact that higher amounts are consumed daily by the individuals (6 x 10-4 kg of food (kg bw day)$^{-1}$). Similar to milk are the results concerning the exposure due to consumption of bacon, cooked and processed meat products and cheese. The highest exposure for the consumers was found to originate from the consumption of dry nuts, French fries, fried snacks and cured minced meat products. Even though dry nuts and cured minced meat products are not consumed in large amounts, both have a mean daily intake of 3 x 10-4 kg food (kg bw day)$^{-1}$. Consequently they are the main sources of MDA exposure due to their high contamination levels.

As a consequence of the lower levels of HNE determined in the samples compared to MDA, the exposure to HNE was found to be significantly lower. Dry nuts and cured minced meat products were defined as the most significant sources of mean HNE intake estimated at the levels of 0.108 and 0.169 μg (kg bw day)$^{-1}$ respectively. Intake of HNE from consumption of plant oils and potato crisps was half compared to MDA. Concerning fried snacks, French fries and cured unprocessed meat products, the exposure to HNE was approximately ten times lower than MDA. The intake of HHE from the analyzed foods was in agreement with the significantly low levels the compound was detected in the samples. Only frozen and fresh salmon contributed to the daily intake of HHE from the consumers; however the level of exposure is as low as 0.031 μg (kg bw day)$^{-1}$. P 99.6 and max intake of MDA and HNE, as estimated form the applied deterministic approach, indicated that the portion of the consumers who are consuming specific foods in large amounts are much more exposed to these aldehydes than the average consumer

Table 6.3 Deterministic approach estimated intakes of MDA, HNE and HHE for the different food categories (Upper bound scenario)

Food groups	Daily intake for upper bound scenario (µg (kg bw day)$^{-1}$)								
	MDA			HNE			HHE		
	Mean	**P 99.5**	**Max**	**Mean**	**P 99.5**	**Max**	**Mean**	**P 99.5**	**Max**
plant oils	0.008	0.429	0.859	0.004	0.056	0.095	0.002	0.053	0.082
dry nuts	0.556	11.752	12.515	0.108	1.846	1.956	0.003	0.044	0.047
potato crisps	0.127	1.438	2.816	0.047	0.075	0.682	0.003	0.026	0.051
French fries	0.521	3.276	3.920	0.062	0.422	0.509	0.003	0.009	0.011
cookies	0.355	5.408	11.981	0.023	0.465	1.053	0.001	0.018	0.041
soya based products	0.284	0.947	0.953	0.052	0.176	0.177	0.002	0.004	0.004
fried snacks	0.481	3.749	3.938	0.045	0.382	0.401	0.002	0.004	0.004
frozen and fresh salmon	0.278	2.283	2.601	0.007	0.097	0.113	0.031	0.138	0.152
smoked salmon	0.213	0.913	0.964	0.005	0.044	0.048	0.001	0.004	0.005
full fat milk	0.023	0.691	0.931	0.019	0.140	0.182	0.003	0.020	0.026
cheese	0.073	0.665	0.860	0.012	0.032	0.041	0.002	0.001	0.006
cured and cooked meat products	0.051	1.535	1.648	0.054	3.380	3.822	0.002	0.083	0.091
bacon	0.033	0.491	0.673	0.002	0.032	0.045	>0.001	0.002	0.002
cured raw ham	0.327	2.204	2.894	0.022	0.190	0.249	0.001	0.001	0.001
cured minced raw meat products	0.549	6.847	7.272	0.169	2.774	2.905	0.015	0.343	0.373

Table 6.4. Best fit distributions, minimum, mean and maximum of MDA and HNE concentrations (µg kg^{-1}) and food intakes (µg (kg bw day)$^{-1}$) for the different food categories.

Food category	Variable	Function	Min	Mean	Max
plant oils	Food intake	RiskPearson5(4.2911,0.0002823, RiskShift(0.0000144805))	0	0.0001	+∞
	MDA content	RiskInvgauss(108.97,25.57,RiskShift(-1,7783))	0	107.179	+∞
	HNE content	RiskLoglogistic(6.41,19.704,1.5723)	0	44.28	+∞
dry nuts	Food intake	RiskLoglogistic(0.000014623,0.00030053,3.9276)	0	0.0004	+∞
	MDA content	RiskPearson5(0.97551,383,RiskShift(47.194))	47.1944	1581.183	+∞
	HNE content	RiskInvgauss(283.74,81.25,RiskShift(24.277))	24.27	308.017	+∞
potato crisps	Food intake	RiskPearson5(4.6459,0.0017115,RiskShift(-0.0000628971))	-0.0001	0.0004	+∞
	MDA content	RiskExtvalue(246.72,101.39)	-∞	305.2452	+∞
	HNE content	RiskPareto(2.2073,69.273)	69.2727	126.6508	+∞
French fries	Food intake	RiskGamma(2.9222,0.00025678,RiskShift(0.00020175))	0.0002	0.001	+∞
	MDA content	RiskExpon(263.73,RiskShift(250.76))	250.7644	514.4924	+∞
	HNE content	RiskExpon(65.458)	0	65.458	+∞
cookies	Food intake	RiskPearson5(3.3467,0.00060902,RiskShift(0.00000181019))	0	0.0003	+∞
	MDA content	RiskInvgauss(1343.7,2513.1)	0	1343.7	+∞
	HNE content	RiskInvgauss(89.771,75.115,RiskShift(-2.2742)	47.778	144.458	+∞
soya based products	Food intake	Mean			
	MDA content	Mean			
	HNE content	Mean			
fried snacks	Food intake	RiskInvgauss(0.00085548,0.00468132,RiskShift(-0.0000735606))	-0.0001	0.0008	+∞
	MDA content	RiskPearson5(2.3714,817.67,RiskShift(47.141))	47.1406	643.3647	+∞
	HNE content	RiskExpon(57.675)	0	57.6749	+∞
frozen and fresh salmon	Food intake	RiskWeibull(1.9658,0.0010109,RiskShift(-0.00000887314))	0	0.0009	+∞
	MDA content	RiskInvgauss(289.13,366.09,RiskShift(23.532))	23.53	312.66	+∞
	HNE content	Mean			

Table 6.4 (Continued)

Food category	Variable	Function	Min	Mean	Max
smoked salmon	Food intake	RiskLognorm(0.00012464,0.0000963702,RiskShift(0.00018315)	0.0002	0.0003	+∞
	MDA content	RiskExpon(580.87)	0	580.87	+∞
	HNE content	RiskExpon(13.029,RiskShift(-1.0857))	3.6979	9.221	+∞
full fat milk	Food intake	RiskPearson5(1.7704,0.00040336,RiskShift(0.00012477)	0.0001	0.0006	+∞
	MDA content	RiskExpon(37.658)	0	37.65	+∞
	HNE content	Mean			
cheese	Food intake	RiskLognorm(0.00026134,0.0001585,RiskShift(0.00011031)	0.0001	0.0004	+∞
	MDA content	RiskExpon(180.96,RiskShift(-1.8016))	-1.8016	179.1554	+∞
	HNE content	Mean			
cured and cooked meat products	Food intake	RiskLoglogistic(0.0000434604,0.00037262,2.8397)	0	0.0005	+∞
	MDA content	RiskInvgauss(168.78,24.028,RiskShift(8.7601))	8.7601	177.5433	+∞
	HNE content	RiskExpon(107.06,RiskShift(-2.7664))	0	104.29	+∞
bacon	Food intake	RiskLoglogistic(0.00000638264,0.00020202,4.1427)	0	0.0002	+∞
	MDA content	RiskExpon(141.99,RiskShift(-8.3522))	0	141.98	+∞
	HNE content	RiskExpon(4.47,RiskShift(1.8765)	4.008	7.848	+∞
cured raw ham	Food intake	RiskLogistic(0.000264472,0.0000404549)	-∞	0.0003	+∞
	MDA content	RiskExpon(1233.5,RiskShift(-82.23))	-82.23	1151.22	+∞
	HNE content	RiskExpon(81.962)	0	81.962	+∞
cured minced raw meat products	Food intake	RiskLoglogistic(-0.0000809502,0.00037252,7.1859)	-0.0001	0.0003	+∞
	MDA content	RiskGamma(0.48519,3552.3)	0	1723.51	+∞
	HNE content	RiskInvgauss(553.6,14.071)	0	553.604	+∞
chilled cooked meals	Food intake	RiskDiscrete			
	MDA content	RiskExpon(188.89)	0	188.89	+∞
	HNE content				

6.3.4 Probabilistic exposure assessment

Probabilistic approach allows a more accurate intake estimation for the targeted aldehydes, since every possible value each variable can have and the weight of each possible scenario is taken into account. For probabilistic analysis the upper bound scenario was taken into account. In practice it means that whereas the analytes were detected below the LOD, the value was replaced with the calculated LOD of the analytical method. Consumption and contamination data for the different food categories were fitted to best distributions which were defined based on chi square statistics and P-P plots attributed in @Risk software ***(Table 6.4).*** Within the table the column titled as function is presenting the function calculating the best fit distribution using the parameters in parenthesis. Columns min, mean and max represent the minimum, mean and maximum values the concentration the aldehydes can have within the defined distribution. Examples of selected distributions, not associated with the experimental data (RiskInvGauss, RisjPearson5 and RiskExponFunction) can be found as an example in the appendix. Distribution fitting was feasible when at least five positive data were available (Vinci et al., 2012). Therefore, probabilistic analysis could not be applied for all HHE data and for the HNE data obtained for fresh and frozen salmon, vegetarian, milk and cheese, due to large amount of non-detects.

Probabilistic estimates of the intake resulting from the consumption of each food category are presented in ***Table 6.5***. Food categories with the highest contribution to the intake of MDA were dry nuts, French fries, fried snacks and cured minced meat products. On the other hand consumption of plant oils, milk, cheese, cured and cooked meat products and bacon had a lower contribution to the intake of MDA. The highest exposure to HNE derived from the consumption of potato crisps and cured minced raw products and the lowest from plant oils, smoked salmon and bacon.

The mean intakes of MDA and HNE estimated with the two approaches are similar, however differences were observed in the P 99.5 (***Table 6.3*** and ***Table 6.5***). The max and P 99.5 of the intakes were found higher when the deterministic approach was applied, indicating an overestimation compared to probabilistic approach outcome.

Table 6.5 Probabilistic approach estimated intakes of MDA, HNE and HHE from the different food categories (Upper bound scenario)

Food groups		Daily intake for upper bound scenario (µg (kg bw day)$^{-1}$)						
		Mean	**StDev**	**P 50**	**P75**	**P 90**	**P 97.5**	**P 99.5**
plant oils	MDA	0.006	0.023	<0.001	0.003	0.011	0.047	0.138
	HNE	0.005	0.021	0.002	0.004	0.009	0.023	0.063
dry nuts	MDA	0.551	1.153	0.203	0.509	1.269	3.397	7.311
	HNE	0.107	0.217	0.043	0.103	0.241	0.630	1.321
potato crisps	MDA	0.124	0.107	0.094	0.153	0.239	0.400	0.654
	HNE	0.051	0.076	0.035	0.057	0.096	0.189	0.392
French fries	MDA	0.490	0.356	0.390	0.607	0.909	1.448	2.146
	HNE	0.062	0.074	0.038	0.081	0.147	0.268	0.429
cookies	MDA	0.350	0.431	0.225	0.414	0.727	1.413	2.701
	HNE	0.023	0.041	0.012	0.025	0.052	0.114	0.225
soya based products	MDA	-	-	-	-	-	-	-
	HNE	-	-	-	-	-	-	-
fried snacks	MDA	0.507	1.017	0.328	0.563	0.964	1.962	4.211
	HNE	0.045	0.054	0.027	0.059	0.106	0.192	0.312
frozen and fresh salmon	MDA	0.277	0.296	0.185	0.345	0.596	1.075	1.777
	HNE	-	-	-	-	-	-	-
smoked salmon	MDA	0.214	0.236	0.142	0.289	0.493	0.835	1.300
	HNE	0.004	0.006	>0.001	0.007	0.012	0,020	0.031
full fat milk	MDA	0.035	0.157	0.016	0.036	0.072	0.167	0.422
	HNE	-	-	-	-	-	-	-
cheese	MDA	0.066	0.079	0.042	0.088	0.155	0.275	0.450
	HNE	-	-	-	-	-	-	-
cured and cooked meat products	MDA	0.045	0.196	0.002	0.022	0.084	0.364	1.149
	HNE	0.052	0.078	0.029	0.065	0.124	0.240	0.438
bacon	MDA	0.033	0.040	0.020	0.043	0.076	0.139	0.226
	HNE	0.002	0.003	>0.001	0.002	0.005	0.010	0.018
cured raw ham	MDA	0.326	0.350	0.214	0.446	0.767	1.268	1.933
	HNE	0.022	0.023	0.014	0.029	0.051	0.085	0.126
cured minced raw meat products	MDA	0.523	0.813	0.217	0.660	1.410	2.807	5.844
	HNE	0.167	1.100	0.008	0.035	0.181	1.304	5.960
chilled cooked meals	MDA	0.224	0.447	0.074	0.240	0.594	1.338	2.654
	HNE	0.037	0.074	0.013	0.040	0.098	0.221	0.445

Consumption of cooked chilled meals contributed to mean daily intake of MDA and HNE at the levels of 0.224 and 0.037 µg kg bw day^{-1} respectively. The results indicated that cooked chilled meals are a higher MDA and HNE intake source than vegetable oils, potato crisps milk and dairy products. However, they contribute less to the intake of the studied aldehydes

compared to French fries and fried snacks which are typical examples of foods not prepared at home.

6.3.5 Risk assessment

Due to the lack of toxicological data of MDA, HNE and HHE the TTC principle was applied. Chemicals are classified into three classes according to Cramer decision tree (Cramer et al., 1978) and therefore the TTC for chemicals are considered 1800, 540, 90 µg person d^{-1} for those belonging to Cramer class I, II and II respectively. Based on this approach MDA was classified in class I while HNE and HHE were classified in class II. Therefore, a TTC level of exposure of 30 µg $(kg\ bw\ day)^{-1}$ was accepted for MDA and 9 µg $(kg\ bw\ day)^{-1}$ for HNE and HHE. Based on the probabilistic analysis of the consumption and the contamination data it can be suggested that, the consumers of the studied food categories are not at risk, since exposure was far below the defined TTC. The exception was the cured minced meat products, where 1.4% of the consumers of this specific food group were found to be exposed to HNE at levels above 8.3 µg$(\ kg\ bw\ day)^{-1}$. Thus a potential risk may occur for this portion of consumers frequently consuming this specific food category. The results are in agreement with what was reported by Surh and Kwon (Surh & Kwon, 2005). The study was carried out in 2005 based on Korean population and similarly suggested that no particular risk was presented due to the intake of HNE and HHE through foods.

6.3.6 Uncertainty evaluation of the exposure assessment

Uncertainties associated to exposure assessments should essentially be considered for the interpretation of the results presented in this work. Under or overestimation of the consumption of the studied food groups resulting from misreporting during the collection of consumption data (e.g. inaccurate consumed quantities reported, foods reported in wrong food groups) could be a limiting factor in the precision of the estimated intakes for the aldehydes. Furthermore, the BNFCS used in this study was conducted in 2004 and therefore it is not clear if the nutritional habits of the Belgian population have changed since then. Concerning chilled cooked meals, the consumption data do not represent the total population residing in Belgium, thus deviations could be expected from the national consumption of these products. The current calculations and interpretations are performed for the individual food categories, no conclusions can be drawn for the total exposure of the Belgian consumers when all included food categories are considered together. Furthermore, the risk from the

exposure to the studied aldehydes is evaluated individually for each compound. In view of their similar reactivity and the fact that in addition to the three aldehydes studied also other reactive aldehydes are produced during lipid oxidation, it cannot be excluded that a combined exposure would put more consumers at risk as predicted from this study considering the individual exposure of only three components respectively.

6.4 CONCLUSIONS

To the best of our knowledge, this is the first study assessing the exposure of a population group to MDA, HNE and HHE through food consumption. We have reported the exposure related to the consumption of the specific food categories included in the study and attempted to evaluate the resulting potential risk. The results indicate that, consumers of the studied food categories are mainly exposed to MDA and less to HNE. Exposure to HHE was not found to be significant. Based on the available methods, no risk could be identified related to the consumption of these foods for the vast majority of the consumers. The only exception was a small proportion (1.4 %) of those who consume cured and minced raw meat products, who could be potentially at risk. However, since no robust toxicological data for MDA, HNE and HHE are available we believe precautions should be taken on the direction of preventing lipid oxidation in foods during processing and storage.

CHAPTER 7

GENERAL DISCUSSION, CONCLUSIONS AND PERSPECTIVES

CHAPTER 7. GENERAL DISCUSSION, CONCLUSIONS AND PERSPECTIVES

The first part of this research included the evaluation of the available, and development of new analytical methods for quantification of MDA, HNE, HHE and 2-butenal in various food matrices. After extensive investigation of the nature of the studied aldehydes, it was decided that distinctive analytical approaches would be applied for the determination of MDA, HNE and HHE, 2-butenal. In **Chapter 2** results from the evaluation of the spectrophotometric TBARS test and the HPLC based analytical method combined with fluorometric detection for determination of free MDA in various food matrices were presented. The results clearly indicated the non-specificity of TBARS towards MDA. More specifically, it was concluded that the TBARS test was reliable only when applied for the determination of MDA in vegetable oils and in unprocessed meat and fish products. In processed beef, pork and fish, dry nuts, cheese and potato crisps TBARS test was overestimating the content of MDA due to interferences of other compounds with TBA. Furthermore the results also indicated that other than secondary lipid oxidation products (i.e. other aldehydes) interfere with the TBARS test. On the basis of these results it was decided that the HPLC method should be applied in the further steps of this research.

With regard to the determination of free HNE and HHE in foods, an evaluation of the analytical methods available in the literature indicated the necessity for developing, implementing and validating a new method for the simultaneous determination of HNE and HHE. As described in **Chapter 3**, the developed stable dilution isotope assay involved the use of deuterated HNE and HHE as internal standards, extraction of the analytes from the matrices followed by derivatization and detection with GC-MS. The outcome was an accurate and reproducible analytical method that could provide reliable quantification of both analytes at concentrations as low as 12 ng g^{-1} of sample. It was necessary to apply dedicated extraction techniques for different food matrices, illustrating the delicate character of the quantitative analysis of these compounds in terms of their interactions with various matrix components and their rather amphiphilic character making their quantitative extraction challenging. It was suggested that milk and dairy products or generally samples containing surface active compounds can be analyzed with the QuEChERS extraction method. The method in which the analytes are extracted with acidified methanolic solution, developed and validated on raw

beef and crisps, could also be applied on cooked meat and meat products, dry nuts and cookies. Oils, butter, margarines and mayonnaise could be analyzed by the same method that involved extraction of the analytes with a methanolic solution. Furthermore, the simplicity of the extraction techniques allowed the method to be applied for routine analysis of a large amount of samples.

A few data are available in the literature regarding formation and determination of 2-butenal levels in foods. Therefore, for determination of 2-butenal in foods a new analytical method was developed. As described in chapter 4, the outcome was the development of a stable isotope dilution assay. For this purpose an appropriate deuterated internal standard, consisting of butenal *d-6,* was synthesized in the lab. The analytical method was based on the extraction of 2-butenal from the food matrix, derivatization of the analyte and analysis with HS-GC-MS. This method was proven accurate and reproducible and could provide reliable quantification data at levels as low as 28 ng g^{-1} of sample. At this point it should be mentioned that derivatization of the analyte with PFBHA created the opportunity of reaching low LODs by using direct head space analysis without involving solid phase micro extraction.

Regarding the analytical methods, the following should be taken into account. When mass spectrometry is used for the detection of the targeted compounds the opportunity of involving isotopicaly labeled compounds as internal standards should always be considered. The necessity of these internal standards was indicated in preliminary experiments and the use of these compounds was proven to be the only reliable way to avoid problems related to the recovery of the analytes during extractions from the matrix. The studied aldehydes are highly reactive molecules interacting with components of the food matrix and especially proteins as it was already observed in initial method development experiments. Therefore it becomes more clear that the use of other compounds as internal standards, even those with similar molecular structure and polarities as the analyte of interest is inappropriate. This phenomena becomes even more relevant when application of SPME is attempted. All the above were well observed in an attempt to develop an analytical method for determination of acrolein in various matrices, which was finally abandoned in view of a lack of commercial availability of a suitable labeled internal standard.

In **Chapter 4**, apart from the development of the analytical method for the determination of 2-butenal, a study on the formation of 2-butenal during frying is presented. It was concluded that 2-butenal is present in oils as a results of heating at frying temperatures at levels between 0.05 to 1 μg g^{-1} of oil. The results indicated that oils containing ω-3 PUFAs demonstrated the highest levels of 2-butenal. Furthermore, it was shown that at the studied temperature the highest level of 2-butenal determined in the oils was already formed after two hours of heating and it was suggested that 2-butenal content is mainly affected by the fatty acid composition than the extent of lipid oxidation. Surprisingly, fried foods purchased from fast food shops did not contain significant amounts of 2-butenal. Similarly, frying of several foods in 2-butenal containing oils did not result in any increase of 2-butenal content of the fried food. In preliminary experiments performed but not presented in this dissertation 2-butenal demonstrated a high reactivity towards amino acids and proteins. Based on the aforementioned observations and given the low levels at which 2-butenal was produced compared to the other aldehydes in the experiments presented in chapter 6, it was decided that determination of free 2-butenal in food matrices may not be relevant. Thus free 2-butenal was not included in the exposure assessment performed. Similarly and although preliminary experiments were performed in the framework of this research, but which were not presented in this thesis, a parallel conclusion for acrolein is suggested. Obviously however both highly reactive α, β- unsaturated aldehydes will react to some extent with various nucleophiles present in the food, so it certainly relevant to investigate the identity and the behavior of these adducts more in detail.

In order to obtain an insight on the formation of these specific aldehydes in food systems, the study presented in **Chapter 5** was carried out. After investigation of the formation of MDA, HNE, HHE and 2-butenal in oil-in-water emulsions during photo- and autoxidation it was concluded that MDA was the major aldehyde formed during photooxidation in the presence of EPA and DHA PUFAs. In addition, HHE was found to be the second most important, while 2-butenal represented only a small portion. HNE was found to be the major aldehyde formed when linoleic acid was the dominant PUFA. When lipid oxidation proceeded in a slower rate in the dark, where only autoxidation is expected to occur, EPA and DHA did not produce any significant amount of MDA, HHE and 2-butenal. This implied that there are other secondary oxidation products formed in more significant amounts than the studied aldehydes. However, samples containing linoleic and linolenic acid produced considerable amounts of MDA, HNE, HHE and 2-butenal when stored under dark. In the experiment

presented in this chapter the two mechanisms of photooxidation were not studied separately. As a future perspective, in order to have a more detailed view on the effect of singlet oxygen in the formation of the studied aldehydes, it is suggested that experiments including a physical singlet quencher such as sodium azide should be performed.

Exposure assessment was one of the main parts of this research as presented in **Chapter 6**. In order to evaluate the dietary exposure of the population to the focusing aldehydes, consumption data of specific food groups as well as their contamination levels were required. Consumption data were obtained from the Belgian National Food Consumption Survey (BNFCS) conducted in 2004 and the levels of the aldehydes were determined in foods purchased from the Belgian market. Once the analytical methods were developed and validated, a screening of specific food groups available in the Belgian market was performed and the contamination with MDA, HNE and HHE was estimated. Based on the consumption data obtained from the BNFCS an exposure assessment was performed. It was concluded that the Belgian population who is consuming the studied food categories is mainly exposed to MDA and less to HNE. Furthermore, exposure to HHE was not found to be significant. Food categories with the highest contribution to the intake of MDA were dry nuts, French fries, fried snacks and cured minced meat products. On the other hand consumption of plant oils, milk, cheese, cured and cooked meat products and bacon had a lower contribution to the intake of MDA. The highest exposure to HNE derived from the consumption of potato crisps and cured minced raw products and the lowest from plant oils, smoked salmon and bacon.

Due to lack of specific toxicological data, the Threshold of Toxicological Concern was applied in order to characterize the risk. Based on the levels of exposure suggested from this approach, it was concluded that the consumers of the studied food categories might not be at risk, since exposure was far below the defined TTC. The exception was the cured minced meat products, where 1.4% of the consumers of this specific food group were found to be exposed to HNE at levels above the value suggested by the TTC (8.3 μg kg bw day^{-1}). Thus a potential risk may occur for this portion of consumers frequently consuming this specific food category. However it has been suggested that since the current calculations and interpretetions are performed for the individual consumption of the studied food categories, no conclusions can be drawn for the total exposure of the Belgian consumers when all included food categories are considered together. Furthermore, the risk from the exposure to the studied aldehydes is evaluated individualy for each compound. In view of their similar

reactivity and the fact that in addition to the three aldehydes studied also other reactive aldehydes are produced during lipid oxidation, it cannot be excluded that a combined exposure would put more consumers at risk as predicted from this study considering the individual exposure of only three components respectively. At this point it should be also mentioned that interestingly the levels MDA, HNE and HHE detected in the screening of the foods from the Belgian market were far below the levels these compounds were produced in the experiments presented in chapter 5. Without underestimating the differences between a model system and a food matrix, this could be an indication that a considerable amount of the aldehydes formed in the analyzed food matrices has reacted with various food ingredients and thus are matrix bound.

As future perspectives in regards to risk assessment of the studied aldehydes, the following can be suggested: Development of a methodology in which a total daily intake for the consumers can be estimated after taking into account all the food groups that are possibly consumed daily. From this perspective a possible underestimation of the exposure could be eliminated. Furthermore, a great contribution for a more accurate risk assessment could be the availability of specific toxicological data, preferably including the combined effect of the potentially hazardous aldehydes formed during lipid oxidation.

In addition, further research on the behavior of the considered reactive aldehydes in food matrices should be carried out. As outlined already above, it is clear that these aldehydes are able to react with several food nucleophilic food matrices forming adducts. The nature and behavior of these adducts during for instance digestion would enable a better risk assessment of the parent aldehydes. In addition however, it should be noted that these and also other aldehydes are typically used as indicators for lipid oxidation in foods. In view of their high reactivity their role as lipid oxidation markers can be questionable Thus, a systematic investigation of the fate of the aldehyde-protein adducts in qualitative and quantitative manner in model systems could potentially result in development of analytical methods more accurate concerning the evaluation of lipid oxidation in foods. Finally, the total amount of aldehydes, free and bound, present in oxidized foods could be evaluated.

Regarding the factors influencing the formation of the studied aldehydes, further research should be performed to gain more knowledge concerning the impact of different storage temperatures, oxygen levels during storage and pH of the food matrices. The levels of free

aldehydes detectable in a model system where proteins are present should also be studied. Furthermore the effect of the two types of light induced oxidation should be investigated in order to have a more detailed picture on the importance of the singlet oxygen related to the formation of toxic aldehydes.

Due to the numerous secondary lipid oxidation products present in foods, apart from those studied, and their so far unclear effects on human health prevention towards lipid oxidation should always be priority in the food chain. It is advised that measures should always be taken during processing, transporting and storing foods, especially those rich in PUFAs.

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SUMMARY & SAMENVATTING

SUMMARY

This PhD thesis is contributing to the risk assessment related to the dietary exposure of the following toxic aldehydes which are formed in foods upon lipid oxidation: malondialdehyde (MDA), 4-hydroxy-2(E)-nonenal, (HNE), 4-hydroxy-2(E)-hexenal, (HHE) and 2-butenal. In the first part of **Chapter 1** an overview of the approaches used in risk assessment is presented. In the second part, lipid oxidation mechanism as well as suggested mechanisms related to the formation of the studied aldehydes and analytical methods for their determination in foods are discussed. Finally, a description of the current status of these aldehydes with regard to the potential hazard they may introduce to humans is elaborated.

In **Chapter 2** results related to the evaluation of the ability of the spectrophotometric thiobarbituric acid reactive substances (TBARS) test to determine MDA in various food matrices are presented. In this study, MDA was extracted from the foods, the extract reacted with thiobarbituric acid (TBA) and the formed TBA-MDA adduct was measured spectrophotometrically at 532nm. In parallel, the TBA-MDA adduct was analyzed with High Performance Liquid Chromatography (HPLC) coupled with fluorescence detection. The results indicated that heated and unheated oils, unprocessed and uncooked meat and fish products, did not exhibit any significant difference in the amount of MDA measured by the two methods , showing that the major substance reacting with TBA and forming an adduct that absorbs at 532nm was malondialdehyde. However, in products such as dry nuts, pork sausages, cooked fish and gouda cheese an overestimation of malondialdehyde was observed, indicating that the TBARS test is unsuitable for accurate determination of MDA in foods. Furthermore, the results in the present work suggest that overestimation of MDA by the TBARS test as it was applied, is related to the interference of other than secondary lipid oxidation products. As an outcome of these results it was decided that MDA determination throughout the present study would be performed with the HPLC method.

The development and validation of an analytical method suitable for the simultaneous determination of HNE and HHE in various food matrices is described in **Chapter 3**. The presented method involved the use of deuterated HNE and HHE as internal standards, extraction of the analytes from the matrices followed by derivatization and detection with gas chromatography - mass spectrometry (GC-MS). Four different food matrices were chosen as

model systems including vegetable oils, unprocessed meat, fried potato crisps and infant formula and three different extraction techniques suitable for the different matrices were applied. The simplicity of the extraction techniques allowed the method to be applied for routine analysis of a large amount of samples. The results confirmed the accuracy and reproducibility of the analytical technique and its ability to provide reliable quantification of both analytes at concentrations as low as 12 ng g^{-1} of sample. Furthermore, in this chapter a short overview of the levels of HNE and HHE in several products available in the Belgian market is presented.

The research presented in **Chapter 4** is related to the quantification and formation of 2-butenal as a result of lipid oxidation. The formation of 2-butenal in oils subjected to temperatures applied in frying as well as in fried foods was evaluated. For this purpose, a stable isotope dilution assay was developed and validated using as an internal standard 2-butenal *d6* synthesized in the lab. The method involved extraction of the analyte from the matrix, derivatization with pentafluorobenzyl hydroxylamine (PFBHA) and analysis of the formed adduct with Headspace GC-MS (HS-GC-MS). Results confirmed the method as reliable, reproducible and very sensitive since LOD and LOQ's were 0.014 and 0.028 µg g^{-1} of sample respectively. With regards to 2-butenal formation, results have shown that heating of corn oil, sunflower oil, extra virgin and refined olive oil, ω-3 frying oil and palm oil at 170 °C for a total of 8 hours resulted in the formation of 2-butenal at levels between 0.05 to 1 µg $g^{-1}$and the highest amounts present in the oils were detected after two hours of heating. The highest levels of 2-butenal were observed in the oil containing ω-3 unsaturated fatty acids indicating that 2-butenal content is mainly affected by the fatty acid composition. No significant amount of 2-butenal was detected in fried foods purchased from fast food shops with the exception of one chicken sample and a pork brochette. Further experiments including several foods did not indicate any increase of 2-butenal content due to frying. Therefore, it was suggested that fried foods may not be a significant dietary source of 2-butenal for humans.

The factors influencing the formation of MDA, HNE, HHE and 2-butenal in oil-in water-emulsions were investigated in **Chapter 5**. Emulsions were prepared with different fatty acid profiles and stored at 4 ^{o}C for a total of thirty days with and without illumination in the presence of riboflavin. The results suggested that the amounts of MDA, HNE, HHE and 2-

butenal in the studied system was strongly affected by the type and extent of lipid oxidation along with the fatty acid profile. In illuminated samples, MDA was found to be the major aldehyde formed in emulsions prepared with fish oil, where eicosapentaenoic (EPA) and docosahexaenoic (DHA) were the major ω-3 poly unsaturated fatty acids (PUFAs). HHE was found to be the second most important, while 2-butenal was produced in only a small portion. HNE was found to be the major aldehyde formed when linoleic acid was the dominant PUFA. When lipid oxidation proceeded in the dark, EPA and DHA did not produce any significant amounts of MDA, HHE and 2-butenal, indicating that there are other secondary oxidation products formed in more significant amounts than the studied aldehydes. In contrast, samples containing linoleic and linolenic acid produced considerably higher amounts of MDA, HNE, HHE and 2-butenal when stored in the dark. On the basis of the results obtained from this study and in combination with the results presented in the previous chapter it was concluded that measuring free 2-butenal in foods may not be relevant. Therefore, 2-butenal was excluded from the study presented in the upcoming chapter.

In the study presented in **Chapter 6**, the concentrations of MDA, HNE and HHE were determined in a total of sixteen food categories available in the Belgian market and in combination with consumption data obtained from a national representative sample of the Belgian population, a quantitative exposure assessment was performed. MDA was detected above the detection limit in 84% of the analyzed samples while HNE and HHE in 63% and 16% of the samples respectively. Consumption of dry nuts, fried snacks, French fries and cured minced meat products(salami type) were found to contribute the most to the intake of MDA and HNE. Intake of HHE from the foods analyzed was not found to be significant. An evaluation of any potential risk related to the intake of the studied aldehydes for the consumers of the specific food categories analyzed was made by applying the Threshold of Toxicological Concern concept. No risk to human health could be identified related to the consumption of these foods for the vast majority of the consumers, with the only exception of a small proportion (1.4 %) of those who consume cured and minced raw meat, that could be at risk. It should be emphasized however that this study dealt with a limited number of toxic compounds and that these compounds may be present in the foodstuff in a bound form. However, the nature and the behavior of these adducts is yet poorly studied. Hence, precautions to protect foods from lipid oxidation should be taken into account.

Finally in **Chapter 7** a general discussion on the results presented throughout chapters 2 to 6, conclusions and future perspectives were elaborated

SAMENVATTING

Dit proefschrift levert een bijdrage aan de risicobeoordeling van de blootstelling via de voeding van volgende toxische aldehyden welke ontstaan in levensmiddelen ten gevolge van lipide-oxidatie: malondialdehyde (MDA), 4-hydroxy-2(E)-nonenal (HNE), 4-hydroxy-2(E)-hexenal (HHE) en 2-butenal. In het eerste deel van **Hoofdstuk 1** wordt een overzicht gegeven van de manier waarop een risicobeoordeling tot stand komt. In het tweede deel wordt het lipide-oxidatiemechanisme voorgesteld, waarbij met name de vormingsmechanismen van de beschouwde componenten worden besproken. Daarnaast wordt ook aandacht besteed aan de analytische methoden die tot hiertoe werden gebruikt om deze componenten in levensmiddelen te bepalen. Tot slot wordt kort het mogelijk risico dat een te hoge blootstelling aan desbetreffende componenten zou kunnen betekenen aangehaald.

In hoofdstuk 2 wordt de toepasbaarheid van de spectrofotometrische methode welke thiobarbituurzuurreactieve verbindingen detecteert (TBARS – Thiobarbituric acid reactive substances) geëvalueerd om het MDA-gehalte in diverse levensmiddelen te bepalenHiertoe werd het MDA uit de matrices geëxtraheerd waarna dit extract onderworpen werd aan een reactie met thiobarbituurzuur (TBA). Vervolgens werd het gevormde TBA-MDA adduct spectrofotometrisch bepaald bij een golflengte van 532 nm. Daarnaast werd parallel het TBA-MDA adduct via hoge druk vloeistofchromatografie met fluorimetrische detectie bepaald. De resultaten toonden aan dat bij de analyse van niet verhitte oliën, vers vlees of verse vleesbereidingen en verse vis, geen significant verschil kon worden waargenomen tussen deze twee technieken . In producten zoals noten, varkensworst, gekookte vis en gouda kaas werd echter een duidelijke overschatting van het MDA-gehalte teruggevonden indien de spectrofotometrische methode werd gebruikt. Bovendien kon op basis van de bekomen resultaten gesuggereerd worden dat deze overschatting te wijten was aan de aanwezigheid van andere componenten dan deze die tijdens de lipide-oxidatie ontstaan. Uit dit onderzoek werd dan ook besloten dat de spectrofotometrische methode voor het bepalen van het MDA-gehalte in een breed gamma van levensmiddelen of andere matrices niet geschikt is en bijgevolg de vloeistofchromatografische methode met fluorescentiedetectie dient gebruikt te worden.

De ontwikkeling en validatie van een analytische methode voor het gelijktijdig bepalen van HNE en HHE in diverse levensmiddelenmatrices wordt voorgesteld in **Hoofdstuk 3**. Deze

methode maakt gebruik van gedeutereerd HNE en HHE als interne standaarden en omvat een extractie van de componenten uit de matrix die vervolgens gederivatiseerd, gescheiden en kwantitatief bepaald worden via gaschromatografie gekoppeld aan massaspectrometrie (GC-MS). Om deze methode te valideren werden vier verschillende levensmiddelenmatrices geselecteerd, namelijk plantaardige olie, vers vlees, chips en zuigelingenvoeding. Voor de eerste twee matrices kon hetzelfde extractieprotocol gebruikt worden, doch voor de andere matrices diende de extractieprocedure te worden aangepast. De verschillende extractiemethoden waren zeer eenvoudig en zijn toepasbaar voor routineanalyse wat de verwerking van een groot aantal stalen mogelijk maakt. De validatiestudie bevestigde de accuraatheid en de reproduceerbaarheid van de voorgestelde methode alsook zijn gevoeligheid gezien beide componenten konden bepaald worden in diverse matrices tot op een niveau van 12 ng per gram staal. Deze methode werd dan ook toegepast voor de analyse van diverse levensmiddelenmatrices. De teruggevonden concentraties werden in dit hoofdstuk gerapporteerd.

Het onderzoek dat voorgesteld wordt in **Hoofdstuk 4** betreft de kwantificatie en vorming van 2-butenal in levensmiddelen, ten gevolge van lipide-oxidatie. De vorming van 2-butenal werd opgevolgd in olie die verhit werd tot temperaturen die typisch worden bereikt tijdens frituren, alsook in producten die gefrituurd werden. Om dit te verwezenlijken werd een stabiel isotoop verdunnings assay ontwikkeld en gevalideerd welke gebruik maakt van 2-butenal *d6* als interne standaard, die werd gesynthetiseerd in het laboratorium. De methode omvat een extractie van de analiet uit de matrix, gevolgd door een derivatisatie met PFBHA waarna het gevormde adduct via een directe gaschromatografische analyse van de kopruimte gekoppeld aan massaspectrometrie (HS-GC-MS, headspace GC-MS) werd geanalyseerd. Deze methode bleek heel betrouwbaar en reproduceerbaar te zijn en vertoonde bovendien een grote gevoeligheid: de detectie- en kwantificatielimiet bedroeg 0.014 en 0.028 µg per gram staal. Voor de vorming van 2-butenal werden verschillende oliën (maïsolie, zonnebloemolie, extra virgin olijfolie, gerafineerde olijfolie, frituurolie met omega-3 vetzuren en palmolie) verhit bij 170 °C gedurende 8 uur. Er werd vastgesteld dat de hoogste concentratie aan 2-butenal reeds bereikt werd na 2 uur verhitten en dat de concentraties, afhankelijk van de olie, varieerden tussen 0.05 tot 1 µg per gram olie. De frituurolie met polyonverzadigde omega-3 vetzuren bleek het meeste 2-butenal te bevatten. Op basis van de resultaten kon besloten worden dat de vorming van 2-butenal voornamelijk afhankelijk is van de

vetzuursamenstelling. Bij gefrituurde levensmiddelen, aangekocht in diverse frituren, kon slechts in twee geanalyseerde monsters de doelcomponent worden aangetroffen en dit in zeer kleine hoeveelheden. Verder werd tijdens frituurexperimenten in het laboratorium geen significante toename in het 2-butenalgehalte van diverse stalen waargenomen. Op basis van deze resultaten werd gesuggereerd dat de 2-butenalconcentraties in deze levensmiddelen wellicht dermate laag zijn. Bijgevolg vormen gefrituurde producten geen significante blootstellingsbron aan deze toxische component.

De vorming van MDA, HNE, HHE en 2-butenal in olie-in water emulsies werd onderzocht in **Hoofdstuk 5**. Hiertoe werden emulsies aangemaakt met mengsels van verschillende oliën om zodoende een relevante variatie in vetzuursamenstelling van de emulsies te bewerkstelligen. De emulsies bevatten riboflavine en werden 30 dagen gestockeerd bij 4 °C, zowel in het donker als onder continue belichting. De resultaten toonden een tijdsafhankelijke vorming van de beschouwde aldehyden aan welke in belangrijke mate afhankelijk was van het type lipide-oxidatie waaraan de monsters werden onderworpen alsook van de vetzuursamenstelling. In de belichte emulsies werd MDA teruggevonden als belangrijkste aldehyde indien de emulsies visolie en dus de zeer sterk onverzadigde langketen vetzuren eicosapentaeenzuur (EPA) en docosohexaeenzuur (DHA) bevatten. HHE werd teruggevonden als tweede belangrijkste aldehyde in deze emulsies, terwijl 2-butenal slechts in kleine hoeveelheden werd gevormd. HNE was het voornaamste aldehyde dat gevormd werd indien linoleenzuur aanwezig was als dominant vetzuur. Bij oxidatie in het donker bleken echter EPA en DHA geen significante hoeveelheden MDA, HHE en 2-butenal meer te produceren, wat er wellicht op wijst dat in deze omstandigheden andere secondaire oxidatiecomponenten preferentieel gevormd worden. Deze bevindingen contrasteerden met deze voor de emulsies die linol- en linoleenzuur bevatten, gezien in deze emulsies duidelijk hogere hoeveelheden MDA, HNE, HHE en 2-butenal werden gevormd. Op basis van de bekomen resultaten uit dit onderzoek en deresultaten uit het vorige hoofdstuk werd besloten dat het meten van vrij 2-butenal in levensmiddel niet relevant is. Daarom werd ook besloten deze component niet mee te nemen in de studie die wordt voorgesteld in het volgende hoofdstuk.

In **Hoofdstuk 6** werden de concentraties MDA, HNE en HHE in zestien verschillende levensmiddelencategorieën bepaald door verschillende producten te selecteren die

beschikbaar zijn op de Belgische markt. In combinatie met de consumptiecijfers uit een nationaal representatief monster van de Belgische consument kon een kwantitatieve blootstellingsinschatting worden uitgevoerd. MDA werd gedetecteerd in 84 % van de onderzochte monsters, terwijl HNE en HHE in 63 % en 16 % van de onderzochte levensmiddelen werd gedetecteerd. De consumptie van droge noten, gefrituurde snacks, frietjes en gerijpte, gepekelde en verkleinde vleeswaren (type salami) bleek op een belangrijke manier bij te dragen tot de blootstelling aan MDA en HNE. De blootstelling aan HHE via de voeding werd als niet significant beschouwd. Een beoordeling van het mogelijk risico dat door de blootstelling aan deze toxische aldehyden kon ontstaan was mogelijk door gebruik te maken van het "Threshold of Toxicological Concern" concept, een concept dat stelt dat boven een bepaalde drempelwaarde naar blootstelling van een bepaalde component een risico ontstaat. Op basis van deze benadering werd besloten dat slechts een klein aandeel van de consumenten, namelijk 1.4 %, blootgesteld is aan te hoge hoeveelheden van de beschouwde componenten en dit met name door consumptie van gerijpte, gepekelde en verkleinde vleeswaren (type salami). Voor het grootste deel van de bevolking blijkt de blootstelling aan de beschouwde componenten geen voedselveiligheidsrisico in te houden. Er werd echter opgemerkt dat de studie enkel betrekking had tot een beperkt aantal toxische componenten die gevormd worden tijdens lipide-oxidatie én dat de beschouwde componenten deels aanwezig kunnen zijn in het levensmiddel onder gebonden vorm. Bovendien zijn deze adducten niet volledig bekend en is hun gedrag onvoldoende bestudeerd. Bijgevolg dient toch geadviseerd te worden levensmiddelen voldoende te beschermen tegen lipide-oxidatie.

Ten slotte wordt in **Hoofdstuk 7** van dit werk een algemene discussie van de resultaten voorgesteld en worden de voornaamste conclusies op een rijtje gezet. Ook worden een aantal perspectieven en suggesties geformuleerd naar toekomst toe.

LIST OF ABBREVIATIONS

LIST OF ABBREVIATIONS

ADI	acceptable daily intake
ANOVA	analysis of variance
AOCS	American oil chemists society
BMDL	benchmark dose lower confidence limit
BNFCS	Belgian national food consumption survey
BSTFA	N,O-bis(trimethylsilyl) trifluoroacetamide
CAR-PDMS	carboxen- polydimethyl siloxane
CV	coefficient of variation
DHA	docosahexaenoic acid
DNPH	dinitrophenylhydrazine
EFSA	European food safety agency
EPA	eicosapentaenoic acid
FAO	food and agriculture organization
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
HHE	4-hydoroxy-2(E) hexenal
HNE	4-hydoroxy-2(E) nonenal
HPLC	high pressure liquid chromatography
HS-GC-MS	headspace gas chromatography-mass spectrometry
IS	internal standard
LD 50	median leathal dose
LOD	limit of detection
LOQ	limit of quantification
MDA	malondialdehyde

MOE	margin of exposure
MS	mass spectrometry
MSD	mass spectrometric detector
MSM	multiple source method
m/z	mass to charge ration
NOAEL	no observed adverse effect level
PE	poly ethylene
PFBHA	pentafluoro benzyl hydroxylamine
PTFE	polytetrafluoroethylene
PUFAs	poly unsaturated fatty acids
QuEChERS	quick easy cheap effective rugged safe
SIDA	stable isotope dilution assay
SIM	selective ion monitoring
SPME	solid phase micro extraction
StDev	standard deviation
TAGs	triacylglycerols
TBA	2-thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
TCA	trichloroacetic acid
TD 50	median toxic dose
TDI	tolerable daily intake
TEP	tetraethoxypropane
TMCS	trimethylchlorosilane
TTC	threshold of toxicological concern
UV	ultra violet
WHO	world health organization

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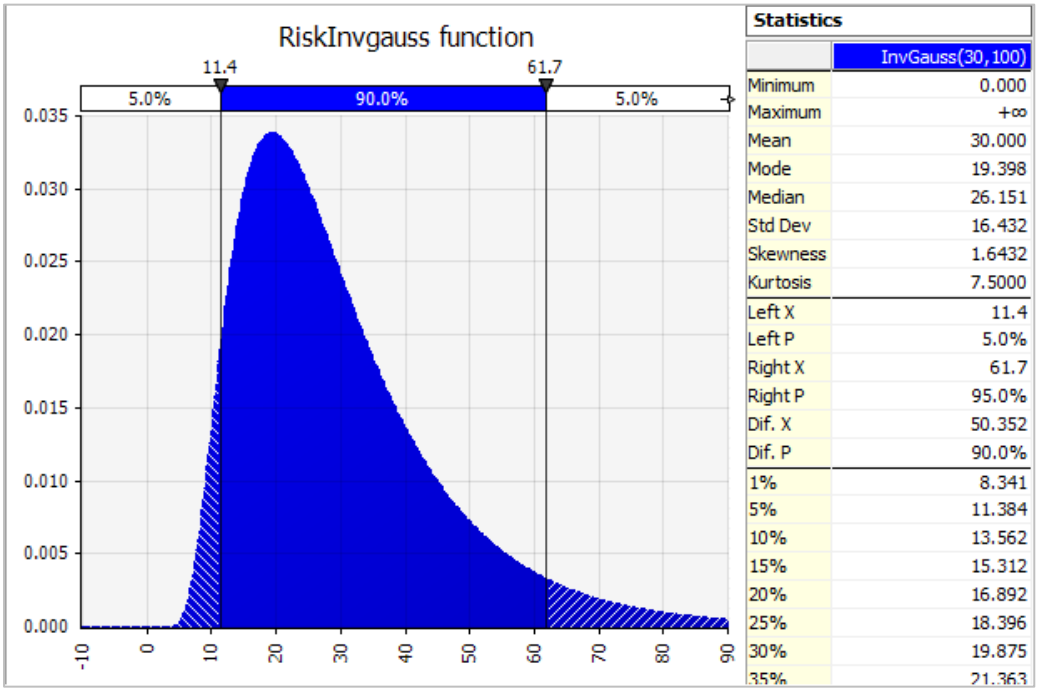
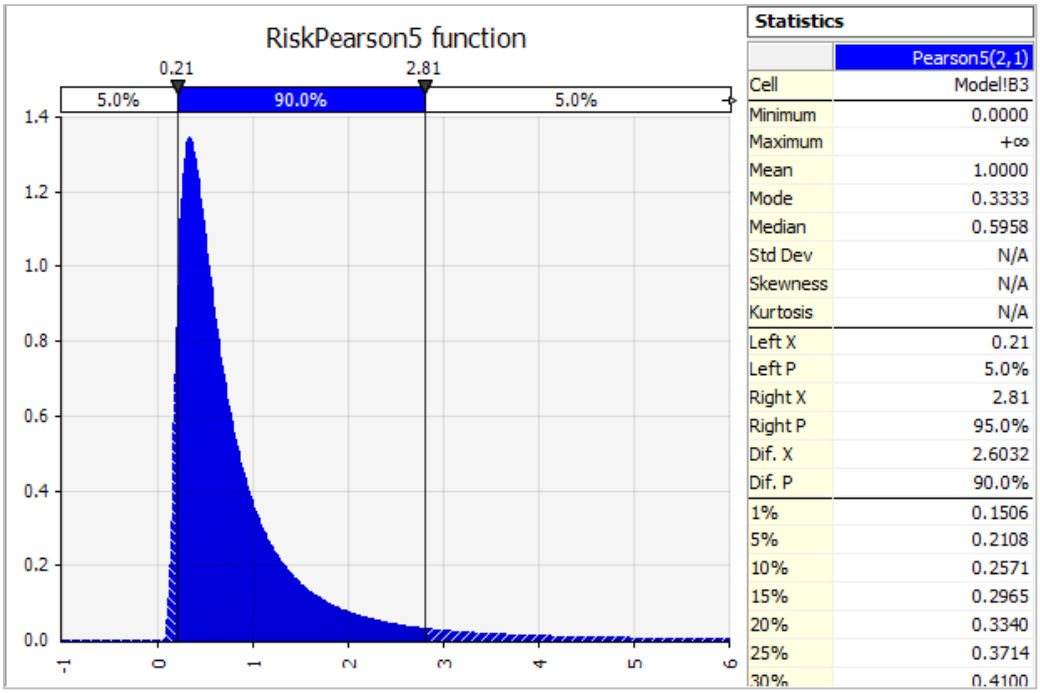
APPENDIX



Statistics	
	InvGauss(30,100)
Minimum	0.000
Maximum	+∞
Mean	30.000
Mode	19.398
Median	26.151
Std Dev	16.432
Skewness	1.6432
Kurtosis	7.5000
Left X	11.4
Left P	5.0%
Right X	61.7
Right P	95.0%
Dif. X	50.352
Dif. P	90.0%
1%	8.341
5%	11.384
10%	13.562
15%	15.312
20%	16.892
25%	18.396
30%	19.875
35%	21.363

Example of RiskInvgauss function. InvGauss(30,100) : 30 ;100 represent the parameters μ and shape parameter λ



Statistics	
	Pearson5(2,1)
Cell	Model!B3
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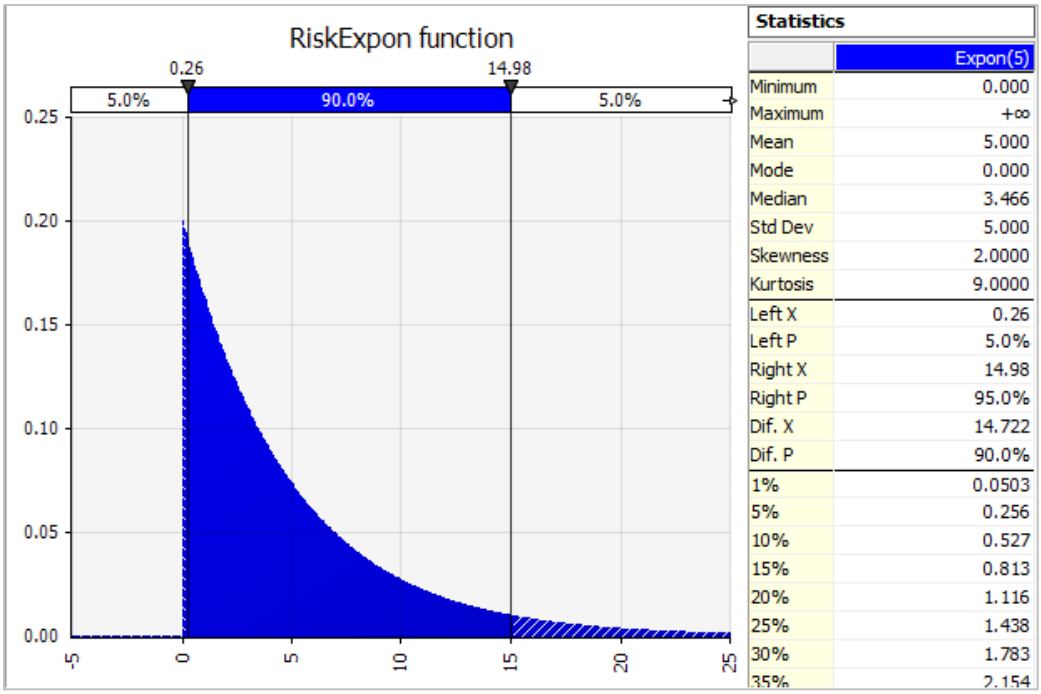
Maximum	+∞
Mean	1.0000
Mode	0.3333
Median	0.5958
Std Dev	N/A
Skewness	N/A
Kurtosis	N/A
Left X	0.21
Left P	5.0%
Right X	2.81
Right P	95.0%
Dif. X	2.6032
Dif. P	90.0%
1%	0.1506
5%	0.2108
10%	0.2571
15%	0.2965
20%	0.3340
25%	0.3714
30%	0.4100

Example of RiskPearson5 function. Pearson5(2,1) : 2 and 1 are the shape parameter α and scale parameter β



Statistics	
	Expon(5)
Minimum	0.000
Maximum	+∞
Mean	5.000
Mode	0.000
Median	3.466
Std Dev	5.000
Skewness	2.0000
Kurtosis	9.0000
Left X	0.26
Left P	5.0%
Right X	14.98
Right P	95.0%
Dif. X	14.722
Dif. P	90.0%
1%	0.0503
5%	0.256
10%	0.527
15%	0.813
20%	1.116
25%	1.438
30%	1.783
35%	2.154

Example of RiskExpon function. Expon(5): 5 is the β parameter which in this case equals the mean

CURRICULUM VITAE

Antonios Papastergiadis was born on February the 15th, 1983 in Thessaloniki, Greece. In 2006 he received his diploma in Food Technology from Technological Educational Institute of Thessaloniki. His thesis, under the title "Physicochemical changes sardine (Sardinops Pilchardus) muscle during storage at -18 ^{o}C and functional properties of produced surimi gels enhanced with Ca^{++} ions and MTGase" included original research results. In 2009 he received his MSc in Food Science from Technical University of Denmark. His MSc thesis titled "Cross-linking of fish muscle protein- Quality and functional properties after treatment with microbial TGase, Laccase and Peroxidase" was a result of original research and results have been presented in international symposia.

Since October 2010 he has been working on his PhD project titled "Exposure assessment in relation to the formation of toxic aldehydes in foods available in the Belgian market as a result of lipid oxidation" under the guidance of Prof. dr. ir. Bruno De Meulenaer. The research has been carried out in the laboratory of the Department of Food Safety and Food Quality of the Faculty of Bioscience Engineering of Ghent University. His research project was funded by the Belgian Federal Public Health Service, Food Chain Safety and Environment. Results of his research were published in a number of peer- reviewed scientific journal and have been presented in international symposia.

Publications of MSc Antonios Papastergiadis:

A1 journals

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