Lipid oxidation in skincare products

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Publication date:
2018

Document Version
Publisher's PDF, also known as Version of record

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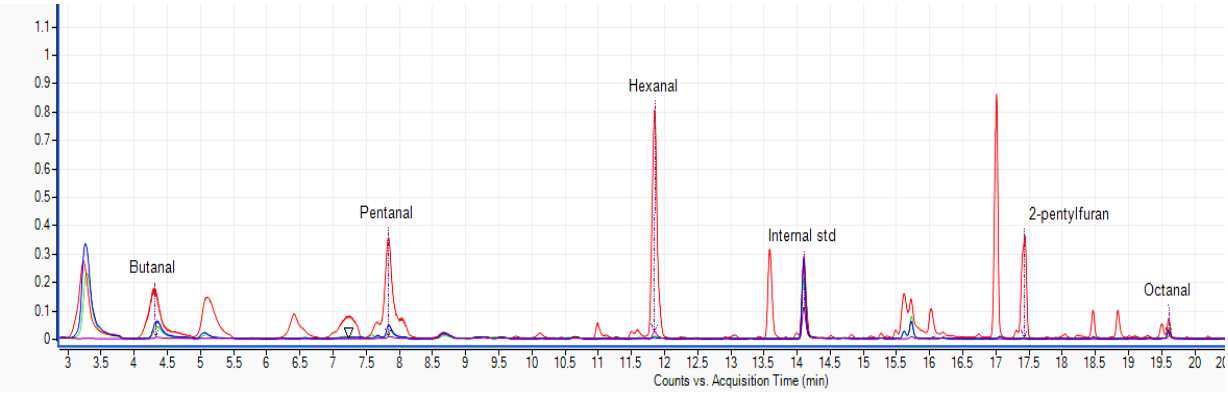

Birgitte Raagaard Thomsen

PhD Thesis

2018

DTU Food
National Food Institute

Preface

The present thesis concludes my PhD project: Lipid oxidation in skincare products. The research project was financed by Glaxo Smith Kline and the Technical University of Denmark.

The research was conducted in the research group for Bioactives – Analysis and Application, National Food Institute, Technical University of Denmark during the period January 2014 to September 2017 including a maternity leave. My main supervisor was Professor Charlotte Jacobsen and co-supervisor was Senior Research Scientist Grethe Hyldig from the same research group. Furthermore, Principal Scientist Richard Taylor at Glaxo Smith Kline acted as co-supervisor. Throughout the PhD project, I have been on two research stay at Glaxo Smith Kline in Brentford, UK.

Several persons have supported me, you know who you are, thank you.

First of all, I am deeply grateful to my main supervisor Professor Charlotte Jacobsen for always supporting me, encouraging me, listening when times were tough and taking time to discuss results. I would also like to thank my co-supervisors Grethe Hyldig and Richard Taylor; I appreciate all our discussions and your inputs for my experimental plans and results throughout the project. My deepest thanks to my colleagues and friends: Senior Scientist Ann-Dorit Sørensen, Postdoc Ditte Baun Hermund, the technicians Lis Berner, Inge Holmberg, Trang Vu and Riuyinosa Igbinovia and several others for being kind, helpful, and making it fun to spend time in the laboratories.

Lastly, I would like thank for my boyfriend Søren Kristensen for understanding that writing a thesis takes time, my daughter Emma Raagaard Kristensen for sleeping early allowing me to write this thesis in the evenings, and my family and friends for help and support through this PhD.

Birgitte Raagaard Thomsen

Summary

Skincare products are functional products containing multiple ingredients that serve various purposes in the formulation. One of the main ingredients in skincare products are lipids. Traditionally, most of the lipids added are saturated lipids to make them less vulnerable to lipid oxidation. However, recent studies have shown that unsaturated lipids improve skin appearance and function more than saturated lipids. Unsaturated lipids are more susceptible to lipid oxidation than saturated lipids. Therefore, unsaturated lipids and use of multiple ingredients challenge understanding and predicting lipid oxidation in the skincare product. Moreover, volatile lipid oxidation products can affect product odour in concentrations below 1 ng/g for some volatile compounds.

The objective of the present thesis was to investigate lipid oxidation in skincare products. Firstly, the extent of lipid oxidation was examined in skincare products, by analysing primary and secondary volatile oxidation products. In addition, sensory evaluation was conducted to examine the odour changes that appeared. The progress of lipid oxidation in all three analyses was compared to find markers for lipid oxidation. Moreover, the complex reactions behind the formation of volatile oxidation products were investigated in detail for the volatile compounds that increased the most. Evaluation of the physical and chemical including oxidative stability can be a bottleneck in product development. To combat this bottleneck, several approaches that can initiate lipid oxidation were examined with the aim to be able to predict oxidative stability faster.

The results showed that two volatile aldehydes, pentanal and heptanal, were good markers for monitoring progress of lipid oxidation in prototype lip care formulations (lip balm), prototype cleansing formulations and facial moisturiser prototypes with seaweed antioxidants added. However, only pentanal could be used as a marker in studies of the prototype skin cream formulation because heptanal was low and stable even though other oxidation products increased. Therefore, pentanal was a universal marker for lipid oxidation in all skincare products used throughout this thesis. Three types of seaweed extracts derived from brown alga, *Fucus vesiculosus*, were added as antioxidants to facial moisturiser prototype to improve oxidative stability. The oxidative stability under photo-activated oxidation was increased most by *Fucus vesiculosus* water extract. The *Fucus vesiculosus* water extract may decrease photooxidation due to a protective effect of the carotenoids present in the extract. However, *Fucus vesiculosus* acetone extract was more efficient in preventing hydroperoxide

decomposition to volatile compounds than the water extract. Furthermore, the *Fucus vesiculosus* acetone extract protected best against thermo-activated oxidation.

The effect of lipid composition on lipid oxidation was evaluated in prototype cleansing and lip care formulations. The prototype cleansing and lip care formulations had lipid content at 8 % and 36 %, respectively. The prototype lip care formulations with the highest lipid content also had the lowest oxidative stability. Thereby, the oxidative stability improved when the lipid content was reduced. However, minor changes in the lipid composition between two prototype cleansing formulation, and storage at 2°C did not show any clear difference. Nevertheless, storage at 20°C with exposure to light gave rise to a significantly higher concentration of heptanal in prototype cleansing formulation 1 than in formulation 2 after 56 days of storage. Furthermore, the intensities of the sensory attributes also increased with increased amount of unsaturated fatty acids.

The effect of storage conditions on lipid oxidation was evaluated by storing skincare products at temperatures ranging from 2°C to 50°C. Overall, a non-linear relationship was observed between the concentration of volatile compounds and temperature. In addition to temperature, the effect of exposure to light was also investigated. Exposure to light resulted in equal or an increased peroxide value and concentration of volatile oxidation products. A sensory profiling showed a clear effect of increased temperature on the sensory attribute intensities in the prototype cleansing formulation. Antioxidant addition resulted in less sensitivity towards photooxidation, a lower concentration of volatile compounds and intensities of attributes. Attributes that increased in sensitivity were suggested by partial least squares regression to be linked to formation of pentanal and butane nitrile. However, addition of pentanal and butane nitrile to prototype cleansing formulation did not result in the suggested attributes, probably because a combination of several volatile compounds gave rise to the attributes.

Butanal and pentanal were initially present or appeared in most lipid containing raw materials. One raw material, isoamyl p-methoxycinnamate, had a high concentration of 3-methyl-1-butanol. The main reaction route leading to the formation of 3-methyl-1-butanol may be direct hydrolysis of the ester-group. Butane nitrile did not appear in any raw materials, but it may be a by-product from azobisisobutyronitrile decomposition or a migrant from the plast packaging material. Lastly, approaches to predict oxidative stability faster were examined. Three initiators were able to accelerate lipid oxidation. However, only $FeCl_2/H_2O_2$

was able to make a fast and correct prediction of the oxidative stability in both prototype skin cream formulations and facial moisturiser prototypes containing *Fucus vesiculosus* extracts.

Resume

Hudplejeprodukter er funktionelle produkter med mange forskellige ingredienser, som alle har et specifikt formål i produktet. Traditionelt, indeholder hudplejeprodukter lipider med mættede fedtsyrer, der har en højere oxidativ stabilitet end umættede fedtsyrer. Men indenfor de seneste år har nye studier vist, at umættede fedtsyrer plejer huden bedre end mættede fedtsyrer. Tilsætning af umættede fedtsyrer og de mange forskellige ingredienser, der anvendes i hudplejeprodukter gør det svært at forstå og prædiktere produktets oxidativ stabilitet. Det er vigtigt at kunne kontrollere lipidoxidation i hudplejeprodukter, da denne giver anledning til dannelse af flygtige oxidationsprodukter, som selv i meget lave koncentrationer påvirker produktets duft under 1 ng/g for nogle flygtige oxidationsprodukter i vand.

Formålet med denne ph.d. var at undersøge lipidoxidation i hudplejeprodukter. Omfanget af lipidoxidation blev undersøgt ved at analysere for primære og sekundære flygtige oxidationsprodukter. Derudover blev der foretaget sensorisk bedømmelse af ændringer i produkternes lugt. Resultaterne fra de tre analyser blev sammenlignet med det formål at finde markører for lipidoxidation. I lipidoxidation kan sekundære flygtige oxidationsprodukter blive dannet via mange forskellige reaktioner. Mulige reaktionsveje blev undersøgt for udvalgte sekundære flygtige oxidationsprodukter. Bestemmelse af hudplejeprodukters oxidativ stabilitet tager ca. seks måneder dette er en flaskehals i produktudviklingen. Denne flaskehals kan muligvis fjernes ved at initiere lipidoxidation hurtigere, således at den oxidativ stabilitet kan bestemmes hurtigere.

Resultaterne viste, at to flygtige aldehyder, pentanal og heptanal, var gode markører for lipidoxidation i prototype rensemælk- og læbepomade-formuleringer, og ansigtsdagcreme tilsat antioxidanter fra tang. Det var dog kun pentanal, der kunne bruges som markør i prototype cremeformuleringen. Derfor var det kun pentanal, der kunne anvendes som en universal markør for lipidoxidation i løbet af dette ph.d. projekt. Med henblik på at øge den oxidativ stabilitet blev ansigtsdagcreme tilsat vand- og acetoneekstrakter fra brunalgen, *Fucus vesiculosus*. Peroxidværdien efter photo-aktiveret oxidation var lavest i ansigtsdagcremer tilsat vandekstraktet. Dette var formentlig relateret til, at vandekstraktet indeholdt karotenoider, som beskyttede imod photooxidation. Det var dog acetoneekstraktet, som var mest effektiv til at modvirke hydroperoxid-nedbrydningen til flygtige oxidationsprodukter. Ligeledes beskyttede acetoneekstraktet bedst imod termo-aktiveret

oxidation. Prototype rensemælk- og læbepomade-formuleringer har et lipidindhold på henholdsvis 8 % og 36 %. De forskellige lipidindhold havde en signikant effekt på lipidoxidation. Prototype læbepomade-formuleringer med det højeste lipidindhold havde en signifikant lavere oxidativ stabilitet end prototype rensemælk-formuleringer. Den oxidativ stabilitet var således signifikant lavere, når lipidindholdet var lavt. Effekten af mindre ændringer i lipidsammensætning blev undersøgt i to prototype rensemælk-formuleringer hvor mængden af umættede fedtsyrer blev varieret. Indholdet af umættede fedtsyrer havde en signifikant effekt under lagring ved 20°C og eksponering til lys. Eksponering til lys og et højere indhold af umættede fedtsyrer gav anledning til en signifikant højere koncentration af heptanal efter 56 dages lagring.

Overordnet var der et ikke-lineært forhold imellem koncentrationen af flygtige oxidationsprodukter og temperaturstigning. En stigning i lagringstemperaturen fra 2°C til 50°C havde signifikant effekt på lipidoxidation. Udover effekten af lagringstemperatur blev effekten af eksponering til lys udforsket. Exponering til lys resultede i samme eller højere peroxidværdi og koncentration af flygtige oxidationsprodukter sammenlignet med opbevaring i mørke ved samme temperatur. Duftprofilerne viste en klar effekt af temperaturstigninger på intensiteten af de fleste attributter i prototype rensemælk formuleringer. I prototype rensemælk formuleringer med højt indehold af umættede fedtsyrer var der en øget intensitet af attributterne. Den øgede intensitet af attributterne skyldtes sandsynligvis, at umættede fedtsyrer var mere udsatte for autoxidation.

Tilsætning af antioxidant resulterede i mindre sensitivitet overfor photooxidation, hvilket kunne ses ved en lavere koncentration af flygtige oxidationsprodukter og lavere intensitet af attributterne. To attributter som steg i intensiteten under lagring blev ved partial least squares regression linket til pentanal and butannitril men attributter steg ikke i intensitet ved tilsætning af pentanal og butannitil. Det er formentlig en kombination af flere flygtige som giver anledning til attributterne. Udover selve formuleringerne blev der foretaget en råvareanalyse hvor de flygtige oxidationsprodukter blev identificeret. Især to flygtige oxidationsprodukter, butanal og pentanal, var til stede og/eller blev dannet i de fleste lipidholdige råvarer under lagring. Udover de to flygtige oxidationsprodukter blev der også målt en høj koncentration af 3-methyl-1-butanol i isoamyl p-methoxycinnamate. Hovedreaktionsvejen til 3-methyl-1-butanol var direkte hydrolyse af ester-gruppen i isoamyl p-methoxycinnamat. Under lagring blev butannitril dannet, men butannitril var ikke til stede i

nogen af råvarerne. Derfor overvejes det, om butannitril målt i dette studie er et bi-produkt fra dekomposition af azobisisobutyronitril. Alternativt kan butannitril være en migrant fra plastpakkematerialet. Evaluering af den oxidativ stabilitet er en tidskævende proces, ca. seks måneder. Derfor blev mulighederne for at prædiktere den oxidativ stabilitet hurtigere ved hjælp af initiatorer udforsket. Alle initiatorerne kunne accelerere lipidoxidation. Det var dog kun $FeCl_2/H_2O_2$, som var i stand til at prædiktere den oxidativ stabilitet i prototype cremeformuleringer og ansigtsdagcremer tilsat antioxidanter fra tang.

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List of Publications

Paper I

Thomsen, B.R., Horn, A.F., Hyldig, G., Taylor, R., Blenkiron, P. and Jacobsen, C. Investigation of lipid oxidation in high- and low-lipid-containing topical skin formulations.
Journal of the American Oil Chemists' Society (accepted August 2017)

Paper II

Thomsen, B.R., Hyldig, G., Taylor, R., Blenkiron, P. and Jacobsen, C. Odour detection threshold determination of volatile compounds in topical skin formulations.
European Journal of Lipid Science and Technology (accepted December 2017)

Paper III

Thomsen, B.R., Taylor, R., Madsen, R., Hyldig, G., Blenkiron, P. and Jacobsen, C. Investigation of lipid oxidation in the raw materials of a topical skin formulation: A topical skin formulation containing a high lipid content.
Journal of the American Oil Chemists' Society (accepted November 2017)

Paper IV

Thomsen, B.R., Taylor, R., Hyldig, G., Blenkiron, P. and Jacobsen, C. Investigation of lipid oxidation and degradation products in raw materials responsible for the presence and development of volatile compounds during storage: Low fat topical skin care formulations
Journal of the American Oil Chemists' Society (submitted December 2017)

Paper V

Thomsen, B.R., Taylor, R., Hermund, D.B., Sørensen, A.-D.M., Heung, S., Hyldig, G., Blenkiron, P. and Jacobsen, C. Prediction of long-term oxidative stability in topical skin formulation using various initiators.
Journal of the American Oil Chemists' Society (submitted December 2017)

Paper VI

Poyato, C., **Thomsen, B.R.**, Hermund, D.B., Ansorena, D., Astiasarán, I., Jónsdóttir, R., Kristinsson, H.G. and Jacobsen, C. Antioxidant effect of water and acetone extracts of Fucus vesiculosus on oxidative stability of skin care emulsions.
European Journal of Lipid Science and Technology 2017, *119*, 1600072(1-11).

Other papers and contributions

Paper VII

Hermund, D.B., Heung, S.Y., **Thomsen, B.R.**, Akoh, C.C. and Jacobsen, C. Application of active ingredients derived from brown algae *Fucus vesiculosus* for skin care products.
In preparation.

Paper VIII

Thomsen, B.R., Yesiltas, B. Sørensen, A-D.M. Hermund, D.B., Glastrup, J. and Jacobsen, C. Comparison of three methods for extraction of volatile lipid oxidation products from food matrices for GC–MS analysis.
Journal of the American Oil Chemists' Society 2016, *93*, 929–942.

Paper IX

Thomsen, B.R., Griinari, M. and Jacobsen, C. Improving oxidative stability of liquid fish oil supplements for pets.
European Journal of Lipid Science and Technology (accepted April 2017)

List of Abbreviations

AAPH:	2,2′-Azobis(2-methylpropionamidine) dihydrochloride
AE:	*Fucus vesiculosus* acetone extract
AIBN:	Azobisisobutyronitrile
AMVN:	2,2'-Azobis(2,4-dimethylvaleronitrile)
D (0.9):	Droplet volume that 90% of the droplets were smaller than
ΔE:	Euclidean distance value
DHS:	Dynamic head space collection
DOD:	Description of difference
DTU FOOD:	National Food Institute at the Technical University of Denmark
EDTA:	Ethylenediaminetetraacetic acid
EE:	*Fucus vesiculosus* ethanol extract
FA:	Fatty acid
GC-MS:	Gas chromatography–mass spectrometry
GSK:	Glaxo Smith Kline
High lipid content	Topical skin formulations with lipid content >15 %
HPLC:	High-performance liquid chromatography
Low lipid content:	Topical skin formulations with lipid content <15 %
NMR:	Nuclear Magnetic Resonance
ODT:	Odour detection threshold
O/W:	Oil-in-water
PA:	Phosphatidic acid
PC:	Phosphatidylcholine
PE:	Phosphatidylethanolamine
PI:	Phosphatidylinositol
PS:	Phosphatidylserine
PV:	Peroxide value
REF:	Reference sample
SPME:	Solid-phase microextraction
TBARS:	Thiobarbituric acid reactive substances
TDU/DHS:	Automatic DHS
UV:	Ultraviolet
UV benzoate:	Hexyl 2-(1-(diethylaminohydroxyphenyl)methanoyl)benzoate
UV cinnamate:	Isoamyl p-methoxycinnamate
UV triazine:	Bis-ethylhexyloxyphenol methoxyphenyl triazine
WE:	*Fucus vesiculosus* water extract
W/O:	Water-in-oil
W/O/W:	Water-in-oil-in-water

Chapter 1: Introduction and hypotheses

1.1 Background for the PhD study and collaboration with GlaxoSmithKline

In the 1960s, western societies experienced significant economic growth, leading to increasing household incomes. This substantially increased income and the youth revolution that characterised this decade influenced consumer product development, as consumer goods companies were quick to accommodate consumers' demands. As a result of the increasing affluence, women began to spend more money on personal care products, including skincare products. The first skincare products that entered the market in the 1960s and 1970s were thin "vanishing day creams" and thick "greasy night creams" containing "magic" ingredients (Elliott, 1984).

Traditionally, lipids were primarily used in skincare products to give the products body and feel. Today, skincare products are functional products formulated for specific target groups with different needs, e.g. to moisturise dry skin and reduce redness. These functional products contain multiple ingredients serving various purposes in the formulation such as spreading, moisturising, cleaning or treating damaged skin. Most of the lipids added are saturated, which is preferred to polyunsaturated lipids to make the skincare products less vulnerable to lipid oxidation (Dederen *et al*., 2012; Singh, 2000; Muggli, 2005; Nicolaou, 2013). Recently, unsaturated lipids have attracted increasing interest because they have been scientifically proven by several researchers to improve skin appearance and function to a larger extent than saturated lipids. Therefore, formulators wish to incorporate unsaturated lipids in skincare products. However, incorporating unsaturated lipids may lead to challenges with maintaining acceptable oxidative stability, because unlike saturated lipids, unsaturated lipids are more susceptible to lipid oxidation (Muggli, 2005; Nicolaou, 2013).

Lipid oxidation is a well-known challenge in food emulsions where numerous approaches to protect the unsaturated lipids have been explored (Let, 2007; Sørensen, 2010; Horn, 2012; Lu, 2013; Cui *et al*., 2016; Liu *et al*., 2016). Lipid oxidation can result in volatile oxidation products. These have been reported to affect product odour in concentrations below 1 ng/g for some volatile compounds in water, e.g. octanal (Buttery, 1988). This off-odour can lead to consumer rejection of the skincare product. In order to prevent economic losses for skincare product manufacturers, action must be taken to prevent lipid oxidation and thereby prevent these off-odours from deteriorating the product quality. When this PhD project was initiated,

an in-depth investigation of lipid oxidation in skincare products from raw materials to formulated product had not been published. Therefore, this PhD project was initiated as a collaboration project between Glaxo Smith Kline (GSK) and the National Food Institute at the Technical University of Denmark (DTU FOOD) on investigating lipid oxidation in skincare products. To comply with GSK terminology the term topical skin formulation will be used instead of skincare product.

1.2 Objectives and hypotheses

The objective of the project is to investigate lipid oxidation with special focus on volatiles in selected topical skin formulations. Earlier studies of topical skin formulations performed at DTU FOOD have shown that previously reported lipid oxidation products in simple emulsions as well as in food emulsions were present in the investigated topical skin formulations. However, other volatile compounds were also found, but the origin of these compounds was not determined.

Volatile oxidation products can result in the development of off-odours. Several studies have determined odour threshold values for the odour-affecting oxidation products and for volatile compounds mostly in water. Compared with odour threshold values determined in water, the multiple ingredients in topical skin formulations may retain volatile compounds and/or mask odour changes. Therefore, the odour threshold values need to be determined in topical skin formulations.

One of the most surprising volatile compounds found in earlier studies at DTU Food was nitrile-containing compounds such as butane nitrile. The origin of these compounds was not known when this thesis work was initiated; however, some topical skin formulations contain raw materials that have amino-group(s) such as ceramide 3, which may be involved in the development of nitrile-containing compounds. Based on the data obtained from previously performed analyses, it seems that nitrile volatiles occur in the topical skin formulations under storage. Butane nitrile has been described in the literature as causing an off-odour in the product.

Lipid oxidation can be decreased by adding antioxidants (AO) because these are able to terminate lipid oxidation. Traditionally, synthetic AOs have been used in topical skin formulations. Though a new “clean label” trend has increased consumer demand for natural AOs. However, the addition of natural antioxidant extracts may introduce some impurities.

Therefore, the effect on both physical and oxidative stability must be evaluated when introducing natural extracts. One source of various AO is extracts derived from brown alga *Fucus vesiculosus*. These extracts have been reported to contain a broad variety of bioactive compounds with antioxidative effects, such as pigments, sulphated polysaccharides, proteins and polyphenols (Farvin and Jacobsen, 2013; Holdt and Kraan, 2011; Hermund *et al*., 2015).

Currently, a storage experiment for up to six months is needed to determine oxidative stability. Developing a method for fast prediction of oxidative stability is desired since this is a bottleneck in product development. Ideally, acceleration methods can be used to reveal unstable formulations faster. However, the studies in which the acceleration methods are used mainly focus on accelerating oxidation and not on testing the ability to reveal long-term product stability fast. Several potential strategies to accelerate lipid oxidation can be used. Amongst the most efficient strategies in emulsions are four acceleration approaches: a water-soluble radical, a lipid-soluble radical and two initiation systems including iron and a reduced agent to regenerate iron (Baron *et al*., 2006; Mosca *et al*., 2010; Matsumuraa *et al*., 2003):

- 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH),
- 2,2′-azobis(2,4- dimethyl) valeronitrile (AMVN),
- $FeCl_3$ and ascorbic acid,
- $FeCl_2$ and H_2O_2

To address all these elements, the thesis is divided into the following sub-parts with a view to:

1. Identifying markers for lipid oxidation products that can be applied in various topical skin formulations both with a high and low lipid content.
2. Determining odour detection threshold (ODT) values and describing selected volatile compounds in topical skin formulations.
3. Evaluating oxidative stability of raw material(s) and identifying reactions that may lead to the formation of the volatile compounds.
4. Determining the effect on physical and oxidative stability of adding *Fucus vesiculosus* extract.
5. Assessing the ability of the four acceleration approaches to accelerate lipid oxidation in topical skin formulations and evaluating the ability of these approaches to reveal oxidative stability within one month.

Throughout this thesis, the following hypotheses were tested:

1. *One or two specific volatile oxidation products can serve as markers for lipid oxidation in various topical skin formulations.*
2. *Odour threshold values in skin products are significantly higher in topical skin formulations than the reported values in water.*
3. *The nitrile-containing volatile compounds occur due to a reaction between lipid oxidation products and amino groups present in the topical skin formulation.*
4. *Volatile compounds both present initially and generated in topical skin formulations can be linked to raw material(s).*
5. *The oxidative stability can be increased by adding a Fucus vesiculosus extract.*
6. *A formulation without AOs can be differentiated from the original formulation with AOs after one month of storage when an accelerator is added.*

The hypotheses gave rise to the following specific objectives to

1. Identify potential markers and evaluate the usability of these for both the development of primary and secondary oxidation products in topical skin formulations under various storage conditions
2. Determine ODT values in both topical skin formulations with a high and low lipid content
3. Describe odour changes that occur due to formation of volatile compounds in both topical skin formulations with a high and with a low lipid content
4. Screen for volatile compounds initially present in raw materials that appear in accelerated storage
5. Develop an acceleration approach that makes it possible to distinguish formulations with and without AOs after one month´s storage.

Chapter 2: Topical skin formulations – a complex emulsion system

This chapter offers an introduction to emulsions, topical skin formulations and the term quality used in this thesis, with special focus on ingredients and purpose.

2.1 Definition of emulsions

An emulsion is a system consisting of "*a dispersion of droplets of one liquid in another liquid with which it is incompletely miscible*" (McClemements, 1998). Due to the immiscibility of the liquids, disrupting forces must be introduced to produce the emulsion by application of pressure or mixing of the solution. There are two types of simple emulsions: oil-in-water (O/W) and water-in-oil (W/O). Whether a simple emulsion is an O/W or W/O depends on the amount of both phases and the type of emulsifier applied. Most topical skin formulations are complex O/W emulsion systems containing multiple ingredients in the three phases: oil, water and interface (Figure 1).

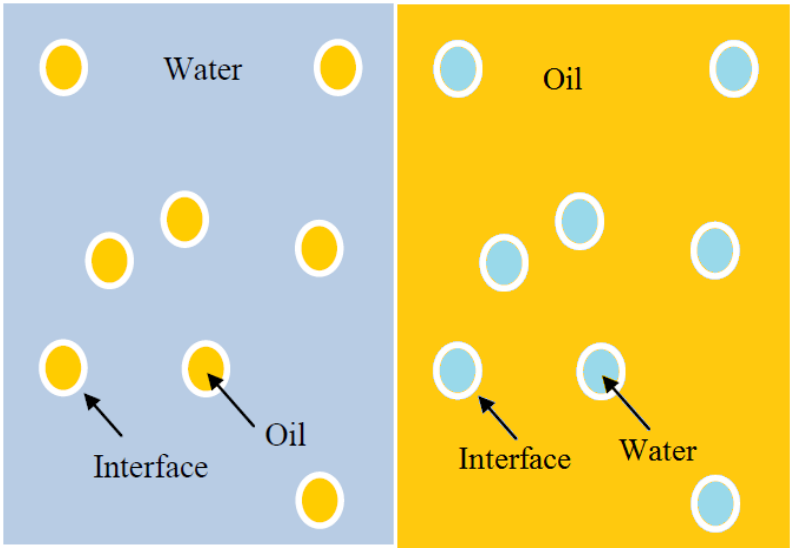


Figure 1. The location of three phases in an O/W and W/O emulsion: oil, water and interface.

In an emulsion solely containing the immiscible liquids, phases separate as water molecules interact with strong hydrogen bonds, however not with oil. Phase separation occurs because the pressure or mixing forces applied to produce an emulsion does not ensure the physical stability of the emulsion. The period when physical stability is maintained depends on the interaction between the two phases in the interface. Therefore, these interactions must be minimised to increase physical stability. The physical stability of the emulsion depends on the emulsifiers. Emulsifiers are surface-active substances with amphiphilic properties, which are present at the interface and prevent droplets from aggregating both under emulsion

formation and storage. An example of an emulsifier is lecithin. Emulsifiers are not thermostable because the hydrophilic head becomes dehydrated with increasing temperature. When the temperature reaches the cloud point, the instability aggregation occurs (McClements, 2005). Emulsion instabilities will be addressed later in this section. In addition to emulsifier, texture modifiers can also be added to improve physical stability even further. Texture modifiers increase the thickness of the interface, thereby decreasing the interactions between the phases. In addition, texture modifiers may also increase the viscosity of the water phase and thereby reduce mobility of the droplets.

Above, a simple emulsion system with few ingredients is described; however, an emulsion can also be a complex emulsion with several ingredients. In a complex emulsion, the complexity of the emulsion is increased further e.g. by the oil droplet containing relatively smaller dispersed droplets. In this way, a water-in-oil-in-water (W/O/W) emulsion is obtained (Figure 2).

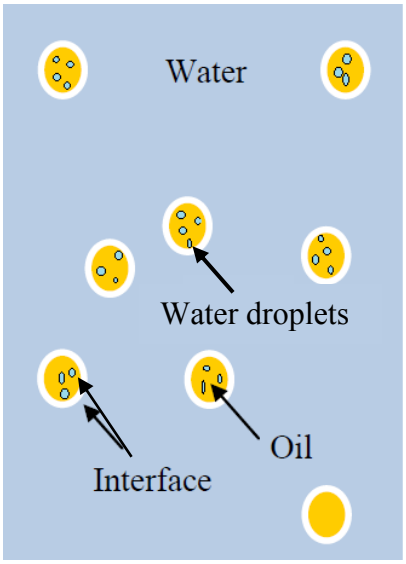


Figure 2. A complex W/O/W emulsion, the location of three phases in a W/O/W emulsion: oil, water and interfaces.

In most cosmetic W/O/W emulsions, complex emulsion systems are used to prevent degradation of a vulnerable ingredient and/or control the release of it (Sonchal *et al*., 2008). Even though application of complex emulsions increases protection of vulnerable ingredients, it also complicates explaining, predicting and understanding the physical and oxidative stability of the cosmetic formulations. Hence, the increased complexity of the emulsion is related to increased interfacial area, the increased interfacial area increases interaction between the phases. As mentioned earlier, the physical stability depends on the interaction at the interface between the phases.

The stability of the emulsion may be challenged by several factors such as gravity, interfacial interactions connecting the droplets and phase inversion (Figure 3). Gravity can separate the emulsion according to the droplet's density to form creaming or sedimentation instability. The connection between the droplets may be related to proteins in the emulsion that can form disulphide bonds; however the droplets´ shape is maintained.

When interfacial interactions connect the droplets, the aggregation leads to flocculation and coalescence. If an emulsion contains too little emulsifier or one which is not able to stabilise the emulsion, droplets may gather to form fewer and larger droplets. This instability is called coalescence. If the droplets are collected together and still maintain their separate droplet form, flocculation has occurred. The last type of instability, phase inversion, arises when a W/O emulsion is transformed into an O/W emulsion or the other way around.

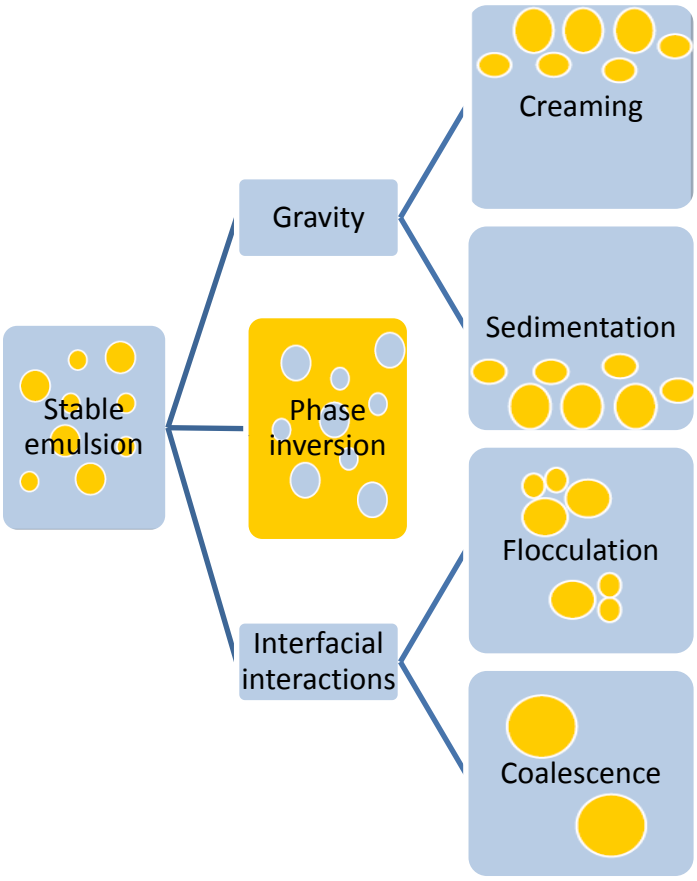


Figure 3. Mechanisms of physical instabilities in an O/W emulsion.

2.2 Definition of topical skin formulations

Topical skin formulations are usually complex O/W, W/O or W/O/W emulsions as described in previous sections. Several types of products are within this category, and even more will most likely be developed in the future.

A variety of definitions of topical skin formulations exists; the regulating authorities do not define topical skin formulations *per se*, moreover cosmetics and drugs are defined based on intended use.

In the EU, the Cosmetics Regulation defines cosmetic products as *"any substance or mixture intended to be placed in contact with the external parts of the human body ... or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance, protecting them, keeping them in good condition or correcting body odours"* (EU Regulation, 2009).

In the US, the Federal Food, Drug and Cosmetic Act complicates the definition further by stating that cosmetic products that cure, mitigate, treat, or prevent diseases are also defined as a drug. In this way, some products are both defined as cosmetics and drugs. Such topical skin formulations must comply with the regulation for both cosmetics and drugs (FD&C Act, 2013).

In this thesis, I define a topical skin formulation as a complex emulsion intended for cleaning, maintaining and moisturising the body and/or face.

Generally, topical skin formulations contain five to seven elements: water and moisturisers, rheology modifiers, perfume, preservatives, special additives, oils and waxes, and emulsifiers (Figure 4).

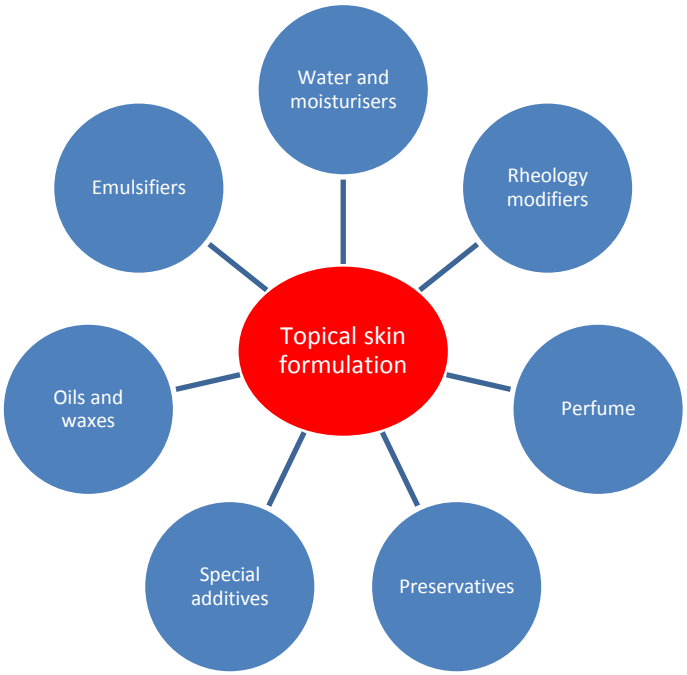


Figure 4. The seven elements used in topical skin formulations.

2.2.1 Water and moisturisers: Water and moisturisers can be divided into three categories: occlusives, humectants and hydrocolloids are raw materials used to keep the product moist and moisturising the skin. The moisturisation is obtained through two main mechanisms: the ingredients provide moisturisation by blocking the water evaporation, and by bringing moisture to the skin itself (Draelos, 2011; Wiechers and Barlow, 1999; Rawlings *et al*. 1994). Moisturisers can be ingredients such as glycerine, sorbitol, propylene glycol, various sugars, acetamide MEA and obviously water.

2.2.2 Rheology modifiers: Rheology modifiers, also known as texture modifiers, are used for several purposes: obtaining and maintaining the product texture and viscosity, however also for gaining the desired rheological properties such as wetting and spreading of the cream when applied on the skin. The product texture must have shear-thinning properties when stress (shear rate) is increased because otherwise the consumer cannot to spread the product on the skin. A product falling to meet spreadability requirements may distribute irregularly on the skin, and therefore consumer acceptance may suffer from this weakness (Montenegro, 2015; Gaspar and Maia Campos, 2003; Bekker *et al*., 2013; Pennick *et al*., 2010). Furthermore, recent studies of creams containing polymers showed that the droplet size in O/W emulsions decreases with increasing polymer concentration (Estanqueiro *et al*., 2016). An increased interfacial area between the water and oil phases is thereby obtained.

Furthermore, polymers increase the viscosity proportional to the concentration of polymer added. Modifiers include ingredients such as isostearyl isostearate, xanthan gum, carbomers, acrylates copolymers and numerous others.

2.2.3 Perfume: Perfumes, essential oils and fragrances are used by some manufacturers to obtain a specific product odour. The topical skin formulations used in this PhD project are perfume free. I have therefore decided not to elaborate further on this ingredient.

2.2.4 Preservatives: When water is present, the product will be prone to microbial spoilage. A preservative system is necessary for preventing microbial growth in the product during manufacturing, on the shelf and from product contamination by the end-consumer during use. However, other ingredients, e.g. AOs with chelating properties such as ethylenediaminetetraacetic acid (EDTA) and factors such as pH and reduced water activity are co-preservatives. Since water is needed for microbial growth, products containing ingredient(s) that inhibit access to water also inhibit microbial growth making the product self-preserving, e.g. in the form of microemulsions. In addition to the microbial preserving effect, some preservatives also have antioxidative effects (Al-Adham *et al*., 2000; Lundov *et al*., 2009; Patrone *et al*., 2010). Preservative ingredients can be parabens, formaldehyde, phenoxyethanol, organic acids etc.

2.2.5 Special additives: A special additive is an ingredient included to obtain a specific property of the topical skin formulation. Examples of special additives are ultraviolet (UV) filters for sun protection, AOs, vitamins, botanic extract and peptides with specific purposes e.g. anti-redness (Draelos, 2011). Most special additives are added to support or enhance the skin´s natural defence mechanism. Addition of AOs reduces the extent of free radical reactions occurring on the skin. In topical skin formulations, the reducing effect on free radical reactions is called “antiaging effect”.
The AOs also protect the raw materials and final products from lipid oxidation. Oils and waxes are widely used in topical skin formulations. These additives contain unsaturated fatty acids that are easily oxidised. The appearance of oxidation products may lead to changes in sensory and physiochemical properties (Briganti and Ricardo, 2003; Stäb, *et al*., 2000).
UV filters are often added to topical skin formulation such as day cream; this is done as sun exposure can lead to skin cancer. For example, as skin cancer is now the most common form

of cancer in the UK, UV filters have become vital raw material for sun protection (British skin foundation, 2017).

2.2.6 Oils and waxes: Oils and waxes are used to give body and feel to the product, called skin sensation. In addition, oils and waxes improve the delivery system of hydrophobicically vulnerable ingredients such as special additives. Frequently used oils and waxes are hydrocarbons, esters, silicone fluids and vegetable oils (Dederen *et al*., 2012; Singh, 2000). Vegetable oils are often applied as fatty base in topical skin formulations. Traditionally, oils and waxes with a low degree of unsaturation have been used because they are less prone to oxidation. Currently, the interest in oils containing polyunsaturated fatty acids is increasing. This increased interest is related to improved skin appearance and function obtained by topical skin formulations containing these unsaturated fatty acids. However, the oils and fats that have a high degree of unsaturation also challenge maintaining oxidative stability because unsaturated fatty acids are susceptible to lipid oxidation (Muggli, 2005; Nicolaou, 2013). Lipid oxidation will be described in more detail in Chapter three.

2.2.7 Emulsifiers: Emulsifiers are required to increase physical stability, as described earlier. Emulsifiers can be anionic, cationic and non-ionic/zwitterion. Emulsifiers' ability to stabilise an emulsion is highly depending on its location. For O/W emulsions mainly anionic and non-ionic emulsifiers are used.
One emulsifier often used in topical skin formulations is lecithin. Lecithin comprises phospholipids, which are amphiphilic because these are composed of a polar and non-polar part. In phospholipids, polar part is the phosphate head and glycerol. The non-polar part is two fatty acid (FA) chains. The amphiphilic properties of phospholipids decrease the interaction between the oil and water phases and hence increase stability. Several types of glycerophospholipids exist: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidic acid (PA) (Wang, 2008; Gbogouri *et al*., 2006).

2.3 Factors important for the quality of topical skin formulations

"Quality" is a commonly used term; however the term has several different meanings. In topical skin formulations, an important quality parameter is product odour. The product odour is known to impact consumer acceptance, and thereby the willingness to re-purchase the product (Moeglin, 2015; Woitalla, 2014). The sensory attributes of topical skin formulations

are vital to consumers (Patravale and Mandawgade, 2008). However, quality is also related to meeting the technical purpose of the product, e.g. does the cleansing formulation provide clean skin or does the cream provide moisture to the skin. Furthermore, the product must remain stable its entire shelf life. Several product characteristics are thus related to quality:

- Product appearance – e.g. packaging, colour and odour
- Product association – e.g. does the product appeal to the consumers´ desires
- Spreading and wetting – e.g. absorption and skin feel (skin sensation)
- Product stability during use – e.g. physical and odour stability

This study focuses on product odour and maintaining the same product odour the entire product shelf life. Therefore, quality is defined as odour stability. The odour stability of the final product is highly related to the quality of the raw materials, manufacturing and storage. Therefore the solution to a quality problem in the topical skin formulation most often must be found in the three above-mentioned quality parameters: ***Quality - what you put in is what you get out!***

Chapter 3: Lipid oxidation in emulsions

Topical skin formulations contain oils and waxes that may be susceptible to lipid oxidation. Lipid oxidation leads to formation of primary and secondary oxidation products. Secondary volatile oxidation products can affect the product odour which is important for product quality. In this chapter, lipid autoxidation and photooxidation are described in detail. Furthermore, the effect on lipid oxidation of various product ingredients is discussed. Lastly, lipid oxidation is put into a broad perspective.

3.1 General autoxidation chemistry and mechanism

Lipid autoxidation occurs through three stages: Initiation, Propagation and Termination (Figure 5).

Initiation:

The reaction between oxygen and unsaturated FA is spin forbidden; therefore an initiator is necessary to remove a hydrogen to obtain opposite spin directions. The initiator acts as a hydrogen absorber to produce an alkyl radical (Figure 5, Reaction 1, the reaction is not balanced). Initiators can be metal ions such as Fe^{2+}, heat, and other radicals present in raw materials or produced during manufacturing. These reactions are slow because these are dependent on the initiator (Schaich, 2005; Frankel, 1998).

Propagation:

An alkyl radical is an unstable intermediate, and it immediately reacts with oxygen to form a lipid peroxyl radical (Figure 5, Reaction 2). The lipid peroxyl radical reacts with the next FA to produce the primary oxidation products, lipid hydroperoxide, and a new lipid radical (Figure 5, Reaction 3). The radical reacts with new fatty acids, and propagation can be repeated (Figure 5, Reaction 4). The hydroperoxide can decompose in the presence of metals, heat, light and radicals (Gebicki and Bielski, 1981; Howard and Ingold, 1967). The reaction catalysed by metal ions forms a lipid alkoxyl or peroxyl radical and an acid or base. Thus, the reaction, being dependent on a catalyst is rate limiting. A decomposition catalysed by heat or light results in two radicals, lipid alkoxyl radical and hydroxyl radical. Hence, lipid oxidation propagation is accelerated even further (Figure 5, Reactions 5-7) (Ingold, 1969; Schaich and Borg, 1988; Schaich, 1992). The propagation reactions of unsaturated fatty acids to hydroperoxides proceed until a termination reaction occurs.

Termination;

Two radicals can react to produce a non-radical product which stops the chain reaction. Several reactions can terminate the chain reaction (Figure 5, Reactions 8) (Frankel, 1998).

Classic Free Radical Chain Reaction

Initiation

$L_1H \rightarrow L^*$ (1)

Propagation

$L_1{}^* + O_2 \rightarrow L_1OO^*$ (2)

$L_1OO^* + L_2H \rightarrow L_1OOH + L_2^*$ (3)

$L_2OO^* + L_3H \rightarrow L_2OOH + L_3^*$ etc. (4)

Free radical chain branching *(initiation of new chains)*

$LOOH \rightarrow LO^* + OH^- \ (reducing\ metals)$ (5)

$LOOH \rightarrow LOO^* + H^+ \ (oxidizing\ metals)$ (6)

$LOOH \rightarrow LO^* + OH^* \ (heat\ and\ uv)$ (7)

Termination *(formation of non-radical products)*

$2\ radicals(L^*, LO^*, LOO^*) \rightarrow Polymer\ and\ non-radical\ products$ (8)

Figure 5. An overview of autoxidation: mechanism of initiation, propagation and termination. Abbreviations: radical-alkyl (L·), lipid alkoxyl radical(LO·), peroxyl radical (LOO·), lipid hydroperoxide(LOOH) and hydroxyl radical (OH·). A modification of Schaich (2005).

Autoxidation is a complex series of reactions leading to various oxidation products which may decompose even further. To exemplify the reactions occuring, lipid oxidation reactions for linoleic acid to volatile secondary oxidation products are described. Starting from C18:2 in the initiation stage, the hydrogen is removed by an initiator allowing two different positions of hydroperoxide group. In the propagation stage, electron resonans favours hydroperoxide located in external positions (Figure 6) (Tallman *et al*., 2001).

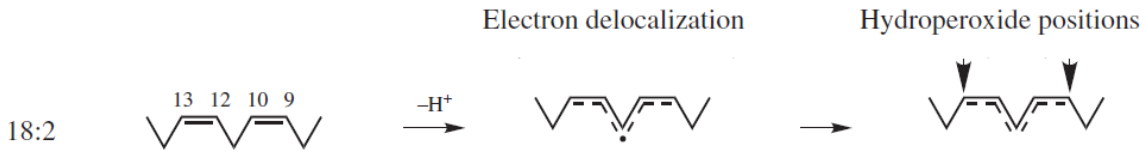


Figure 6. Formation of hydroperoxide on C18:2. Doubly allylic H abstraction sites, electron resonance and positions and hydroperoxide group. A modification of Schaich (2005).

The major product is 9-hydroperoxyoctadecadienoic acid and 13-hydroperoxyoctadecadienoic acid, these two hydroperoxides are relatively unstable, and they can decompose further into secondary oxidation products (Tallman *et al*., 2001). The decomposition occurs through hydrolytic cleavage followed by α- or β-scission (Figure 7).

The α- or β-scission refer to cleavage side of the alkoxyl radical, where α-scission is cleavage on the acid side. Multiple factors affect whether a α- or β-scission occurs. This has yet to be fully understood. However, a combination of both scissions is often detected. The scission leads to a mixture of carbonyl products and free radicals, most often aldehydes, alkanes, and oxo-esters. The scission of the carbonyls continues until stable saturated products are reached (Frankel, 1987; Schaich, 2005). Scission results in a complex mixture which includes volatile secondary oxidation product associated with rancidity (these will be described in the section; "Volatile secondary oxidation products").

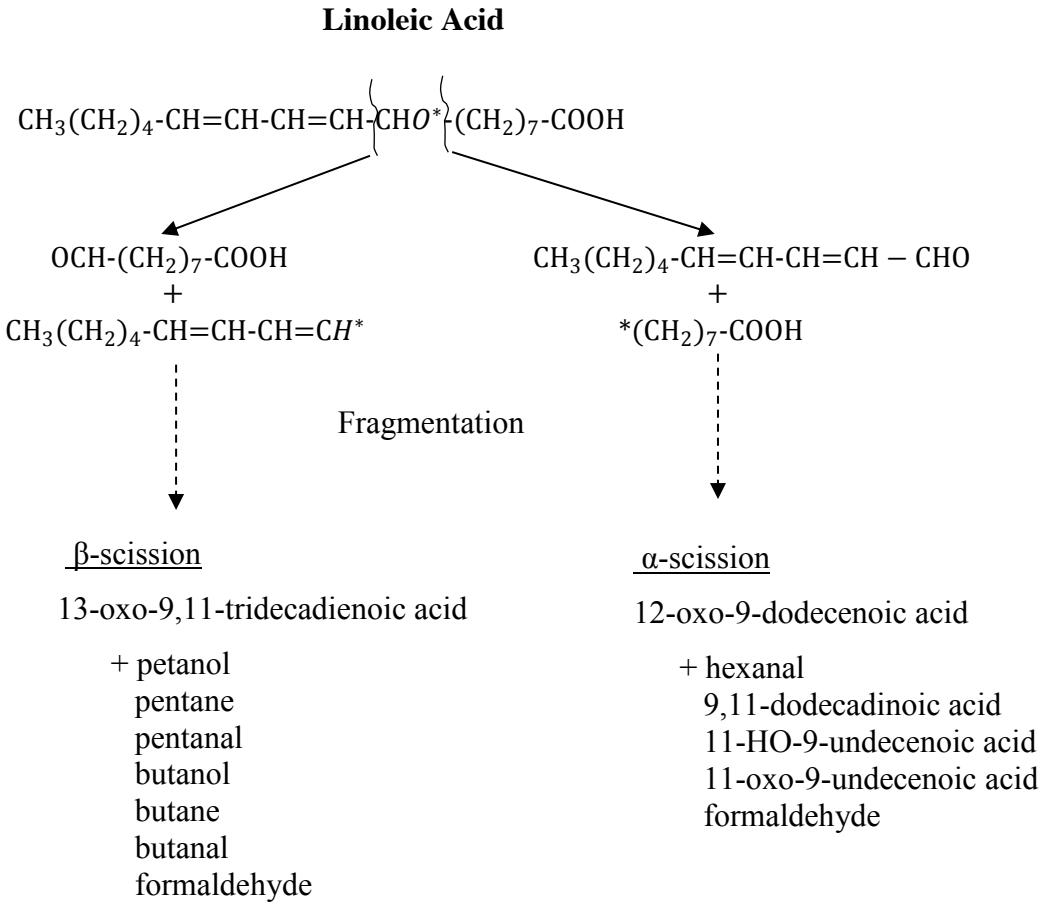


Figure 7. Scission decomposition of 9-hydroperoxyoctadecadienoic acid and 13-hydroperoxyoctadecadienoic acid from linoleic acid occurring in the propagation stage. Parentheses indicate unstable intermediates; square brackets denote products from secondary scissions. A modification of Schaich (2005).

The scission may also lead to termination because the formation of furan ends propagation. As in the propagation stage, a complex mixture of scission products is produced. The formation of volatile secondary oxidation products such as 2-pentyl furan terminates the radicals' propagation when the double bond in the furan ring is formed instead of a radical (Figure 8) (Frankel, 1987).

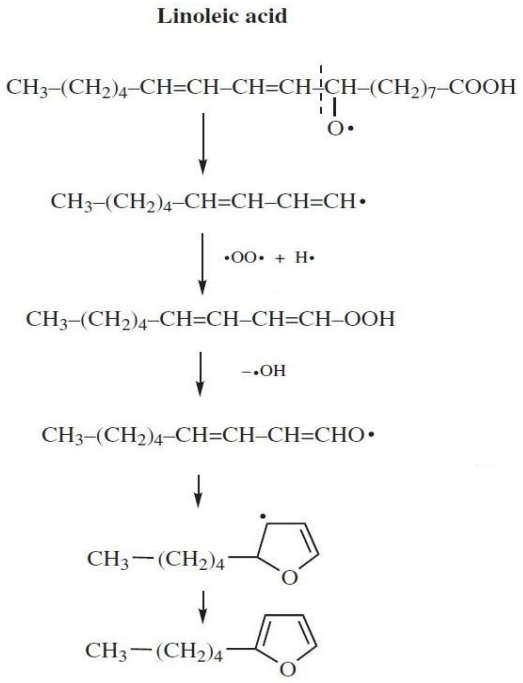


Figure 8. Terminating secondary scissions of intermediate products that occurred from linoleic acid. A modification of Schaich (2005).

Lipid oxidation of linoleic acid leads to a complex mixture of various oxidation products. In addition to the terminating secondary scissions, a hydrogen donating AO such as α-tocopherol can also terminate the propagation (this will be described in the section; "Factors affecting lipid oxidation").

3.2 Photooxidation chemistry and mechanism

Photooxidation is lipid oxidation initiated by light; this initiation can be indirect and direct. Direct light exposure means direct exposure to sunlight or fluorescence without protection from glass or plastic packaging. In this PhD study, the topical skin formulations were stored in bottles, and therefore not exposed to direct light having wavelengths below 220 nm. Hence, as the topical skin formulations were only exposed to indirect light, only indirect photooxidation will be elaborated. Indirect light at wavelengths above 220 nm cannot produce radicals directly (Frankel, 1998). However, the quantum energy of visible light is absorbed by molecules that can activate the photooxidation. The quantum energy can be transformed by two pathways. Both pathways provide the energy required to obtain opposite spin directions. For type one, the excited sensitiser transfers the excitation energy to absorb an electron or a hydrogen to form a free radical (Figure 9, Reaction A). Photooxidation type two occurs with approximately the same reaction rate as autoxidation. However type two is an oxygenation which occurs 1500 times faster. For type two, the excited sensitiser transfers

the excitation energy to oxygen and transfer triplet oxygen to singlet oxygen (Figure 9, Reaction B) (Rawls and Santen, 1970; Foote, 1968; Foote, 1991).

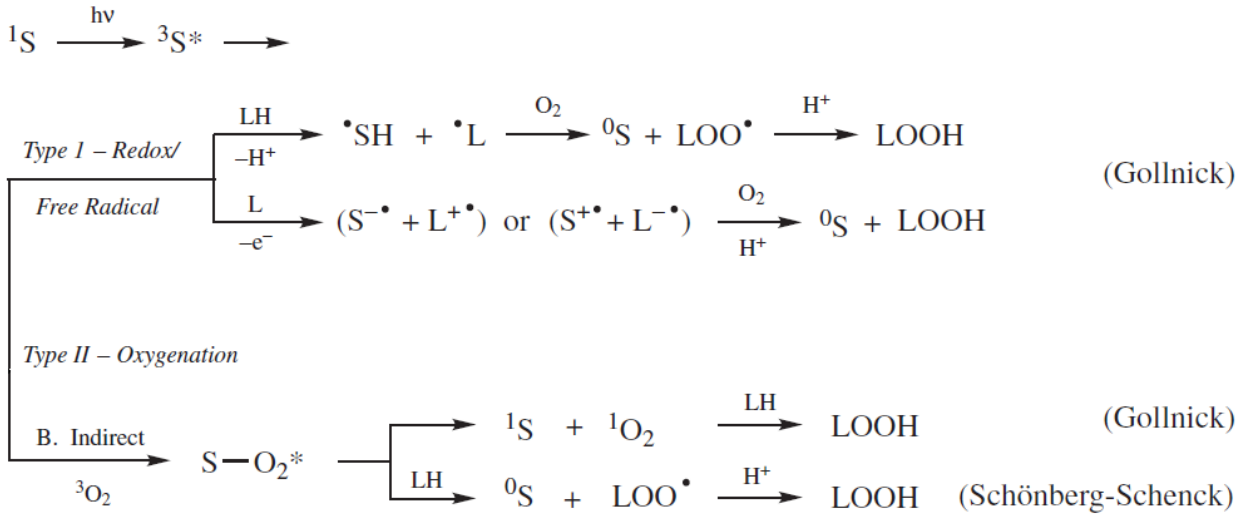


Figure 9. Triplet sensitiser directs the photooxidation by type one and type two initiations. A modification of Schaich (2005).

While singlet oxygen is not a radical itself, it produces hydroperoxides that are precursors for formation of radicals as shown in Figure 5. The reaction between singlet oxygen and unsaturated FA can occur by two mechanisms: attachment to a carbon having a double bond and abstraction of an allylic proton. In addition, in a reaction between singlet oxygen and conjugated double bonds a cyclic endoperoxide can be formed e.g. photooxidation of oxidised linoleic acid. The hydroperoxides produced under photo-activated oxidation decompose by the same mechanism described for hydroperoxides in the section 3.1 (Schaich, 2005).

3.3 Odour detection thresholds of volatile secondary oxidation products

The primary oxidation products, hydroperoxides, are colourless, odourless and flavourless. Therefore, only the volatile secondary oxidation products formed in lipid oxidation of unsaturated fatty acids can affect product odour. The volatile secondary oxidation products are widely studied in foods. The volatile secondary oxidation product can affect colour, odour and flavour even when present at extremely low levels (ng/g). Since this PhD study is about lipid oxidation in topical skin formulations, the flavour changes are not relevant. However, lipid oxidation products effect on product odour is highly important for the product quality. The volatile secondary oxidation products that often have been reported to affect product quality are aldehydes, ketones, acids and alcohols.

The concentration at which an effect on product odour is observed depends on the individual volatile compounds´ odour threshold value (Frankel, 1984; Frankel, 1998). The odour threshold value can be very low, even below 1 ng/g in water, which is below the detection limit of gas chromatography–mass spectrometry (GC-MS) analysis (Frankel, 1998). However, the odour threshold value for volatile compounds present in matrices other than water may be higher because they must be released from the matrix.
In complex emulsions, the volatile compounds can either solubilise or bind to lipids and compounds such as stabilising gums, polymers and carbohydrates. These interactions with the matrix increase are called matrix retaining of the volatile compounds. Matrix retaining increases the odour threshold values. The matrix retaining can occur through four types of interactions: 1) Covalent bonds can be formed between a ketone or aldehyde and an amino group (Le Thanh *et al*., 1992; McGorrin, 1996). 2) Hydrogen bond interaction can retain a polar volatile compound such as an alcohol with compound having the heteroatoms N, S and O (McGorrin, 1996; Solms *et al*., 1973). 3) The matrix retains the volatile compounds by most-probable binding site in hydrophobic pocket of lipid or non-polar pocket of the protein (McGorrin, 1996; Guichard, 2002). 4) Complexes can be formed between gums and aldehydes such as hexanal (McGorrin, 1996; Naknean and Meenune, 2010). Therefore, volatile compounds are expected to have higher odour threshold values in a skin product matrix vs. in a water matrix. In addition to retaining the volatile compounds, the base odour of the product may also have a masking effect, which further increases the odour threshold values.

3.4 Factors affecting lipid oxidation

The large complexity of skin products makes it very difficult to predict oxidative stability due to the large number of ingredients which each may affect oxidative stability. The ingredients' effects on oxidative stability can be prooxidative, antioxidative and systemic (e.g. steric hindrances, viscosity and pH). In addition, the location of the compound is highly important for its effect on lipid oxidation. Thus, lipid oxidation is initiated at the interface between water and oil and the compound must be located in the interface to have an impact on the initiation of lipid oxidation (Figure 10). In the following sections, the effect of the ingredient, droplet size and processing/storage conditions are discussed.

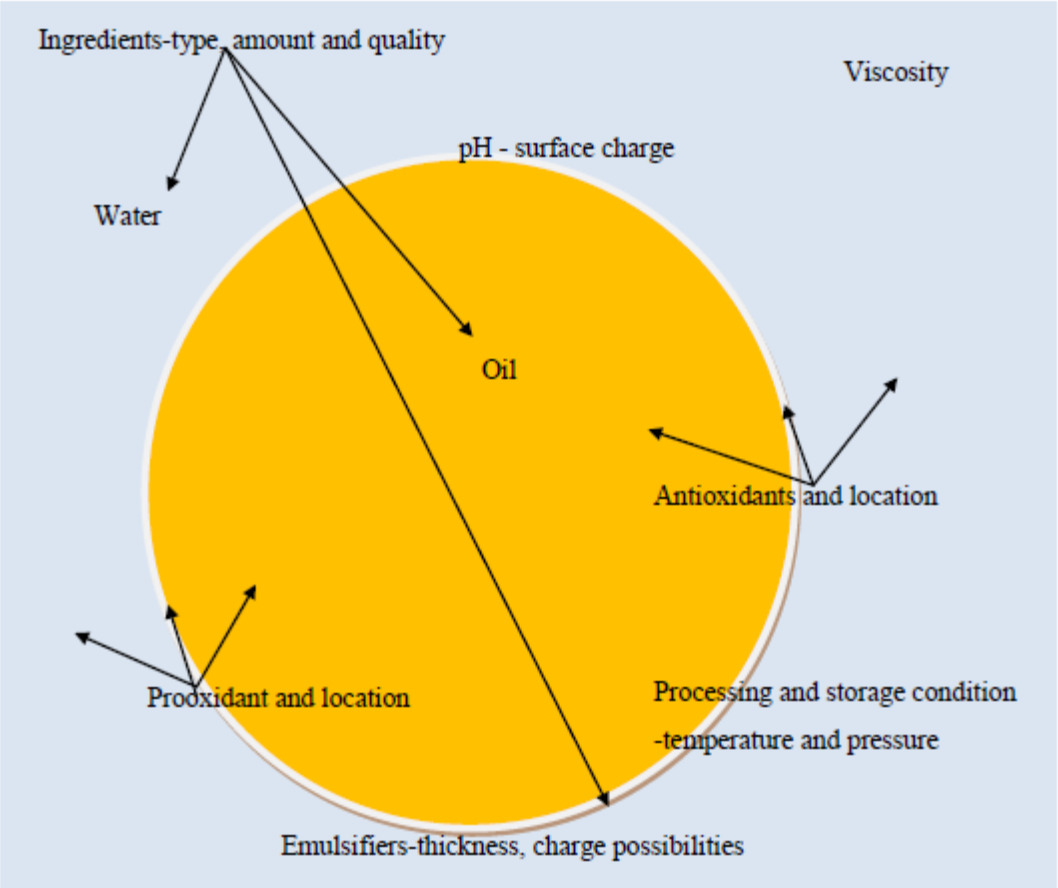


Figure 10. The factors that can affect the oxidative stability of the enriched product. A modification of Jacobsen and Nielsen (2007).

3.4.1 Ingredients

The fatty acids in oils and waxes can have different resistance to oxidation depending on their degree of unsaturation. Although the natural AO content in the oil can also increase the resistance to oxidation. For example, tocopherols are natural AOs which are often present in vegetable oils (Frankel, 1998). Furthermore, the oil concentration in the product can also have an impact on the oxidative stability. A study by Osborn and Akoh (2004) found that the oxidation stability of an emulsion having a low oil concentration resulted in more oxidation than emulsions having a high oil concentration.

Several studies have demonstrated that the quality of oils and waxes used for production of product significantly affected the oxidative stability. Poor quality oils and waxes may initiate and propagate lipid oxidation faster due to a high hydroperoxide concentration. In a study by Let *et al*., (2005a; 2005b), a product produced with a high-quality oil was possible to distinguish from one containing a poor-quality oil. However, another study found that the quality of the oil had less impact in yoghurt than in milk. These results indicate that products with high viscosity have higher oxidative resistance than those with low viscosity (Jacobsen

and Nielsen, 2007). Ingredients in topical skin formulations, such as the moisturisers and rheology modifiers, may contribute to increasing the thickness of the interface and the viscosity of the product (Estanqueiro *et al*., 2016). In this way, moisturisers and rheology modifiers may decrease the diffusion of prooxidants between the phases.

Since a topical skin formulation is stored for up to one year, an effective preservation system is needed to prevent microbial growth. In addition to spoiling the product and posing a health risk to the consumer, microbial growth is known to result in volatile compounds. These volatile compounds can also affect product odour (Kai *et al*., 2007). Preservatives, such as rosemary, oregano and mint essential oils, may also have an antioxidative effect (Patrone *et al*., 2010).

The ingredient group "special additives" may provide antioxidative effects; these will be elaborated in section 3.4.2. However, they may also contain impurities that can initiate lipid oxidation. Nevertheless no literature about this area has been found. The presence of prooxidants and AOs can either initiate or limit lipid oxidation. Prooxidants are often trace metals that can initiate oxidation (Farvin *et al*., 2010b; Frankel, 1998; Nielsen *et al*., 2009). The effect of prooxidants and AOs are highly dependent on their solubility, partitioning and diffusion between the oil, water and interface (Waraho *et al*., 2011). The partitioning and diffusion of prooxidants and oxygen are highly affected by the viscosity of the product because high viscosity can decrease the diffusion of oxygen and prooxidants between the three phases. If the prooxidants cannot reach the unsaturated FA lipid oxidation is inhibited or at least limited (Farvin *et al*., 2010a; Nielsen *et al*., 2009). In addition, the unsaturated FA can also be protected by a thick interface. A thick interface protects the unsaturated FA against oxidation. Amphiphilic polymers can unfold in the interface and increase the interfacial thickness. The unfolding of polymers is dependent on the pH. The pH can also affect the droplet charge, a positive charge can repulse transition metal ions such as Fe^{2+} and a negative charge can attract them. The location of transition metal ions can affect the extent of lipid oxidation, the closer location the more likely is initiation of lipid oxidation. The optimal pH is highly dependent on the ingredients added and the interactions between these (McClements, 2005; Berton-Carabin *et al*., 2014; Hu and Jacobsen, 2016).

3.4.2 Antioxidant

Most special additives are AOs added with the purpose of antiaging effect. In addition, the AOs can help limit lipid oxidation in the topical skin formulation itself. However, the location in three phases and concentration of the AO is crucial for promoting its antioxidative

effect. Thus, too high concentrations might result in prooxidative effects instead of antioxidative (Kamal-Eldin, 2006; Let *et al*., 2005b).

The AO resulting in the best protection has been suggested by several authors to be consistent with "the polar-paradox". The polar-paradox means that the polarity of AO is the opposite of the media. Consequently, lipophilic AOs are more efficient in O/W than in bulk oil, whereas the opposite was the case for the hydrophilic AOs (Frankel *et al*., 1994). Using this paradox seems fairly simple. However, selecting AOs for more complex emulsions cannot be done by solely applying the "polar-paradox" because numerous other factors can influence partitioning and diffusion of AOs (Pinedo *et al*., 2007).

Recently, the polar paradox has been expanded with the cut-off effect. The cut-off effect describes the effect of the chain length on the location and thereby effectiveness of AOs in the interface. A study by Laguerre *et al*. (2009) found a non-linear relationship between the AO capacity and the chain length in an emulsified medium. The AO capacity increases with increasing alkyl chain length until a threshold is reached at a C12-chain length (dodecyl). Chain lengths beyond the chain length of dodecyl lead to a drastic decrease in the AO effect of the chlorogenic acid esters.

The most often used AOs in topical skin formulations are vitamins such as tocopherols, carotenoids and ascorbic acid, and metal chelators such as EDTA and caprylhydroxamic acid (Patravale and Mandawgade, 2008; Draelos, 2011).

The Icelandic brown algae *Fucus vesiculosus* may be a natural source of AO compounds. The antioxidative properties of brown algae are related to the content of a broad variety of bioactive compounds with anti-oxidative effects, such as pigments, sulphated polysaccharides, proteins and polyphenols. The most important polyphenols in brown algae is phlorotannins, which are responsible for the antioxidative effect (Farvin and Jacobsen, 2013). Furthermore, AOs from brown algae have shown great potential in improving oxidative stability for fish-oil enriched mayonnaise and granola bars (Honold *et al*., 2015; Karadag *et al*., 2017)

3.4.3 Emulsifier

As mentioned earlier, lipid oxidation is initiated at the interface. Thus, an emulsifier is located at the interface to stabilise the emulsion's physical stability, however emulsifiers also have a key role in the oxidative stability of emulsions (Waraho *et al*., 2011; McClements, 2005). Emulsifiers are surface-active molecules with amphiphilic properties that reduce the surface tension.

The emulsifier lecithin (natural and synthetic) is a widely used emulsifier for emulsion systems, including topical skin formulations (Patravale and Mandawgade, 2008). Commercially available lecithin is phospholipids as described in section 2.2.7. Several other authors have reported increased oxidative and physical stability in salmon oil model systems, in dairy products generated by adding phospholipid. Several mechanisms have been suggested for antioxidative effects. In the following the two most common are outlined.

1) Some phospholipids are able to provide a positive charge of the oil droplet that can repel metal ions. The positive charge of the oil droplet depends on the pH of the product (Mei *et al*., 1998; Mei *et al*., 1999). PC has a net positive charge at low pH below the pK_a of the phosphate group (Carlsson, 2008).
2) Some phospholipids are able to regenerate AOs. For example, PE and PS can easily donate a hydrogen atom from the primary amino group to the alpha-tocopheroxyl radical (Figure 11), and can thereby regenerate alpha-tocopherol (Hildebrand, 1984; Doert *et al*., 2012). This synergy results in a higher oxidative stability than phospholipids or α-tocopherol were able to provide separately (Bandarra *et al*., 1999; Weng and Gordon, 1993).

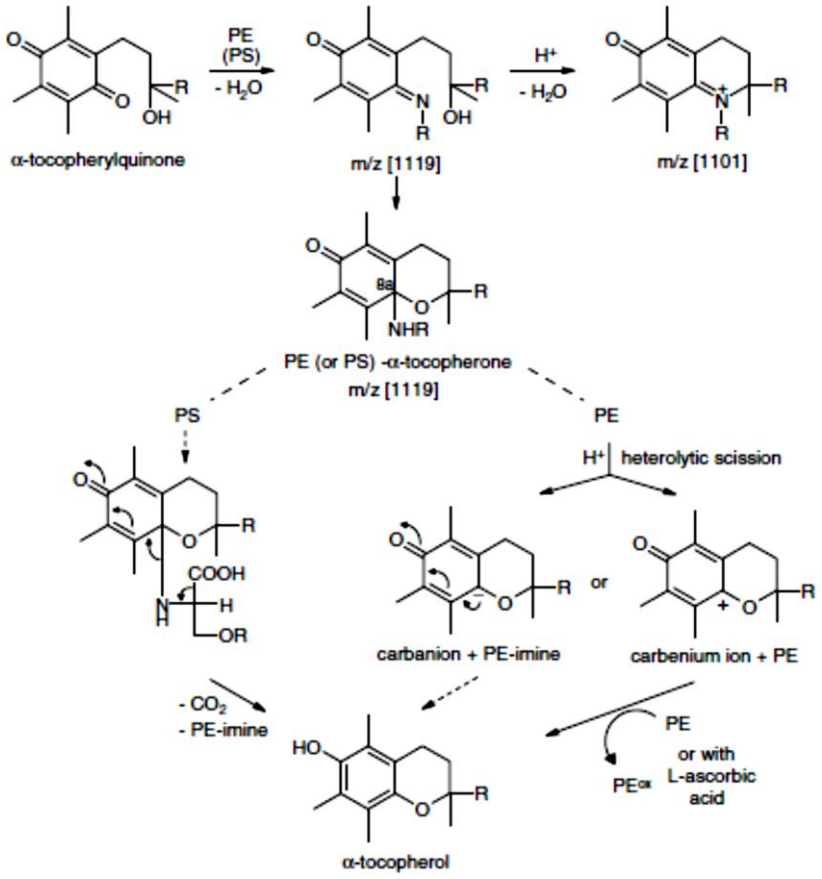


Figure 11. Suggested mechanism for regeneration of alpha-tocopherol by PE and PS. Adopted from Doert *et al*., 2012.

3.4.4 Droplet size

Since the droplet size directly affects interfacial area, it also affects lipid oxidation because initiation occurs at the interface. In theory, a small droplet size and consequently a larger interfacial area results in elevated oxidation risk. Despite the fact that several other factors also can affect the degree of lipid oxidation. Osborn and Akoh (2004) studied the effect of the droplet size in O/W emulsions containing sucrose FA ester or whey protein isolate as emulsifier, no clear effect was observed on the oxidative stability. Another study concluded the opposite than theory, namely, the smaller droplet, the higher oxidative stability of the fish oil enriched milk emulsion (Let *et al*., 2005a). However, the higher oxidative stability was related to the production conditions. During homogenisation to smaller droplets, high temperature and pressure were applied to the emulsion, which changed the composition of the interface. The high pressure and temperature resulted in more β-lactoglobulin and less casein in the interface (Sørensen *et al*., 2007). The optimal droplet size is highly product-dependent consequently the droplet size has a marginal influence on the oxidative stability alone (Waraho *et al*., 2011).

3.4.5 Processing and storage conditions

Before and during the production, the raw materials must be protected as much as possible to ensure a low initial content of oxidation products that can initiate oxidation. Protection is needed to minimise the product contact with oxygen and light. Moreover, optimal temperature and pressure during processing are required to limit oxidation. The exposure to oxygen and light can be limited by producing under vacuum using containers that are resistant to light and air.

After production, lipid oxidation can be limited by storing the product with a modified atmosphere headspace in a packaging material impermeable to oxygen and light (Frankel, 1998). Again, temperature can affect oxidation. Let (2005a) studied the effect of small changes in storage temperature in fish-oil enriched milk. The temperature range from 2-9ºC was investigated. As the temperature increased, the secondary volatile oxidation products appeared faster.

3.5 Lipid oxidation in complex emulsion systems

Multiple factors can affect lipid oxidation in a complex emulsion system. The autoxidation and photooxidation reactions presented in section 3.1-2, are not inaccurate just incomplete for

complex emulsion systems containing polyunsaturated FAs. In theory, the major volatile oxidation product after hydroperoxide decomposition is alcohols; however, the GC-MS detected other volatile compounds. The volatile compounds produced in a complex emulsion system are dependent on multiple side-reactions depending on reaction condition and composition of the emulsion. The side-reactions can be scission reactions in parallel to the traditional reaction route, which may result in the formation of aldehydes (Figure 12). Therefore, the side-reactions occurring in the complex emulsion system and the effect of raw material cannot be ignored when studying lipid oxidation.

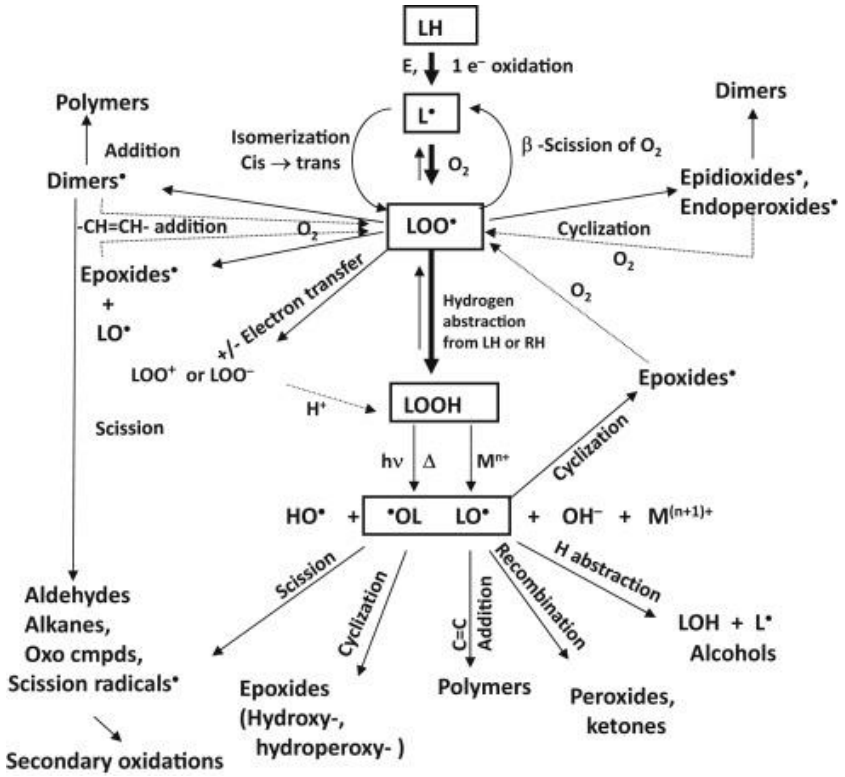


Figure 12. Lipid oxidation in a broader perspective. Adopted from Schaich (2005).

As the hydroperoxide decomposition can result in a complex mixture, these alternative pathways increase the complexity of both the kinetics and the end-product formed even further. Therefore, understanding lipid oxidation in complex emulsion systems should be considered as a new process for each product. Our understanding has increased significantly due to fast and sensitive analysis such as GC-MS. GC-MS makes it possible to measure the development of the individual volatile compound and provide us with knowledge beyond the traditional peroxide value (PV) analysis. At present, we do not fully understand why, when and which factors shift the balance between pathways. However, even more detailed knowledge about the individual side-reactions is needed in order to consider and understand the multiple pathways possible in complex emulsion systems, making it possible to control

lipid oxidation. Thus, the multiple pathways beyond the classical autoxidation in complex emulsion systems may contribute to product deterioration and, in the worst case, toxic side reactions (Schaich, 2005; Jacobsen, 2016).

3.6 Methods selected to determine the extent of lipid oxidation

The extent of lipid oxidation can be measured by various methods. Starting from the beginning of autoxidation, the radicals produced in the initiation stage can be detected by electron spin resonance (Jacobsen, 2010a).

The primary lipid oxidation product formed in the propagation stage can be measured by several approaches two of those methods are PV and conjugated diene (Jacobsen, 2010a).

The secondary lipid oxidation products include both volatile and non-volatile compounds. The volatile compounds were interesting from a product odour point of view. Volatile compounds can also be measured by spectrophotometric methods such as thiobarbituric acid reactive substances (TBARS) value and anisidine value. In contrast to GC-MS, which measures all volatile compounds, these methods only measure the aldehydes and other compounds that react with TBARS and Anisidine reagents added. The secondary volatile oxidation products and volatile compounds formed in other reactions/decompositions can also be detected by GC-MS. Prior to GC-MS analysis, the volatile compounds are collected from the sample; this can be done through several approaches. Often used methods for collection of volatile compounds are dynamic head space collection (DHS), also known as purge and trap, and solid-phase microextraction (SPME). Recently, a new version of automatic DHS has been developed (TDU/DHS). The volatile compounds are collected from the headspace of neat sample (Figure 13). TDU/DHS must not be confused with DHS where nitrogen is purged directly through the sample dissolved in water.

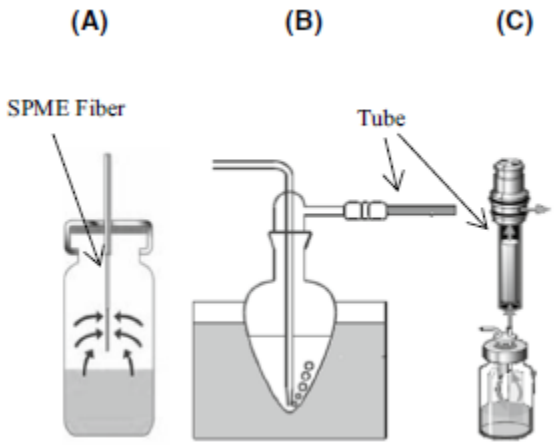


Figure 13. Schematic description of the three collection methods: SPME (A), DHS (B) and TDU/DHS (C). Adopted from Thomsen *et al*., 2016.

The DHS collection is widely used and suitable for a complex mixture. Furthermore, it allows collection of compounds that are only available as trace amounts. DHS collection has been reported to be more efficient at collecting long-chained volatile compounds than short-chained ones (Contarini and Povolo, 2002; Fabre *et al*., 2002; Frankel, 1998).

The second collection method considered was SPME. The volatile compounds are collected on a fiber from neat sample. The method is an automatic collection procedure, and it does not require a substantial manual work. SPME has been reported to collect short-chained volatile compounds more efficiently than long-chained ones (Pawliszyn, 1997; Contarini and Povolo, 2002; Fabre *et al*., 2002; Frankel, 1998). However, a number of authors have reported problems with detecting the volatiles compounds from lipid oxidation. Several of these have suggested that competition between the volatiles "on gaining a spot on the fiber" was the issue (Lu *et al*., 2012; paper VII).

In TDU/DHS collection, the volatile compounds are collected from the headspace above the sample. The collection of volatile compounds can be conducted on the topical skin formulation without addition of any solvents. Another advantage is that TDU/DHS only requires one gram of sample and a limited amount of manual sample preparation. However, the TDU/DHS is a relatively new and complex system, and therefore a massive number of hours must be invested in maintaining and repairing the system, including optimising the collection method (paper VIII).

In addition to the above mentioned chemical analysis, sensory analysis can be applied using the senses of human subjects as instruments to measure odour changes. Various product attributes can be described and assessed by sensory evaluation: appearance, odour, texture, flavour and sound. This thesis will only focus on sensory evaluation of product odour as

odour affecting oxidation products are of main interest for this PhD study. Odour of a product can be detected when volatiles enter the nasal passage and are perceived by the olfactory system. The volatility is highly dependent on temperature: the higher temperature, the more volatile compounds are released from the product. Therefore, temperature control of the product presented/served to panellists is essential to ensure reproducibility (Meilgaard, 1991). Even though the thesis is limited to odour, several different types of sensory assessment can be explored. Sensory evaluation can be applied to investigate several product properties including odour acceptance, ODT values, odour description etc. The use of humans as the instrument constitutes a key challenge in sensory science. A variety of approaches can be used to calibrate the "human instrument" to obtain reproducible results from human subjects. The most important parameter in all approaches is to ensure the panellists' capability to perform the analysis. One approach is to use panel trained to use e.g. an intensity scale to describe a product (trained panel). Various approaches can be applied in descriptive analysis, including quantitative, time-intensity and free-choice profiling. The training of the panel differentiates from method to method. In free-choice profiling, the panel is trained in describing and recognising the odour(s) wished to evaluate. Hereafter, the intensity of odour in different concentration is tested and discussed in plenum of all panellists. A panel leader guides the panellists through this plenum discussion to ensure progress (Wortel and Wiechers, 2000).

Descriptive sensory analysis is used to describe product compared to a target product, changes, track and relate attributes to chemically measured changes etc. (Meilgaard, 1991, Dooley et. al, 2009). In descriptive sensory analysis, profiling is used to evaluate product attributes. In a descriptive profiling analysis, the characteristics that are selected in plenum to describe the product odour are both olfactory and nasal attributes. These are assessed on an intensity scale; the linear scale is used in this thesis on a line from 0-15 to pick up small differences. Most often, a panel of five to ten judges is used, which was also the case in this thesis, where eight to ten assessors were involved.

Often sensory evaluation is used for comparing samples either to investigate effect of storage or different composition. A trained panel is needed to obtain reproducible results (Cortez-Pereira, 2009). Using a trained panel, a reproducibility of ± 20 % is possible to obtain. Threshold values can be used to identify the concentration where a change in odour (and flavour) is observed and can be described. Depending on compound and product matrix, threshold value can vary widely. Threshold value can be divided into four types: absolute,

recognition, difference and terminal thresholds. The difference threshold value is the concentration at which samples can be differentiated (Meilgaard, 1991).
Threshold values can be determined using three approaches, mainly two are used: method of limit and method of average error. In the method of limit, panellists are exposed to increasing odour intensity. The concentration starts at a level below odour detection limit, and then the level is gradually increased until panellists report that they can detect a change. The opposite approach is applied for the method of average error, panellists alter sample until the odour of interest is barely detectable against the control (Meilgaard, 1991).

Chapter 4: Experimental Approach

The purpose of this PhD was to increase knowledge about the extent of lipid oxidation in real topical skin formulations and not merely in simple model emulsions. The topical skin formulations used in this study were produced by GSK in their facilities. Moreover, the topical skin formulations had the same level of complexity as real topical skin formulations (papers I-V). Due to the high complexity of the topical skin formulations studied and the high number of ingredients that all can affect lipid oxidation, interpretation of the results obtained was complicated.

In addition to investigating lipid oxidation, the purpose was also to explore ways to combat potential bottlenecks in product development caused by time-consuming stability tests. Currently, the stability tests take at least six months.

Lipid oxidation was explored in four steps (Figure 14): 1) The progress of lipid oxidation for six months at various storage conditions to simulate different storage conditions in the home of consumers (papers I-III); 2) Sensory evaluation of odour changes at storage and individual effect of selected volatile compounds on odour (papers I, II and VI); 3) The raw materials´ effect on oxidative stability. Moreover, the link between raw materials and volatile compounds that appeared in topical skin formulations was studied (papers III and IV); and 4) Initiation of lipid oxidation by radicals or other initiators with the purpose of fast prediction of long-term oxidative stability (papers I-V).

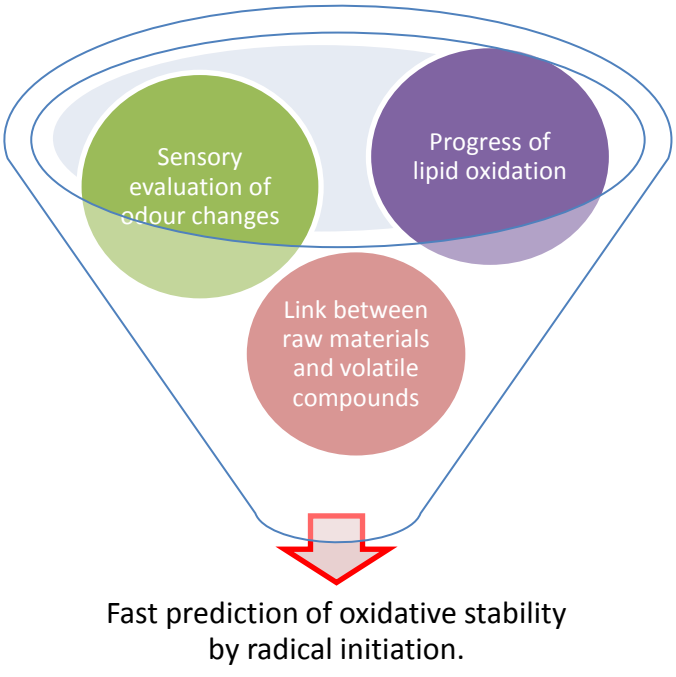


Figure 14. The working approach of the PhD.

4.1 Materials: Topical skin formulations

The experiments were conducted on two main types of formulations representative of topical skin formulations with low and high lipid content: three prototype cleansing formulations and one prototype serum formulation (low lipid), and four prototype cream formulations and four prototype lip care formulations (high lipid) (Figure 15).

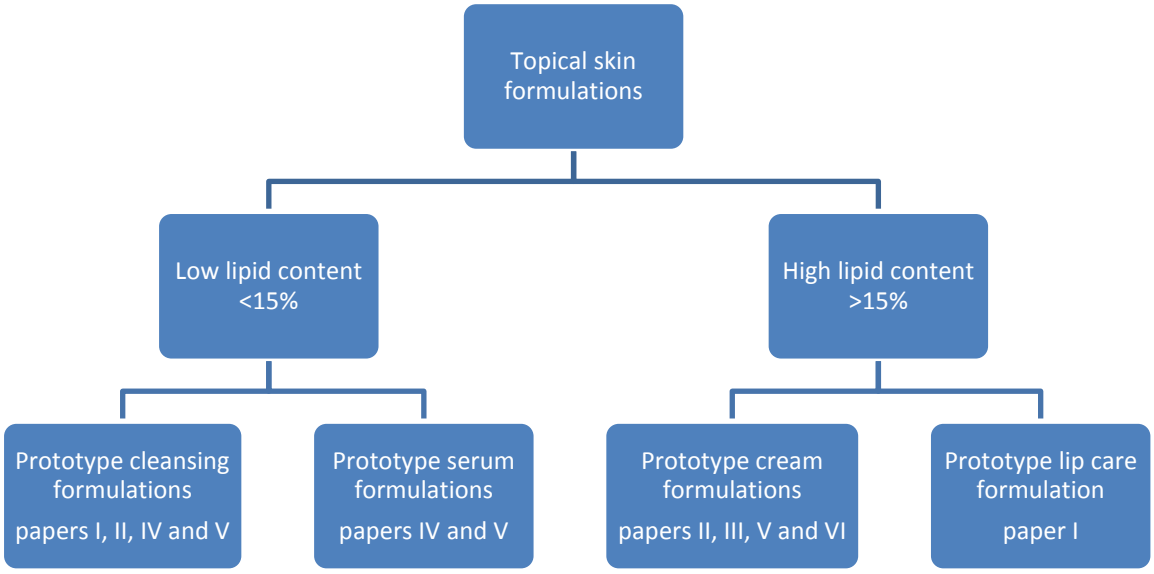


Figure 15. Distinction between high and low lipid content and formulations within each category.

The topical skin formulations used in papers I-V were produced by GSK. The physical stability of the topical skin formulation has an impact on oxidative stability. The physical stability of the topical skin formulation produced by GSK was assessed in their laboratories. Therefore, the physical stability of the topical skin formulations produced by GSK was not evaluated. Moreover, the exact composition cannot be shared due to a confidentiality agreement with GSK. However, the lipid-containing ingredients and details listed below help to explain the difference in the oxidative stability between e.g. lip formulations can be shared.

4.1.1 Prototype cleansing formulations (papers I, II and IV)

The prototype cleansing formulations contained agents that clean the skin. The prototype cleansing formulations have a low lipid content. Four prototype cleansing formulations were examined (Table 1).

Table 1. Ingredients in prototype cleansing formulation constituents pertinent to lipid oxidation investigation (papers I, II and IV).

Product	Prototype cleansing formulation 1 (paper I)	Prototype cleansing formulation 2 (paper I)	Prototype cleansing formulation 3 (paper I)	Prototype cleansing formulation 4 (papers II and IV)
Product contained several other raw materials including the listed.	Caprylic/capric triglyceride Cocos nucifera oil Hydrogenated lecithin Caprylyl glycol Butyrospermum parkii butter Squalane Ethylenediamine disuccinate Ceramide-3	Caprylic/capric triglyceride Cocos nucifera oil Hydrogenated lecithin Olea europa (olive oil) Caprylyl glycol Elaeis guineesis oil Squalane Vegetable oil PMEA Ethylenediamine disuccinate Ceramide-3	Caprylic/capric triglyceride Cocos nucifera oil Hydrogenated lecithin Olea europa (olive oil) Caprylyl glycol Elaeis guineesis oil Squalane Vegetable oil Caprylhydroxamic acid PMEA Ethylenediamine disuccinate Ceramide-3	Caprylic/capric triglyceride Hydrogenated starch Hydrolysate Cocos nucifera oil Hydrogenated lecithin Olea europaea fruit oil Caprylyl glycol Elaeis guineensis oil Olus oil Squalane Caprylhydroxamic acid Palmitamide MEA Sarcosine Ethylenediamine disuccinate Ceramide NP

4.1.2 Prototype serum formulation (papers IV and V)

The prototype serum formulation has a higher concentration of performance ingredients for targeting specific skin-care concerns compared with other topical skin formulations with a low lipid content (Table 2).

Table 2. Selected raw materials in prototype serum formulation constituents pertinent to lipid oxidation investigation (papers IV and V).

Product	Prototype serum formulation (papers IV and V)
Product contained several other raw materials including the listed.	Glycerine Isostearyl isostearate Acetamide MEA Lecithin Palmitamide MEA

4.1.3 Prototype cream formulations (papers II, III, V and VI).

The prototype day, night and skin cream formulations produced by GSK contained several raw materials including those presented in Table 3.

Table 3. Ingredients (relevant to lipid oxidation) and approximate lipid contents for prototypes day and skin cream formulations (high lipid content) (papers II, III and V).

Product	Prototype day cream formulation (paper II)	Prototype skin cream formulation (papers III and V)	Prototype night cream formulation (paper V)
Product contained several other raw materials including the listed.	Dicaprylyl carbonate Butyrospermum parkii butter Isostearyl isostearate Caprylic/capric triglyceride Hydrogenated lecithin Oryza sativa cera Tocopheryl acetate Squalane Trisodium ethylenediamine disuccinate Ascorbyl glucoside Ceramide NP	Rice bran wax Glycerine Isostearyl isostearate Palmitic acid monoethanolamine	Butyrospermum parkii butter Glycerin Olus oil Dicaprylyl carbonate Niacinamide Hydrogenated lecithin Caprylic/capric triglyceride Squalane Behenic acid Oryza sativa cera Tocopheryl acetate Ascorbyl tetraisopalmitate Caprylhydroxamic acid

In addition, one facial moisturiser prototype was produced in DTU laboratories based on a simple recipe; the complete ingredient list is available in Table 4.

Table 4. Ingredients used for producing the facial moisturiser prototype (paper VI).

Water phase	
Demineralized water	52 or 51 g/ 100 g
Aloe vera water	10.0 g/ 100 g
Glycerine	6.3 g/ 100 g
Sodium Stearoyl Lactylate	3.6 g/ 100 g
Natriumbenzoat	0.6 g/ 100 g
F. vesiculosus extract (water or acetone)	1 or 2 mg/g
Oil phase	
Almond oil	21.8 g/ 100 g
Lanette wax	2.0 g/ 100 g
Glyceryl Stearate	1.8 g/ 100 g
Vitamin E	0.9 g/ 100 g

4.1.4 Prototype lip care formulations (paper I)

The prototype lip care formulations were included as a case study of topical skin formulations that have a high lipid content (Table 5).

Table 5. Ingredients in prototype lip care formulation constituents pertinent to lipid oxidation investigation (paper I).

Product	Prototype lip care formulation 1-3	Prototype lip care formulation 4
Product constituents pertinent to lipid oxidation investigation.	Caprylic/capric triglycerides Butyrospermum parkii butter Olus oil Ethylhexyl salicylate Oryza sativa cera Butyl methoxydibenzoylmethane Behenyl alcohol Capryloyl glycine Caprylyl glycol Ascorbyl palmitate Trisodium ethylenediamine disuccinate Tocopherols Hydrogenated lecithin PMEA Oryza sativa bran oil Squalane Phytoshingosine Ceramide-3 Aroma (only lip care formulation 2 and 3)	Butyrospermum parkii butter Olus oil Elaeis guineensis oil Oryza sativa cera Behenyl alcohol Oryza sativa bran oil Hydrogenated lecithin Capryloyl glycine Caprylyl glycol Tocopherols Trisodium ethylenediamine disuccinate Ascorbyl palmitate Behenyl alcohol Squalane Ceramide-3 Phytoshingosine PMEA

4.2 Measuring lipid oxidation products in topical skin formulations

In this PhD, primarily the odour-affecting volatile oxidation products were of interest. However, in order to map the route of reactions in lipid oxidation, the primary oxidation products were also measured. PV analysis was also selected because PV is often used as industry standard method. Therefore, the capability of PV as a marker for the extent of lipid oxidation was relevant to evaluate in the selected topical skin formulations.

Moreover, the choice of GC-MS for this thesis work was obvious because the GC-MS can measure the odour affecting volatile oxidation product formed in oxidation. In addition to the volatile oxidation product, GC-MS also measures other volatile compounds that can affect product odour.

However, the choice of collection method was not as straightforward. The purpose was to select a collection method, which collected various volatile compounds. Yet, mild collection conditions were needed to prevent new volatile compounds from being formed under the collection. Accordingly, DHS collection was performed under mild conditions at a temperature of 45ºC. However, the viscosity was to be decreased by adding water. To avoid water addition, two other collection methods were considered; TDU/DHS and SPME. However, SPME has been reported to have undesirable challenges with competition problems

for space on the fiber. Having the possibility of competition problems as an unknown-factor was undesirable (Contarini and Povolo, 2002; Fabre *et al*., 2002). Therefore, SPME was not selected to collect volatile compounds from a complex matrix; the DHS collection was preferred for this experiment.

Initially, DHS collection was used. DHS purging requires that the topical skin formulation was dissolved in water containing anti-foaming agent to prevent servere foaming from reaching the Tenax® tube. Addition of water changes the viscosity of the topical skin formulation, and thereby the matrix retaining of volatile compounds may be affected. Therefore, the method was changed to TDU/DHS where the headspace collection of volatile compounds was conducted on the topical skin formulation without addition of any solvents. Another advantage of changing collection method was reducing the amount of manual sample preparation.

The extent of lipid oxidation was measured by PV and GC-MS (volatile compounds) for six months' storage (Figure 16).

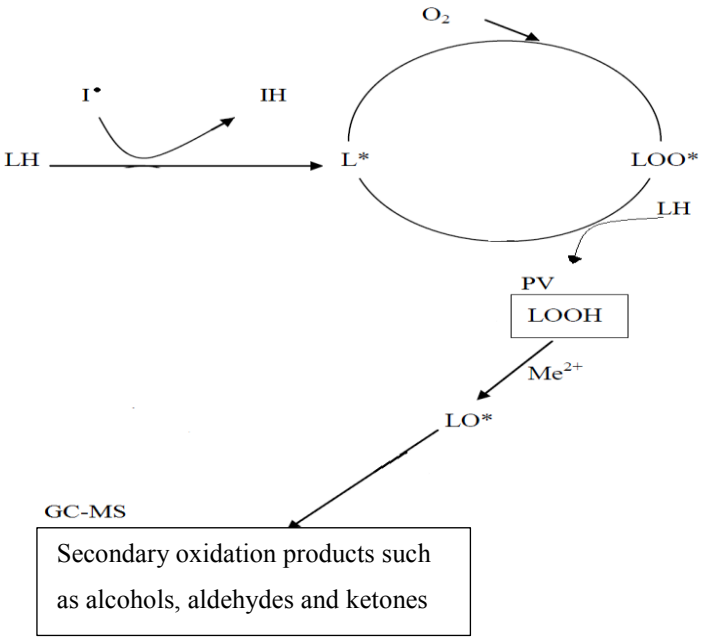


Figure 16. The approach for measuring the extent of lipid oxidation.

In addition to the chemical methods, sensory evaluation was conducted to describe the odour profile of the topical skin formulations and storage related changes. Moreover, sensory evaluation is an important part of this PhD. Hence, the instrumental methods result in a number and do not describe the impact of the volatile compounds. An odour threshold value is needed for evaluating the concentration from which an impact of volatile compounds can

be detected. Even though several studies have reported odour effect and ODT values for most volatile compounds, these ODT values cannot be applied to topical skin formulations unless the ODT determination was carried out in a similar matrix. Since such articles have not been found in literature, we have conducted three sensory studies with four purposes: 1) Initial screening for significant differences (paper I); 2) Scaling and describing; the changes related to storage conditions (paper I); 3) Description of difference (DOD) scale (not published data); and 4) ODT value determination and effect on product odour of spiking selected volatile compounds individually (paper II).

1) Initial screening for significant differences

An initial screening to reveal significant changes related to the storage conditions was conducted using a triangle test. In triangle test, the samples were spread as a 2 mg/ cm^2 layer on a neutral surface with a lid (plastic cup). Then, the samples were brought to room temperature and given a random three-digit number. Moreover, the sample was presented to the assessor together with the reference sample (REF). The assessor was asked to determine whether they differ from one another and, if they do, which sample was different. Thus, the sensory triangle test can only determine whether there is a difference, not what the difference is. Moreover, the triangles for each sample was conducted at least 30 times and served in random order in accordance with ISO 4120.

The evaluation was conducted by a trained sensory panel consisting of a number of assessors who were trained in a standardised way of handling and assessing the samples. The samples included in the triangle test were the prototype cleansing and lip care formulations listed in Tables 1 and 5. The prototype cleansing and lip care formulations were examined after eight weeks of storage at 2°C (REF), at 20°C, at 20°C with exposure to light and at 40°C. In addition to the samples stored for eight week, samples stored for two weeks at 50°C were also analysed.

2) Scaling and describing; the changes related to storage conditions

The sensory attributes that were selected for profiling are described in Table 6.

Table 6. Attributes used in the sensory profiling of prototype cleansing formulation1-3.

Profiling Attributes	Description
Soap flake	Soap flake without any additions
Carbamide cream	Carbamide lotion 10%, without perfume. Ingredients: Aqua, glycerine, urea, parafilm liquidum, propylene glycerol, cetearyl alcohol, PEG-40 hydrogenated castor oil, sodium ceteranyl sulphate, citric acid, sodium gluconate, dimethicone, caprylyl glucol, phenoxyethanol.
Lanoline	Wool
Sour	Sour dishcloth/ sour sock
Stale	Stale, musty, cardboard, dusty
Sourish	Acidic, acetic acid, citric acid
Caramel	Caramel
Peppermint	Peppermint
Sweet	Sweet
Almond peel	Peel from almonds

All relevant attributes were trained to ensure that the assessors were able to use the whole scale for each attribute. At each evaluation session, panellists were served ten samples in random order. Moreover, the sensory profiling was performed on the prototype cleansing and lip care formulations listed in Tables 1 and 5. The prototype cleansing and lip care formulations were examined after eight weeks' storage at 2°C (REF), at 20°C, at 20°C with exposure to light and at 40°C. In addition to the samples stored for eight week, samples stored for two weeks at 50°C also were analysed.

3) Description of difference scale

In addition to the time-consuming scientific sensory methods, a fast industry standard method was used to assess the odour changes as well. In this method, the sample odour was graded versus a REF. The samples were ranked from one to five based on the DOD scale depending on the difference compared with the REF (Table 7).

Table 7. Description of difference scale.

DOD Scale	Description of Difference
1	No differences in character or intensity noted
2	Reasonably sure difference exists, though difference may be too subtle to accurately describe
3	Definite difference, can describe difference with reasonable surety
4	Product or material out of expected range. Moderate or large intensity differences or ANY character differences.
5	Outside normal range. Large intensity and/or character differences.

Note: DOD = Degree of Difference

4) Odour detection threshold value determination and effect on product odour of spiking selected volatile compounds individually

In this thesis, the odour threshold value was determined using the method of limit in triangles. Moreover, the triangles were served until the difference odour threshold value (hereafter referred to as ODT value) was reached. This specific approach was selected because the point of interesting is when the volatile compounds changes the product odour. In addition to odour threshold value, the panellists were asked to describe the overall difference caused by the volatile compounds at the last session. The ODT value was determined in both topical skin formulations with high and low lipid content. Topical skin formulations with high and low lipid content were represented by the prototype day cream formulation and prototype cleansing formulation 4.

Determination of ODT values for all the volatile compounds that appeared would be relatively resource demanding. Therefore ODTs for only six selected volatile compounds were determined in the prototype day cream formulation and prototype cleansing formulation 4. The following six volatile compounds were selected for odour evaluation: 2-ethyl furan, 2-pentyl furan, butanal, pentanal, 1-heptanol and 3-methyl-1-butanol. Moreover, the ODTs were determined based on standard addition. The standards were added by spiking volatile standard in the prototype day cream formulation and prototype cleansing formulation 4 in six concentrations. The concentration of the volatile compound was selected using the following approach: The lowest concentration (Conc. 1) was the concentration closest to the ODT reported in water (Fors, 1988; Buttery *et al*., 1988*;* Guadagni *et al*., 1963; Fazzalari, 1978;

Choe and Min, 2006; Takken *et al*., 1975*;* Teranishi *et al*., 1974; Giri *et al*., 2010; Kaseleht *et al*., 2010; Buttery, *et al*., 1969; Buttery *et al*., 1990). Then, the following concentrations were increased gradually, ending above the ODT value determined by the expert panel (Tables 8 and 9).The highest concentration (Conc. 6) was above the ODT determined in the prototype day cream formulation and prototype cleansing formulation 4 in a pre-experiment using an expert panel.

Table 8. Concentrations applied to determine the odour detection threshold value in the prototype day cream formulation for 2-ethyl furan, 2-pentyl furan, butanal, pentanal, 1-heptanol and 3-methyl-1-butanol.

Concentration selected for prototype day cream formulation						
Volatile standard/ Conc. [ng/g]	**Conc. 1**	**Conc. 2**	**Conc. 3**	**Conc. 4**	**Conc. 5**	**Conc. 6**
2-Ethyl furan	10	20	30	40	55	70
2-Pentyl furan	10	30	50	65	80	100
Butanal	10	25	40	55	65	80
Pentanal	10	30	50	65	80	100
1-Heptanol	10	25	40	55	65	80
3-Methyl-1-butanol	200	240	290	320	360	400

Table 9. Concentrations applied to determine the odour detection threshold value in the prototype cleansing formulation 4 for 2-ethyl furan, 2-pentyl furan, butanal, pentanal, 1-heptanol and 3-methyl-1-butanol.

Concentration selected for prototype cleansing formulation 4						
Volatile standard/ Conc. [ng/g]	**Conc. 1**	**Conc. 2**	**Conc. 3**	**Conc. 4**	**Conc. 5**	**Conc. 6**
2-Ethyl furan	10	20	30	40	55	70
2-Pentyl furan	10	25	40	55	65	80
Butanal	20	60	100	130	160	200
Pentanal	10	25	40	55	65	80
1-Heptanol	10	25	40	55	65	80
3-Methyl-1-butanol	200	240	290	320	360	400

4.2.1 Effect of storage conditions

Since consumer behaviour is difficult to predict, the oxidative stability of the formulations was evaluated over a wide range of storage conditions. A wide range of storage conditions were included to simulate the extremes of consumer behaviour, e.g. exposure to sunlight. The effect of exposure to sun light was studied by exposure to artificial light (approximately 3500 lux). Moreover, the effect of temperature was examined by storing the topical skin formulations at various conditions at 2/5°C, 20°C, 40°C and 50°C for three to six months (papers I-V).

In addition to the topical skin formulations, the storage conditions effect on facial moisturiser prototype containing *Fucus vesiculosus* were evaluated as well. The facial moisturiser

prototype containing *Fucus vesiculosus* was exposed to artificial light or darkness at room temperature, and 40°C for 56 days (paper VI).

The progress of lipid oxidation and changes in product quality were measured based on changes in colour, droplet size, FA composition, tocopherol content, PV and GC-MS (Table 10). The analytical methods are described in papers I- VI.

Table 10. Overview of the storage experiments for topical skin formulation and the analysis conducted: colour changes, droplet size, fatty acid composition (FA), tocopherol content, peroxide value and GC-MS (collection method).

Prototype skin formulation	Storage experiment	Droplet size	Colour	PV	GC-MS	Tocopherol	FA
Lip care paper I	2°C, 20°C(± light), 40°C and 50°C for 84 days.	-	-	x	DHS	-	-
Cleansing 1-3 paper I	2°C, 20°C(± light), 40°C and 50°C for 84 days.	-	-	x	DHS in I	-	-
Cleansing 4 papers II and IV	5°C, 20°C(± light), 40°C and 50°C for 6 months.				TDU/DHS (II) DHS (IV)		
Day Cream paper II	5°C, 20°C(± light), 40°C and 50°C for 6 months.	-	-	x	TDU/DHS	-	-
Cream papers III and V	5°C, 20°C(± light), 40°C and 50°C for 6 months.	-	-	x	TDU/DHS	x(only V)	-
Serum Paper IV	5°C, 20°C(± light), 40°C and 50°C for 6 months.	-	-	x	TDU/DHS	-	-
***Fucus vesiculosus* extracts in facial moisturiser prototype** paper VI	20°C(± light) and 40°C for 56 days.	x	x	x	DHS	X	x

4.2.2 Lipid oxidation in topical skin formulations with a high and low lipid content

Lipid oxidation is a complex series of reactions that easily are affected by several factors. The purpose of measuring lipid oxidation in the first experiment (paper I) was not to gain a deeper understanding of why lipid oxidation occurred rather to investigate to which extent it occurred. Furthermore, the study was conducted to investigate the effect of the overall lipid content on the oxidative stability of topical skin formulations (paper I). The two main types of topical skin formulations (high and low lipid content) were stored for six months at a wide range of storage conditions (Table 7). The study was conducted on "real" topical skin formulations meaning that the composition of lipids was not exactly the same in the two formulations.

Prototype cleansing formulations were used in the case study of topical skin formulations with low lipid content. Three prototype cleansing formulations (formulations 1, 2, and 3, see Table 1) were selected to investigate the effect of the lipid composition and a metal chelator on extent of lipid oxidation. The first cleansing formulation contained a proprietary lipid

blend: caprylic/capric triglyceride, coconut oil, butyrospermum parkii butter and squalane (cleansing formulation 1). The second cleansing formulation also contained a control containing proprietary lipid blend. Moreover, butyrospermum parkii butter was removed and the caprylic/capric triglycerides content was reduced. In addition, it contained various oils (more unsaturated oils): olea europa (olive oil), elaeis guineesis oil, vegetable oil and one antistatic agent PMEA (cleansing formulation 2). The third cleansing formulation had a composition similar to cleansing formulation 2. However the content of caprylyl glycol was reduced and a metal chelator caprylhydroxamic acid was added (cleansing formulation 3).
In addition to exploring topical skin formulations that have a low lipid content, study 1 also explored lipid oxidation in topical skin formulations that have a high lipid content. The case study was lip care formulations, one of which contained no aroma compounds, and two prototype lip care formulations (2 and 3) were added aroma compounds. The aroma compounds added were a mint to lip care formulation 2 and a creamy flavour to lip care formulation 3. These prototype lip care formulations were included to explore whether the aromas had a masking effect on odour profile and to assess their effect on oxidative stability. Moreover, the lipid content of the prototype lip care formulations 1-3 was 36 %. A fourth lip care formulation was also included that contained no aroma compounds. Although it contained more shea butter and palm oil, and less glycerine than the three other prototype lip care formulations (lip care formulation 4). The total lipid content was at 39 % which was slightly higher than the other three lip care formulations.

4.2.3 Effect of raw materials

As mentioned in the section "Factors affecting lipid oxidation", the quality of the topical skin formulation highly depends on the quality of the raw materials used to produce it. Therefore, the oxidative stability of the topical skin formulation is often related to the raw material. To determine the quality of the raw materials, the content of volatile compounds was analysed. Available lipid-containing raw materials present in more than one percent were examined. Furthermore, to investigate where butane nitrile originated from, raw materials that may contain nitrogen were included as well (papers III and IV).
Moreover, the selected raw materials were stored at 40ºC for three months, with sampling each month. The samples were stored at 5ºC until GC-MS analysis. The GC-MS analysis was conducted to determine the volatile compounds present. The volatile compounds were identified and quantified using external standards.

Furthermore, the volatile compounds present in the raw materials were linked to the volatile compounds present in the topical skin formulations. The topical skin formulations included were prototype skin cream formulation (high lipid content, Table 3, paper III), prototype cleansing formulation and prototype serum formulation were studied (low lipid content, Tables 1 and 2, paper IV). These three topical skin formulations were included in the study because the lipid content in topical skin formulations varies widely depending on the product type. Moreover, two topical skin formulations were included with a low lipid content as the raw materials included were quite different depending on the product purpose: cleansing or moisturising.

4.2.4 Effect of antioxidants

In addition to the studies carried out with GSK's topical skin formulations, a study with a facial moisturiser prototype produced at DTU was carried out. In this study the antioxidative effect of *Fucus vesiculosus* extracts was investigated (paper VI). Three *Fucus vesiculosus* extracts, acetone extract (AE), ethanol extract (EE) and water extract (WE) were added to a facial moisturiser prototype in two concentrations: 1 or 2 mg/g (only AE and WE).
The physical stability of the facial moisturiser prototype added AE or WE were assessed by measuring the droplet size at the beginning and at end of the experiment. The droplet size was measured to explore physical stability because it influenced the oxidative stability.

4.3 Fast prediction of long-term oxidative stability

The shelf life of topical skin formulations is from six months to three year. Thus, the evaluation of the oxidative stability is a time-consuming procedure. Currently, the oxidative stability test takes up to six months plus time for analysis, all adding up to approximately eight months. The eight months spend on oxidative stability test are a bottleneck in product development. An approach to combat the bottleneck is to initiate oxidation fast. Moreover, the fast initiation must result in the same sample ranking or oxidation rate of the oxidative stability. Otherwise, the initiation cannot remove the bottleneck. Several effective initiators of lipid oxidation have been reported in literature. However, these studies mainly focused on initiating oxidation and not the ability to reveal long-term product stability fast. For this study, four initiator approaches were selected. The selected approaches were effective initiators in emulsions: AAPH, AMVN, $FeCl_3$/ascorbic acid and $FeCl_2/H_2O_2$. The effective initiator concentrations in the emulsion studies were (Baron *et al*., 2006; Mosca *et al*., 2010; Matsumuraa *et al*., 2003):

- 50 mM AAPH
- 50 mM AMVN
- 0.10 mM $FeCl_3$ and 25 mM ascorbic acid
- 1 mM $FeCl_2$ and 2 mM H_2O_2

In this study, the capability to predict product stability fast was explored in three steps:
(1) Oxygraph was applied for identification of potential initiators, determination of the reaction kinetics and estimation of the required concentration to initiate lipid oxidation;
(2) Hereafter, the effect on physical stability by adding potential initiators in the effective concentration to topical skin formulations was examined. Only, the initiators that were stable in topical skin formulations were included in the storage experiment. In the storage experiment, the oxidative stability was assessed for one month. The assessed oxidative stability was compared to neat topical skin formulations stored for six months;
(3) Finally, the initiators that were able to predict oxidation stability within one month were selected for the crucial test. The crucial test was to evaluate their ability to reveal products with low oxidative stability.

4.3.1 Initial screening for potential initiators using Oxygraph

The reactions in the initiation stage of lipid oxidation consume oxygen. The oxygen consumption is measured by Oxygraph. Moreover, the oxygen consumption is determined by measuring the extent of an oxygen dependent reaction using an oxygen electrode. The oxygen electrode is an electrochemical cell that consists of two electrodes. The two electrodes are immersed in an electrolyte solution (KCL). The electrolyte ionises when a polarising voltage of 700 mV is applied, which initiates a series of electrochemical reactions. The electrochemical reactions depend on oxygen. Therefore, the oxygen concentration in the sample affects the rate of the electrochemical reactions. The oxygen consumption rate was measured in a control sample and samples added initiators. Moreover, the different oxygen consumption rates between control sample and sample with initiator is assumed to be related to lipid oxidation. The different oxygen consumption rate was calculated as $\alpha_{control}$- α_{sample} [nmol/mL] in triplicates.
Due to the high viscosity of the topical skin formulations, the evaluation of the four initiators was done in diluted prototype skin cream, night cream and serum formulations (1:3). Initially,

a blank sample was analysed. Hereafter, the concentration of initiator was gradually increased until a clear effect was observed or phase separation occurred in the sample.

4.3.2 Acceleration of lipid oxidation by initiator addition to topical skin formulations

The initiators were added to topical skin formulations to evaluate the physical stability and their ability to initiate lipid oxidation. The initiator concentration was selected based on the result of the literature (Baron *et al*., 2006; Mosca *et al*., 2010; Matsumura *et al*., 2003) and adjusted in accordance to the results from Oxygraph study:

- 50 mM AAPH
- 50 mM AMVN
- 10 mM $FeCl_3$ and 25 mM ascorbic acid
- 1 mM $FeCl_2$ and 2 mM H_2O_2

The four initiators were added to three topical skin formulations: prototype serum, night cream and skin cream formulations. The three topical skin formulations were stored at 20°C for 1 month with sampling points after: 0, 3, 6, 9, 12, 16, 21, and 30 days. The physical and oxidative stability were examined. In addition, a six months storage experiment at 20°C was conducted to determine the oxidative stability of the neat topical skin formulations. The oxidative stability was assessed by the oxidation rate of volatile compounds. Oxidation rate = $((c_{end}-c_{start})/c_{start})*100\%$.

4.3.3 Ability to reveal products with low oxidative stability

A good initiator must be able to rank the topical skin formulations in the same order as observed after six months for PV and the volatile compounds. Besides, the same rank, the physical stability must be maintained. The most promising initiators were selected for this study. The following initiators and concentrations were used to accelerate lipid oxidation:

- 10, 25 and 50 mM AAPH
- 10, 25 and 50 mM AMVN
- 1 mM $FeCl_2$ and 2 mM H_20_2

The initiators were added to a prototype skin cream formulation (Table 3) with and without AO. The effect of initiators and AO on oxidative stability was examined in a storage experiment. The storage experiment was conducted at 20°C for one month with sampling points after: 0, 3, 6, 9, 12, 16, 21, and 30 days. In addition, a three months storage experiment at 40°C was conducted to determine stability of neat prototype skin cream formulation with

and without AO. After sampling, the samples were stored at 5°C until analysis. The samples were analysed for the concentration of volatile compounds using TDU/DHS GC-MS. The odour changes were assessed using DOD Scale.

4.4 Statistical analysis

In all experiments, statistical methods were employed. The statistical method was selected carefully for each type of data.

Statistics on peroxide values and volatile oxidation products data;

The statistical program Graph pad prism version 6 (graph pad, La Jolla, USA) was used. A two-way analysis of variance followed by a Bonferroni multiple comparison test was employed to evaluate significant changes in PV and volatile oxidation products.

4.6.1 Sensory odour data

The statistical program Panel Check V1.4.0 was used to analyse the sensory profiling. The performance of the assessors was evaluated by a two-way analysis of variance. A one-way analysis of variance was used to analyse the triangle tests.

4.6.2 Partial least squares regression for linking data

The statistical program Unscrambler version 10.3 (Camo, Oslo, Norway) was used to perform a partial least squares regression model. The partial least squares regression model was built on the average of the measured data and full cross validation was used to validate the model. The x-variable was the volatile compounds and the y-variable was the sensory attributes after 56 days of storage.

Chapter 5: Results and Discussion

5.1 The effect of storage conditions and lipid content on lipid oxidation products in topical skin formulations (papers I-VI)

The results from the first experiment showed that lipid oxidation occurred in topical skin formulations with both a high or low lipid content. The results from PV and GC-MS analyses were used to identify appropriate markers. Appropriate markers for lipid oxidation were pentanal and heptanal in prototype lip care and cleansing formulations (paper I). Several factors can have a significant effect on lipid oxidation. Some of these effects will be elaborated upon in the following sections about the effect of storage conditions and lipid content.

5.1.1 Effect of storage conditions – temperature and exposure to light (papers I-VI)

Several storage temperatures ranging from 2-50°C were explored. Compared with the REF stored at 2 or 5°C, elevated temperature resulted in the same or a slightly higher PV and concentration of pentanal, heptanal and 3-methyl-1-butanol (Table 11).

Table 11. The effect of exposure to light and increasing temperature to 40°C is summarized for papers I-VI. The effects compared with the reference are significant (+), not significant (–) and not determined (ND).

Paper	Formulation	PV		Pentanal		Heptanal		3-Methyl-1-butanol	
		Light	40°C	Light	40°C	Light	40°C	Light	40°C
I	Cleansing 1-3	+	-	+	-	+	-	ND	ND
	Lip care 1-4	+	+	+	+	+	+	ND	ND
II and IV	Cleansing 4	+	-	+	-	-	-	ND	ND
II	Day cream	+	-	-	-	ND	ND	-	-
III and V	Skin cream	+	-	+	+	+	+	+	+
IV and V	Serum	+	+	+	+	ND	ND	ND	ND
V	Night cream	+	-	+	-	ND	ND	ND	ND
VI	Facial moisturiser prototype	+	+	+	+	+	+	ND	ND

A new group of volatile compounds was found in this study that has not previously been reported to occur in lipid oxidation in emulsions, namely nitriles: butane, pentane, hexane and octane nitrile (exemplified by butane nitrile in Figure 17D). Butane nitrile was the most important nitrile-containing volatile compound because it may cause skin irritation. Toxicological studies on rabbits showed that exposure to butane nitrile gave rise to skin irritation. Skin irritation occurred at levels above 395 mg in an open irritation test and the lethal dose was 10 mg/kg for rabbits (NIOSH, 2009). None of the evaluated topical skin formulations contained butane nitrile at these high levels. The concentration of all four

nitriles increased with increasing temperatures. The effect of elevated temperature on the formation of volatile compounds is shown for topical skin formulations (Figure 17).

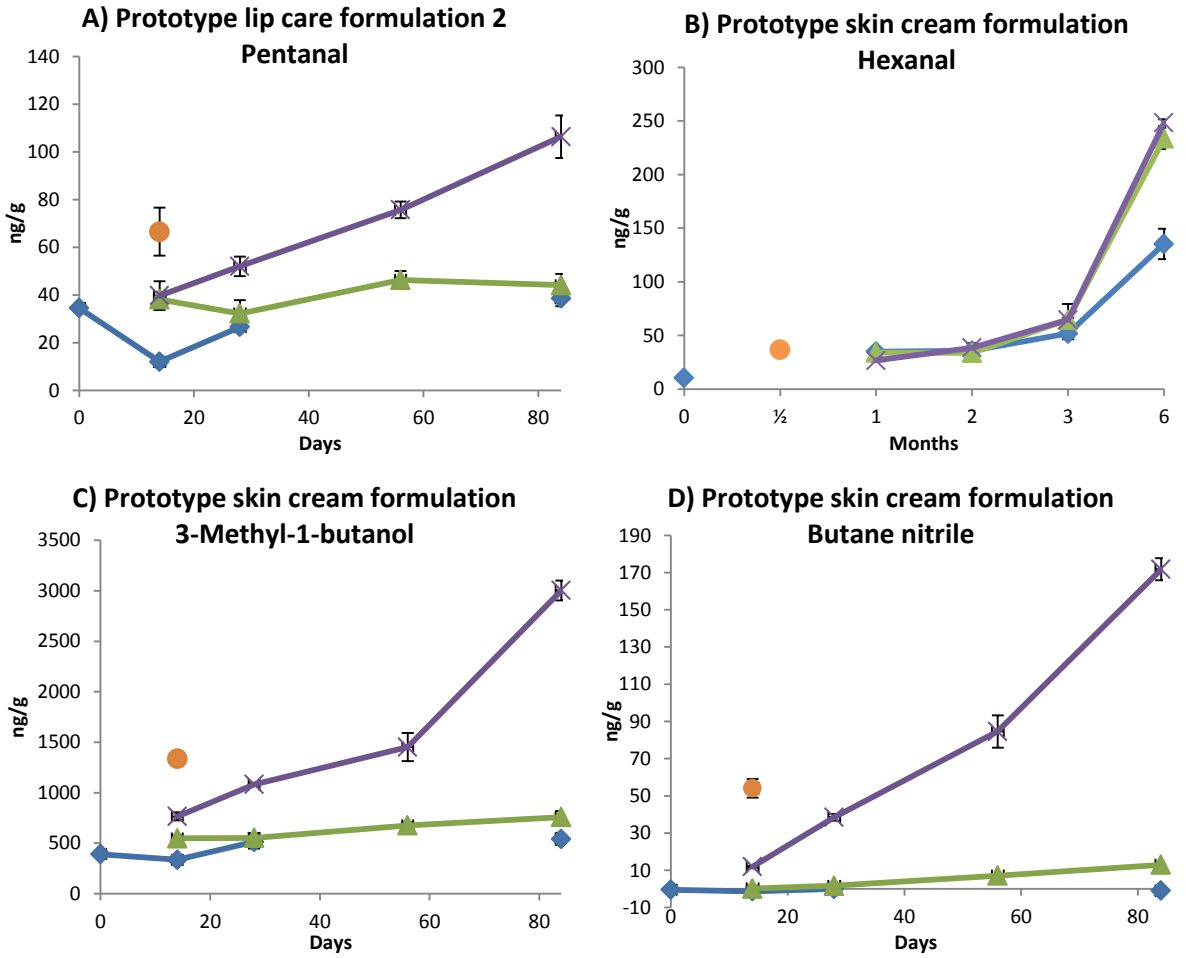


Figure 17. The development of volatile compounds: pentanal (A) in prototype lip care formulation, hexanal (B), 3-methyl-1-butanol (C), and butane nitrile (D) in prototype skin cream formulation [ng/g]stored at 2°C (◆), at 20°C (▲), at 40°C (×) and at 50°C (●) (papers I and III).

For most volatile compounds, a non-linear relationship was observed between the concentration of volatile compounds and the temperature. The effect of the temperature was largest when it increased from 20°C to 40°C.

Exposure to light resulted in an equal or higher PV and concentration of volatile oxidation products in the prototype formulations (Table 11). Apart from the above mentioned volatile oxidation products, furans were also found. Overall, the concentration of furans followed the same pattern. However, in one case exposure to light resulted in decomposition or a further reaction of 2-ethyl furan and 2-pentyl furan. 2-ethyl furan and 2-pentyl furan decreased after exposure to light compared with storage in darkness (data not shown).

The effect of storage conditions and the composition of the topical skin formulation on the product odour was examined. The changes in product odour were assessed using a triangle test. Most of the significant odour changes were observed in the prototype cleansing formulations. In the prototype lip care formulations, only a few significant changes were observed due to storage conditions and the formulations´ composition. However, the addition of aroma compounds to the prototype lip care formulations may have concealed the odour changes that are caused by volatile compounds formed due to lipid oxidation (paper I).

The significant changes observed in the prototype cleansing formulation 1-3 were examined in more details in an odour profiling. In prototype cleansing formulations 1 and 2, the odour profiling investigated the effect of lipid source on the product odour. The odour profiling also examined the effect of the metal chelator, caprylhydroxamic acid, on the product odour in prototype cleansing formulations 2 and 3 as described in section 4.2.2.
To investigate the effect of storage, odour profiles for the three prototype cleansing formulations 1-3 at various storage conditions are shown separately (Figures 18-20).
When comparing the effect of storage conditions on the attribute intensities in the prototype cleansing formulations, the effect of temperature and exposure to light were not the same in all three prototype cleansing formulations. Temperature had a larger effect on the attribute intensities in the prototype cleansing formulation 2 than in prototype cleansing formulation 1. Hence, elevated temperature gave rise to higher intensity in formulation 2. This difference was probably related to the different lipid sources used for the two products. Thus, prototype cleansing formulation 2 contained oils with more unsaturated FAs, which was more susceptible to autoxidation and therefore led to higher concentrations of volatile compounds. The higher concentrations of volatile compounds in prototype cleansing formulation 2 increased the intensity of the attributes (Schaich, 2005). Prototype cleansing formulation 2 developed the highest intensity of “carbamide” at 40°C and 50°C.

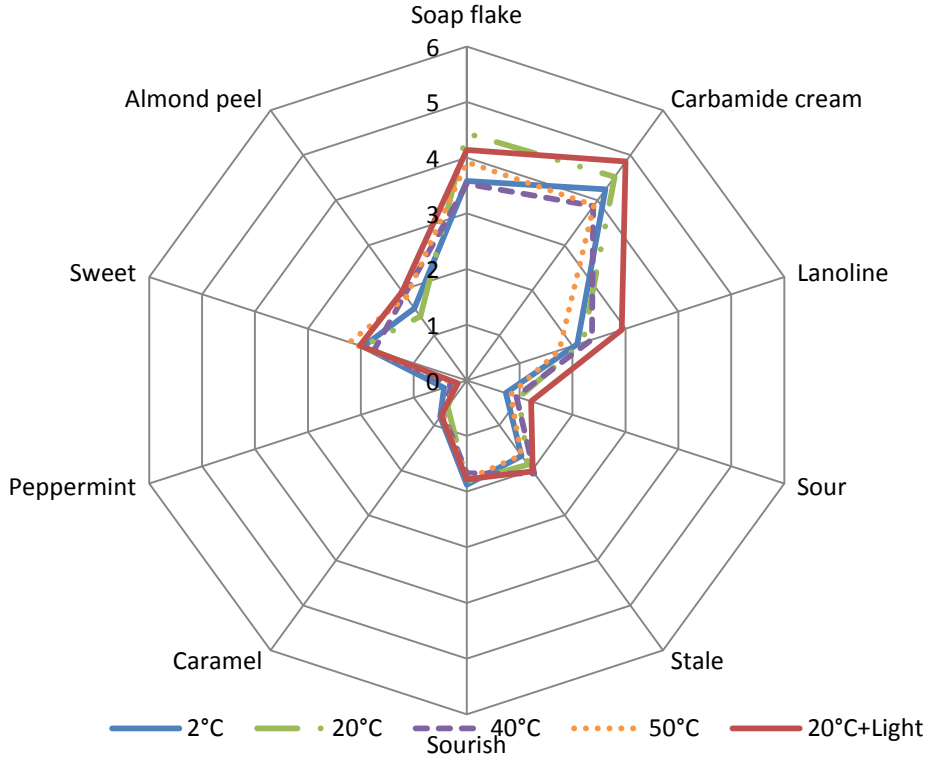


Figure 18. Odour profiling for prototype cleansing formulation 1, the intensity of attributes after eight weeks at 2°C, at 20°C, at 20°C with exposure to light, at 40°C and after two weeks at 50°C.

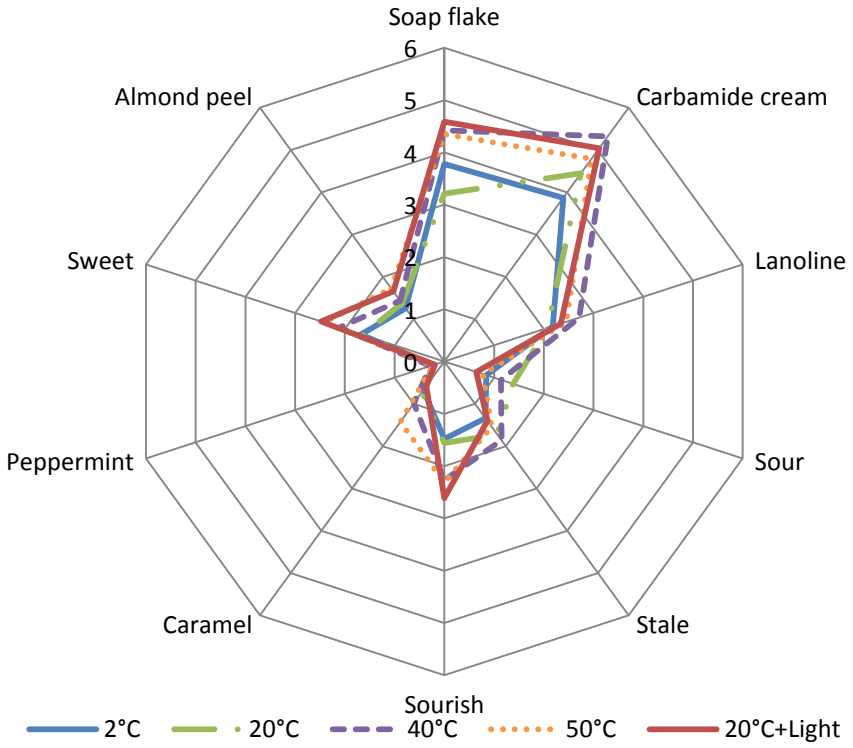


Figure 19. Odour profiling for prototype cleansing formulation 2, the intensity of attributes after eight weeks at 2°C, at 20°C, at 20°C with exposure to light, at 40°C and after two weeks at 50°C.

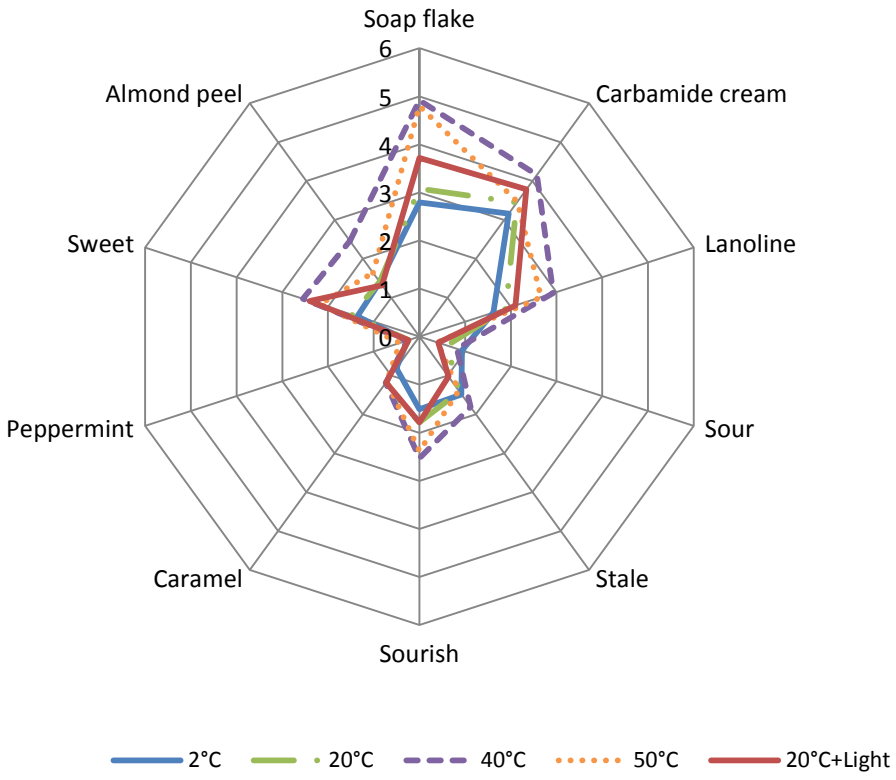


Figure 20. Odour profiling for prototype cleansing formulation 3, the intensity of attributes after eight weeks at 2°C, at 20°C, at 20°C with exposure to light, at 40°C and after two weeks at 50°C.

Similar to prototype cleansing formulation 1 and 2, the odour profile for prototype cleansing formulation 3 was affected by increased temperature to 40°C and 50°C. Both when comparing with storage at a lower temperature and when comparing with prototype cleansing formulations 1 and 2, prototype cleansing formulation 3 had the highest intensity for "soap flake", "sourish", "sweet" and "almond peel".

In prototype cleansing formulation 3, exposure to light resulted in the highest odour intensity of "caramel" and "sweet". Moreover, the dominating attributes in prototype cleansing formulation 1 and 2, "soap flake", "carbamide cream" and "lanoline", were not affected by light to the same extent as in formulation 3. Other studies have found antioxidative effects of metal chelators on photooxidation. Lee and Decker (2011) found a concentration-dependent effect of the metal chelator EDTA on the oxidative stability in O/W emulsions under photo-activated oxidation. In the study by Lee and Decker (2011), addition of a metal chelator led to a decreased formation of lipid hydroperoxides, hexanal, 2-heptenal, and 1-octen-3-ol. Consequently the same conclusion can be drawn in this study, the different attribute intensities observed between prototype cleansing formulation 2 and 3 stored with exposure to

light agree with the findings by Lee and Decker (2011). Accordingly, the presence of a metal chelator in prototype cleansing formulation 3 decreased photo-activated lipid oxidation.

5.1.2 Effect of lipid content (paper I, III and IV)

The effect of the overall lipid content was investigated by comparing two topical skin formulations: prototype lip care formulation 3 with a high lipid content at 36 % and prototype cleansing formulation 1 with a low lipid content at 8 %. Besides the different overall lipid content, the prototype lip care and cleansing formulations had a different lipid composition and contained different special additives depending on the formulation type. In addition to the effect of the overall lipid content, the different special additives and lipid composition can also affect the PV and volatile compounds. The PV was slightly yet significantly higher in prototype lip care formulation 3 after 84 days of storage at 2°C than in prototype cleansing formulation 1. In contrast to the pattern observed at 2°C, PV in prototype cleansing formulation 1 increased significantly more when exposed to light (Figure 21). No significant increase was observed in dark storage.

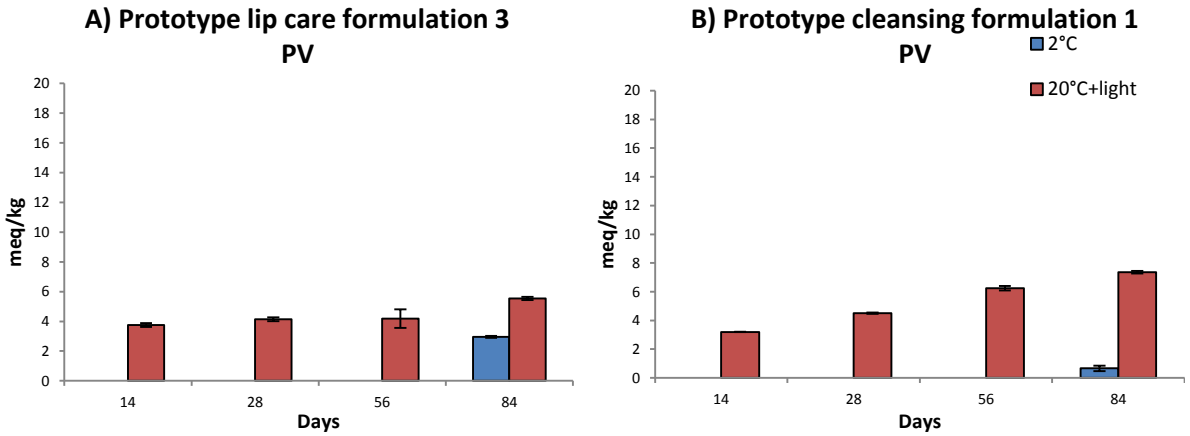


Figure 21. The progress of lipid oxidation measured by PV [meq/kg oil] in (A) prototype lip care formulation 3 and (B) prototype cleansing formulation 1. Samples were continuously taken for 84 days of storage at 2°C and at 20°C with exposure to light: after 14, 28, 56 and 84 days of storage.

The lower oxidative stability of the topical skin formulation with a low lipid content under storage with exposure to light was in accordance with results reported by Osborn and Akoh (2004). They found that decreased lipid content resulted in increased lipid oxidation in simple O/W emulsions. However, the opposite pattern was observed for secondary volatile oxidation products exemplified by pentanal when calculated in ng/g product (Figure 22).

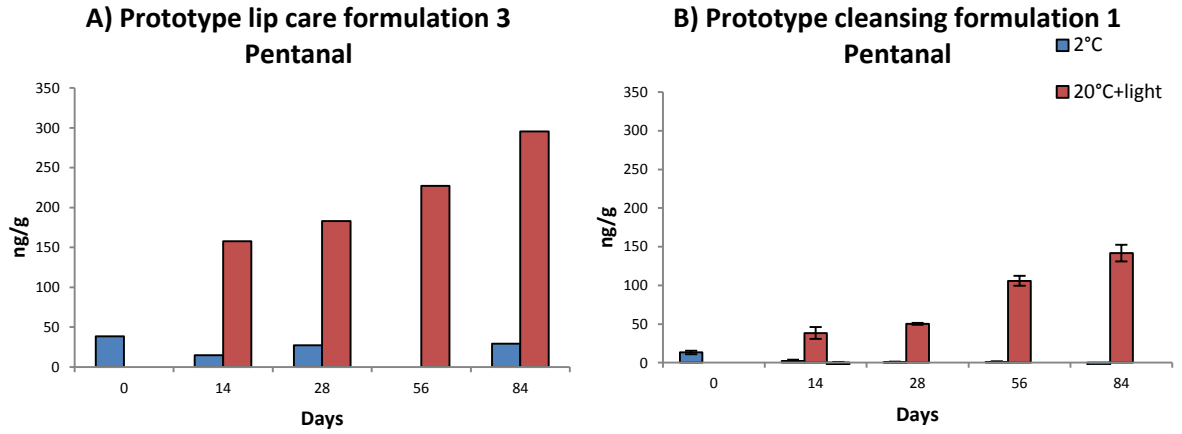


Figure 22. The progress of lipid oxidation measured by pentanal [ng/g product] in (A) prototype lip care formulation 3 and (B) prototype cleansing formulation 1. Samples were continuously taken for 84 days of storage at 2°C and at 20°C with exposure to light: after 14, 28, 56(only prototype cleansing formulation 1) and 84 days of storage.

There was a significant difference in the pentanal concentration between the topical skin formulations with high and low lipid content stored at 2°C and at 20°C with exposure to light. Moreover, the pentanal concentration increased most stored at 20°C with exposure to light in the topical skin formulations with high lipid content. The change in the pattern from PV to secondary volatile oxidation products for topical skin formulations may be related to a faster conversion from primary to secondary oxidation products in prototype lip care formulation 3. Therefore, oxidation was highest in the formulation that has a high lipid content. Yet, the opposite was observed per gram lipid. Nevertheless, a recalculation of the pentanal concentration at day 84 from ng/g product to ng/g lipid resulted in a change in the ranking of the samples. The concentration of pentanal for prototype lip care formulation 3 and prototype cleansing formulation 1 changed from 295.7 ng/g product to 8.2 ng/g lipid and from 141.7 ng/g product to 17.7 ng/g lipid, respectively. Consequently, the additional conclusion is that the unsaturated FAs were better protected in the oil droplets of the formulation having a high lipid content.

Moreover, comparison of PV results from prototype cream, cleansing and serum formulations (papers III and IV) showed the same result, namely, that the oxidative stability was lower for topical skin formulations that have a low lipid content. In the topical skin formulations with a low lipid content, PV increased to 21.9 meq/kg oil, whereas in the topical skin formulation with a high lipid content PV only increased to 1.44 meq/kg oil. Again, the volatile oxidation and degradation products revealed that the oxidative stability was lowest in topical skin formulations with a high lipid content.

The effect of the lipid content in O/W emulsions has been investigated in several studies (Nielsen *et al*., 2013; Horn *et al*., 2011; Osborn and Akoh, 2004). In simple emulsions, they found increasing oxidation with decreasing lipid content. Moreover, these studies found that various factors such as pH and emulsifier also affect the extent of lipid oxidation significantly and this could also be the in topical skin formulations. Hence, the results were the opposite result to the studies in simple emulsions. In the present study, oxidation was highest in the formulations with a high lipid content. This may be related to other factors than lipid content. Hence, topical skin formulations were produced using a high number of different raw materials, which may make it difficult to compare the effect of the lipid content in different types of formulations. Therefore, more studies that investigate the effect of each raw material in topical skin formulations are needed to explain the observed results.

In addition to the effect of the overall lipid content, the effect of small variations in lipid type was also investigated in prototype cleansing formulations 1 and 2. Compared with prototype cleansing formulation 1, prototype cleansing formulation 2 contained no butyrospermum parkii butter and a reduced amount of caprylic/capric triglycerides. Instead, vegetable and olive oils, which contained more unsaturated FAs, were added to prototype cleansing formulation 2. More details are available in section 4.2.2.
There was no significant difference in the PV between the prototype cleansing formulations stored at 2°C. However, storage at 20°C with exposure to light resulted in a significant difference was observed between prototype cleansing formulations 1 and 2 (Figure 23).

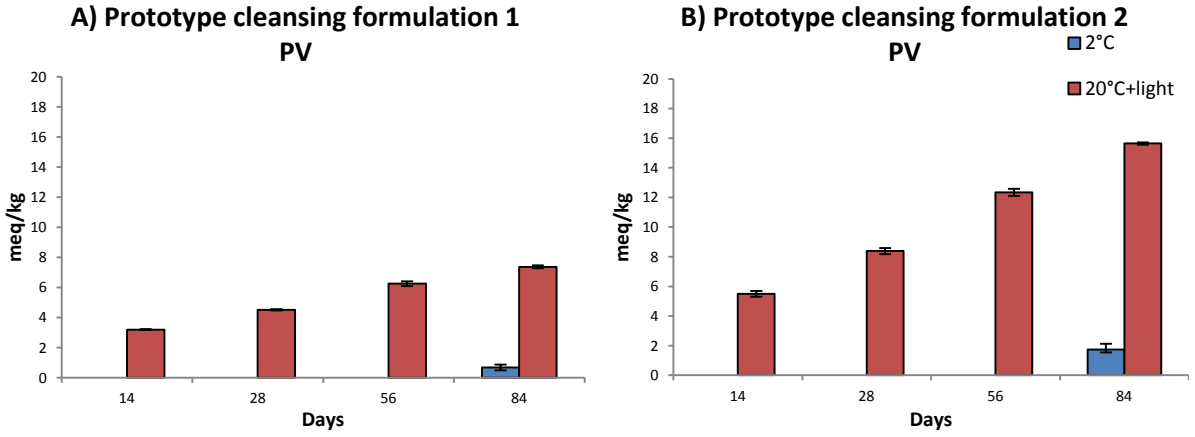


Figure 23. The progress of lipid oxidation measured by PV [meq/kg oil] (A) prototype cleansing formulation 1 and (B) prototype cleansing formulation 2. Samples were continuously taken for 84 days of storage at 2°C and at 20°C with exposure to light: after 14, 28, 56 and 84 days of storage.

As with the effect of the overall lipid content, the opposite ranking was observed for the secondary volatile oxidation products exemplified by heptanal (Figure 24). This point

towards that PV alone did not offer an adequate assessment of the extent of lipid oxidation. Hence, PV did not provide the knowledge about the development of odour-affecting volatile compounds. The conversion rate from primary to secondary volatile oxidation products may differ between topical skin formulations. Consequently, PV cannot be used as the only marker for lipid oxidation.

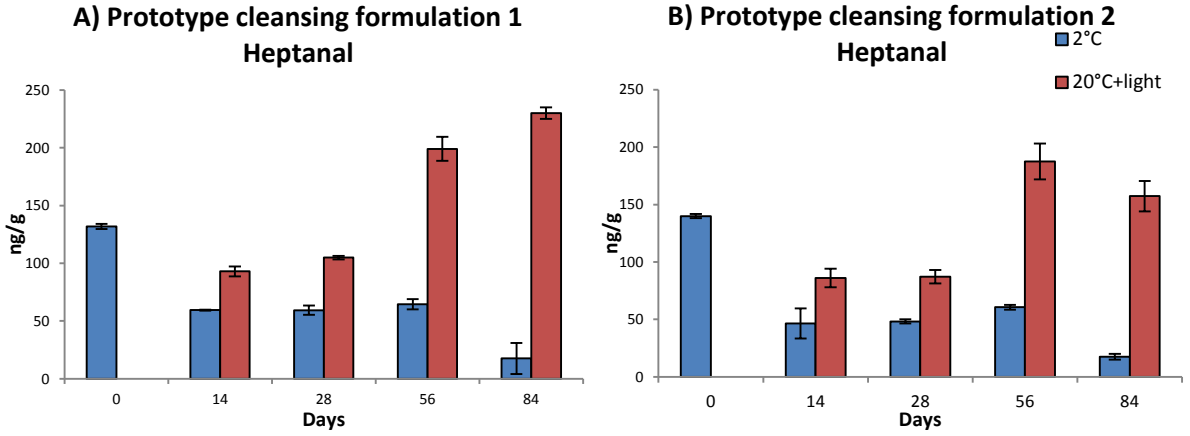


Figure 24. The progress of lipid oxidation measured by heptanal [ng/g] in (A) prototype cleansing formulation 1 and (B) prototype cleansing formulation 2. Samples were continuously taken for 84 days of storage at 2°C and at 20°C with exposure to light: after 14, 28, 56 and 84 days of storage.

The concentration of heptanal was not significantly different in prototype cleansing formulations 1 and 2 stored at 2°C. Moreover, until day 56, no significant difference was observed between the prototype cleansing formulations stored at 20°C with exposure to light. After 84 days of storage, a significantly higher concentration was observed for heptanal in prototype cleansing formulation 1.

Overall, the odour attributes for the three prototype cleansing formulations were the same (Figure 25). Prototype cleansing formulation 1 contained more saturated FAs than the two other protype cleansing formulations. Unsaturated FAs are more suceptible to lipid oxidation. The prototype cleansing formulations 1 and 2 stored at 2°C were similar in the sensory attributes. These findings fit with the PV and concentration of heptanal at this temperature (Figures 23 and 24). Furthermore, these results show that a small increase in the degree of unsaturated FAs does not increase lipid oxidation significantly when stored at low temperature.

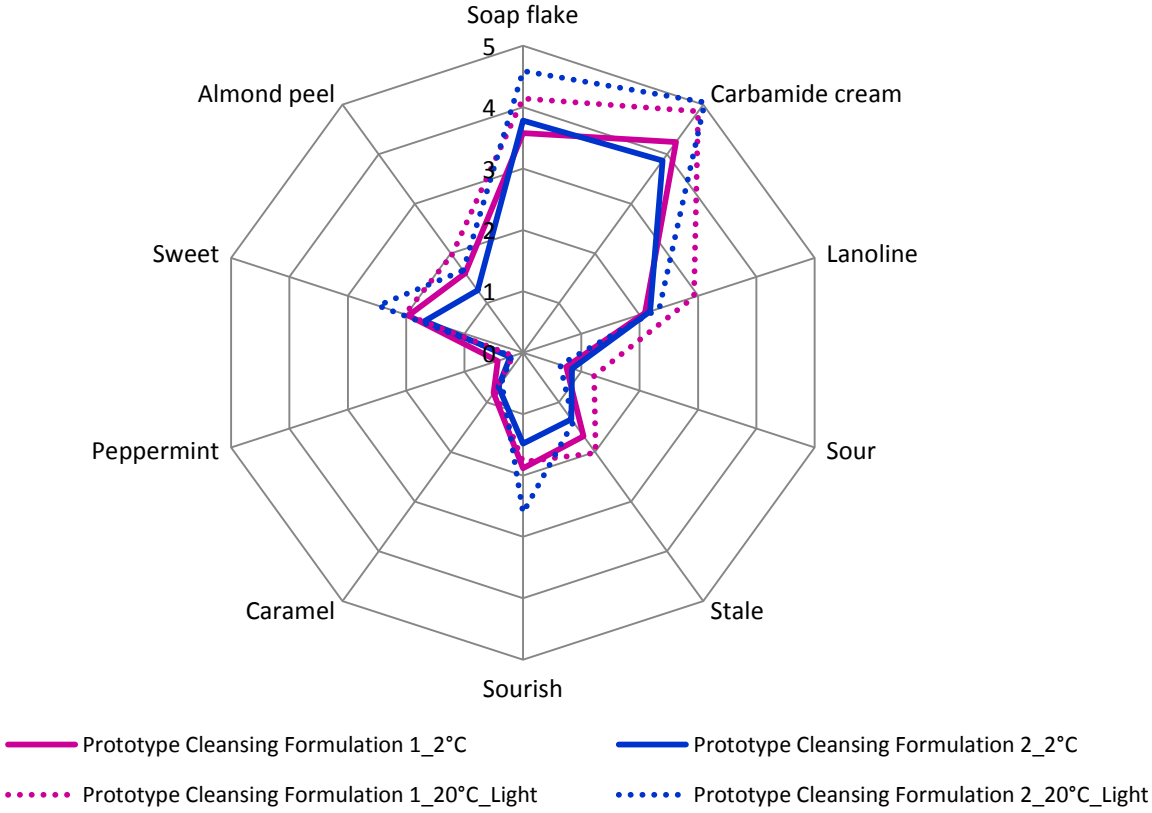


Figure 25. Intensity of attributes in prototype cleansing formulations 1 and 2 after eight weeks at 2°C and at 20°C with exposure to light.

In general, exposure to light increased the intensity of the attributes both in prototype cleansing formulations 1 and 2. The highest intensities of odour were observed for "soap flake" and "carbamide" in prototype cleansing formulation 2. The intensity was fairly similar for prototype cleansing formulations 1 and 2 stored at 2°C. However, exposure to light changed the attributes´ intensities. Small differences in attributes´ intensities were observed between prototype cleansing formulations 1 and 2. Namely, an increase detected in intensities of "lanoline" and "sour" for prototype cleansing formulation 1 and "sourish" for prototype cleansing formulation 2, respectively.

Partial least squares regression analysis related the attributes that increased in intensity to volatile compounds that increased in concentration. Two tendencies were observed (Figure 26, markers A and B): Figure 26A, pentanal was associated with the attribute "carbamide" and Figure 26B, butane nitrile was related to the attributes "sourish" and "peppermint".

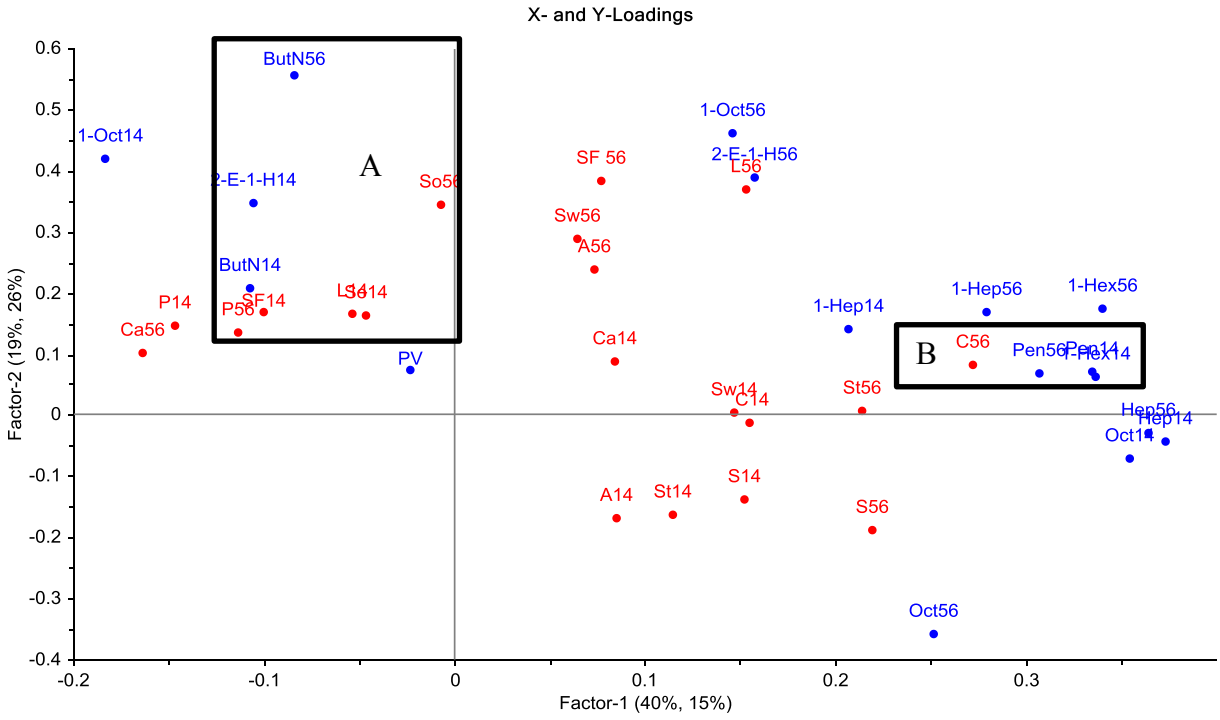


Figure 26. Partial least squares regression where all data were included. Data from day 14 are marked 14, and volatile compounds are 56 on day 56. Abbreviations used in the plot for sensory attributes; Lanoline(L), Sour(S), Sourish(So), Soap flake(SF), Peppermint(P), Caramel(Ca), Sweet(Sw), Almond peel(A), Carbamide cream(C) and Stale(St). Abbreviations used in the plot for volatile compounds: 2-ethyl-1-hexanol(2-E-1-H), 1-octanol(1-Oct),Butane nitrile(ButN),1-heptanol(1-Hep), 1-hexanol(1-Hex), pentanal(Pen), Heptanal(Hep) and Octanal(Oct). Adopted from paper I.

The odour change related to pentanal addition has been reported as "woody", "pungent" and "fruity" (Fenaille *et al.*, 2003). This was quite different from "carbamide". Therefore, pentanal was added to a prototype cleansing formulation to evaluate changes caused by pentanal addition. The attribute "carbamide" did not appear when 150 ng/g was added, which was above the concentration measured in the prototype cleansing formulations. Accordingly, pentanal was not related to the appearance of "carbamide" alone.

The odour change related to butane nitrile addition has been reported as a "bitter almond-like odour" (Wright and Serenius, 1954; Pollak *et al.*, 2012). Butane nitrile seemed to be associated with "sourish" and "peppermint" attributes. Again, a simple evaluation where 100 ng/g of butane nitrile was added to a prototype cleansing formulation was conducted. The evaluation showed that butane nitrile addition resulted in a sweet almond odour. Consequently, butane nitrile was not related to the appearance of the attributes "sourish" and "peppermint" alone. The attributes "carbamide", "sourish" and "peppermint" may be due to a combination of several volatile compounds, which gave rise to these attributes. A combination of several volatile compounds was also found to cause the attributes changing in

fish-oil-enriched milk in a study by Venkateshwarlu *et al*. (2004). This may also be the case in this study.

5.2 The effect of volatile compounds on product odour and the formation of off-odour (paper II)

The chemical methods such as PV, high-performance liquid chromatography (HPLC) and GC-MS only result in a number without an odour description or impact. The individual description or impact on product odour of selected volatile compounds that increased under storage was interesting to examine. To investigate the odour description and impact, sensory evaluation was performed. The sensory studies were designed to determine the effect on the overall odour and to describe the odour profile of volatile compounds in topical skin formulations. Moreover, the ODT value was determined in both topical formulations with high and low lipid content to evaluate the impact of specific volatile compounds when added alone.

Six volatile compounds: two alcohols (3-methyl-1-butanol and 1-heptanol), two furans (2-ethyl furan and 2-pentyl furan) and two aldehydes (butanal and pentanal) were selected for ODT determination (Table 12). The ODT value and odour description were determined both in topical skin formulations with a low and high lipid content because the matrix effect and initial concentration of the volatile compound may be different.

Table 12. Odour detection threshold values for the prototype day cream formulation and the prototype cleansing formulation 4.

Volatile	**Prototype day cream formulation**		**Prototype cleansing formulation 4**	
	ODT avg (added ng/g[1])	GC-MS (total ng/g[2])	ODT avg (added ng/g[1])	GC-MS (total ng/g[2])
3-Methyl-1-butanol	315.65	1926 ± 316	203.10	394 ± 17
1-Heptanol	56.05	155 ± 24	47.90	170 ± 23
2-Ethyl furan	59.35	125 ± 15	52.65	75 ± 9
2-Pentyl furan	77.60	79± 13	53.85	292 ± 41
Butanal	46.20	72 ± 3	112.55	130 ± 10
Pentanal	45.75	87 ± 5	19.90	100 ± 6

1. The amount added to the formulation. 2. The total amount including both initial concentration and added amount of the volatile compound measured by GC-MS

The first alcohol, 3-methyl-1-butanol, resulted in a more sharp and unpleasant odour resembling cleaning agent in the prototype day cream formulation compared to the prototype cleansing formulation 4 (Table 13). The more sharp and unpleasant odour may be related to an initial high concentration in the prototype day cream formulation if the added concentration was compared to the concentration determined by GC-MS (Table 12). The trained sensory panel´s description of 3-methyl-1-butanol in topical skin formulation was quite different from the odour description reported in other studies. Other studies described the odour of 3-methyl-1-butanol in water as balsamic, whiskey, malt or burnt (Acree and Arn, 2016; Fukami *et al*., 2002; Giri *et al*., 2010; Burdock, 2002). Similar to the odour description, the ODT value was almost ten times higher in the prototype day cream formulation than in water. This large difference highlighted the importance of considering the matrix effect and considering not to apply ODT for water or oil in complex systems.
The second alcohol, 1-heptanol, delivered a sweet coconut odour in both topical skin formulations with a high and low lipid content (Table 13). These pleasant sweet odours described for 1-heptanol were different to the mushroom odour in water (Acree and Arn, 2016). GC-MS analysis showed that the total concentration measured in the topical skin formulations was more than three times higher than the added amounts (Table 12).

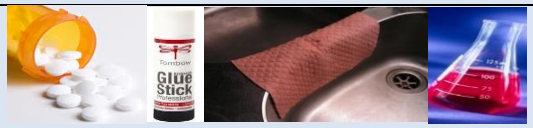
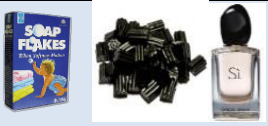
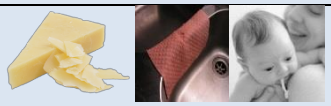
The first furan, 2-ethyl furan, gave rise to a sweet vanilla odour in both topical skin formulations. In the prototype day cream formulation, it also contributed with a stearin odour. The pleasant odour effect of 2-ethyl furan on product odour in topical skin formulation was not comparable to the odour effect in water, though the odour descriptor stearin may be associated to rubber and burnt, however not associated with pungent (Buttery *et al*., 1969; Giri *et al*., 2010). Moreover, GC-MS analysis showed that the total concentration in the topical skin formulation was more than two times higher than the added amount for prototype cleansing formulation 4. Nevertheless, the total concentration was almost the same for prototype day cream formulation (Table 12).
In contrast to the sweet odour of 2-ethyl furan, 2-pentyl furan provided an earthy, moss and mushroom odour in the prototype day cream formulation. However, it resulted in a markedly different odour in the prototype cleansing formulation 4. Here, it provided a perfumed, soap flake and liquorice odour (Table 13). These odour effects in topical skin formulation were similar to the odour effects reported in water. In water, the odour effects were specified as being grassy, liquorice, green, bean and butter (Buttery *et al*., 1969; Acree and Arn, 2016; Giri *et al*., 2010; Yan *et al*., 2010). The ODT value determined by GC-MS was the same as

the spiked amount in the prototype day cream formulation, yet four times higher than the spiked amount in the prototype cleansing formulation 4 (Table 12).

The first aldehyde, butanal, provided a cheese-like and slightly sour odour in both topical skin formulations. However, the sourness odour was an unpleasant sourness described as sour dishcloth and baby regurgitation in the prototype day cream formulation. Meanwhile in the prototype cleansing formulation 4, the sourness odour was described as a pleasant citrus sourness (Table 13). Both descriptions are slightly different from the pungent and green odour reported in water (Acree and Arn, 2016). The ODT value determined by GC-MS was almost equal to the added amount in the prototype day cream formulation, yet almost two times higher in the prototype cleansing formulation 4 (Table 12).
The second aldehyde, pentanal, gave rise to a green basis odour in both topical skin formulations. However, it also provided two rather different side odours: flower in the prototype cleansing formulation 4 vs. milk acidic raw milk/colostrum and cowshed in the prototype day cream formulation, respectively. The rather different side odours may be related to the products' basis odour, which also may explain why the odour differs from its description in water. In water, the odour effects were described as almond, malt, strawberry, fruit and tomato (Buttery *et al*., 1969; Acree and Arn, 2016).
The odour descriptions of the alcohols, 2-ethyl furan and the aldehydes were quite different compared with the descriptions in literature of the pure volatile compounds in water. However, for 2-pentyl furan, the odour effects in topical skin formulation were similar to the odour effects reported for water in literature.

Overall, the matrix had an impact both on the odour descriptors and the ODT value of volatile compounds. The same conclusion was also reached by Costa *et al*. (2016) that the release is strongly influenced by the cosmetic matrices.
The ODT values for 1-heptanol, 2-ethyl furan and 2-pentyl furan were higher than the concentration in topical skin formulations. Therefore they will not be considered further in this thesis.

Table 13. Odour description by the trained sensory panel of the six volatile standards when added to prototype day cream formulation and prototype cleansing formulation 4.

Volatile standard	Prototype day cream formulation	Prototype cleansing formulation 4
3-Methyl-1-butanol	 Glue, rubber, chemical, medicine and cleaning agent	 Medicine, glue, sour dishcloth and chemical
1-Heptanol	 Perfume, lime / citrus juice, sweet and coconut	 Coconut, dried banana, caramel and condensed milk
2-Ethylfuran	 Stearin, white chocolate and artificial vanilla	 Crème anglaise, artificial vanilla, white chocolate and caramel
2-Pentylfuran	 Mushroom, moss, soil and forest soil	 Soap flakes, liquorice and perfume
Butanal	 Parmesan, sour dishcloth and baby regurgitation	 Parmesan, sour and sickly sweet
Pentanal	 Green, milk acidic, raw milk and cowshed	 Flower, green and bark

5.3 Raw materials oxidative stability (papers III and IV)

The following section investigates the initial content and formation of volatile oxidation products in lipid-containing raw material used at concentrations above one percent. In addition, raw materials that may contain nitrile groups, or impurities that can be related to the appearance of butane nitrile, were investigated.

5.3.1 Volatile compounds that appeared under accelerated storage in prototype serum and skin cream formulations

Several volatile compounds appeared in prototype serum and skin cream formulations as shown and discussed in section 5.1 and 5.2. Especially, the concentration of one alcohol 3-methyl-1-butanol and several volatile aldehydes, including butanal and pentanal, increased.

The concentrations of butanal and pentanal in prototype serum formulation (Figure 27) were above odour threshold values determined in topical skin formulation with a low lipid content. Moreover, the concentration of 3-methyl-1-butanol in prototype serum formulations (Figure 17) was above odour threshold value determined in topical skin formulation with a high lipid content.

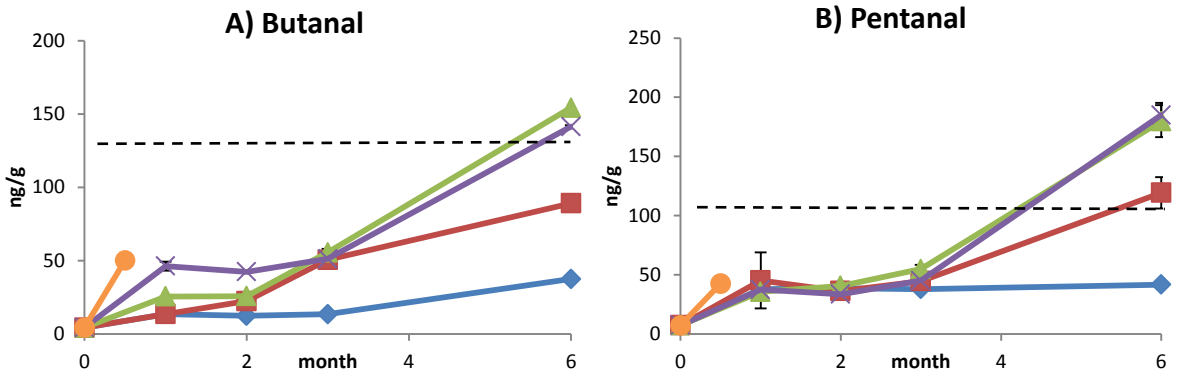


Figure 27. The development of volatile compounds: butanal (A) and pentanal (B) in prototype serum formulation [ng/g] stored at 2°C (◆), at 20°C (■), at 20°C+ligth (▲), at 40°C (×) and at 50°C (●). The dotted line indicates the exact ODT value [ng/g].

In addition to the well-known volatile compounds nitrile-containing compounds appeared in prototype skin cream formulation, as mentioned earlier. These nitrile-containing compounds have not been reported to occur in earlier studies of lipid oxidation in emulsions. One of the nitrile-containing compounds was butane nitrile. The butane nitrile concentration increased more with increasing temperature (Figure 17). Since, butane nitrile may cause skin irritation at high levels (above the detected level) the raw material responsible must be identified.

5.3.2 Effect of raw materials on the formation of volatile compounds responsible for off-odour in topical skin formulations

A large number of volatile compounds appeared under accelerated storage of the raw material (papers III and IV). However, only the volatile compounds present above the ODT value and butane nitrile will be discussed in this thesis: Butanal, pentanal, 3-methyl-1-butanol and butane nitrile.

Butanal appeared in several raw materials. Initially, the largest concentration was observed in caprylic/capric triglyceride, squalane and isostearyl isostearate (Figure 28).

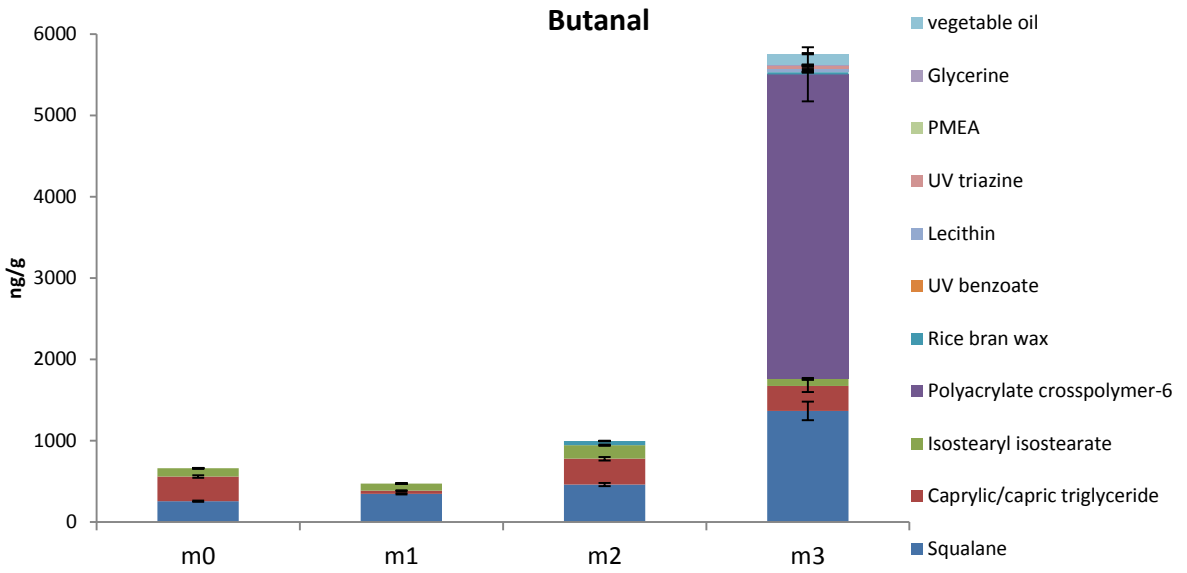


Figure 28. Butanal appeared and increased in several raw materials stored for three months (m). The bars show both the amount in each raw material (colour codes) and the total amount [ng/g] (papers III and IV).

Under accelerated storage, concentrations of butanal increased in several raw materials, especially, polyacrylate crosspolymer-6, squalane and vegetable oil to concentrations above ODT value and 100 ng/g.

However, butanal also appeared in glycerine, bis-ethylhexyloxyphenol methoxyphenyl triazine (UV triazine) and hexyl 2-(1-(diethylaminohydroxyphenyl)methanoyl)benzoate (UV benzoate), lecithin, PMEA and rice bran wax at concentrations lower than 100 ng/g.

The second aldehyde, pentanal, was initially present in lecithin, caprylic/capric triglyceride and isostearyl isostearate (Figure 29). At the end of the storage period, concentrations of pentanal in rice bran wax, polyacrylate crosspolymer-6, isostearyl isostearate, caprylic/capric triglyceride, butyrospermum parkii butter, vegetable oil and coconut oil increased above ODT value and 100 ng/g.

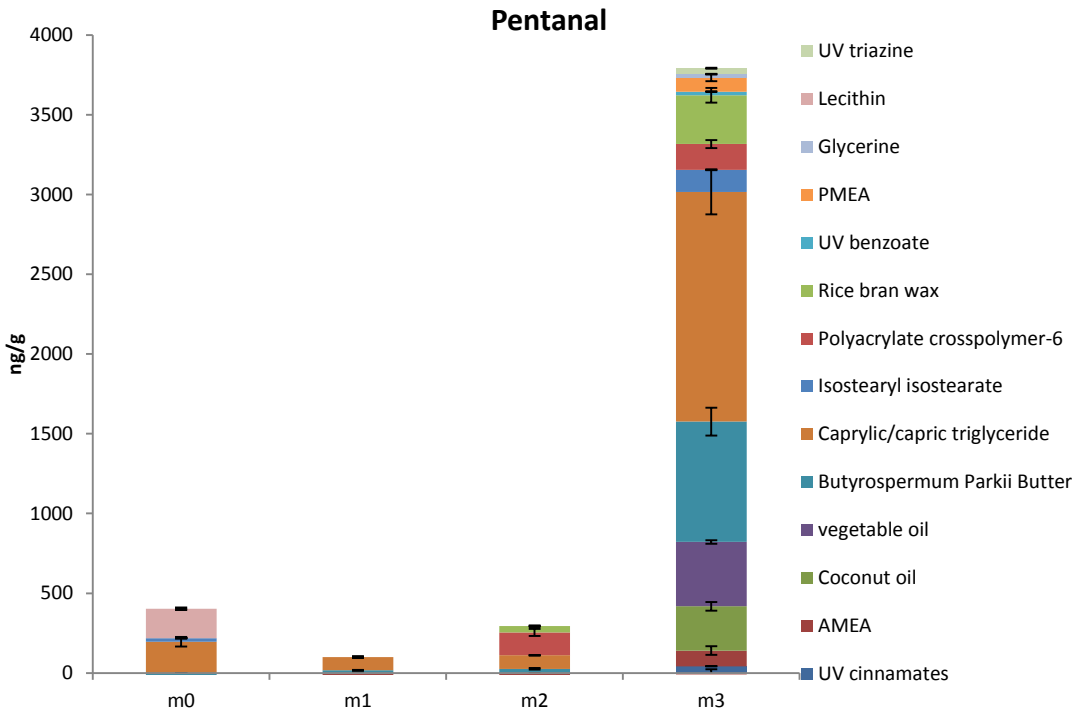


Figure 29. Pentanal appeared and increased in several raw materials stored for three months (m). The bars show both the amount in each raw material (colour codes) and the total amount [ng/g] (papers III and IV).

Although, the concentration of the remaining raw materials in which butanal appeared did not increase above the ODT value, they may contribute to the concentration of butanal in the topical skin formulations.

The same pattern that the concentration of the volatile compound increased under accelerated storage was observed for other aldehydes: 3-methylbutanal, hexanal, octanal and benzaldehyde (data not shown).

The alcohol, 3-methyl-1-butanol, was present initially above ODT value and increased even more after accelerated storage in topical skin formulations. The topical skin formulations' content of 3-methyl-1-butanol was linked to the raw material, isoamyl p-methoxycinnamate (UV cinnamate). UV cinnamate had a large concentration initially and increased even further under accelerated storage. Moreover, the raw material, glycerine, also had a low concentration of 3-methyl-1-butanol. However, the concentration of 3-methyl-1-butanol remained low in glycerine under accelerated storage in. Furthermore, 3-methyl-1-butanol appeared in a few other raw materials; isostearyl isostearate, caprylic/capric triglycerides, butyrospermum parkii butter, AMEA and glycerol. However, the concentrations were

remarkably lower than in UV cinnamate (barely noticeable on the bars) and below ODT value (Figure 30).

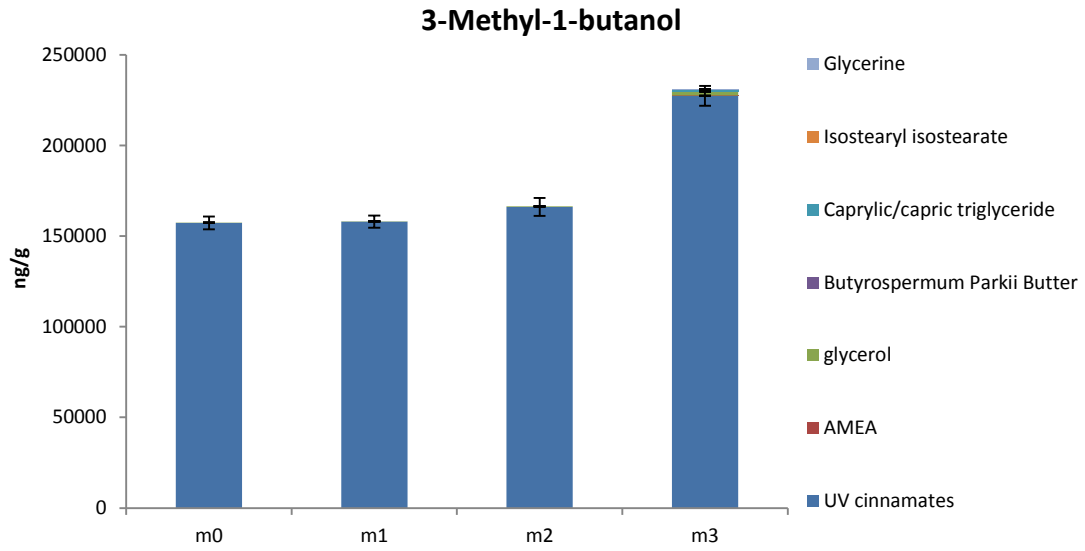


Figure 30. 3-Methyl-1-butanol appeared and increased in several raw materials stored for three months (m). The bars show both the amount in each raw material (colour codes) and the total amount [ng/g] (papers III and IV).

Butane nitrile did not appear in any of raw materials. However, a nitrile-containing compound, tetramethylbutanedinitrile, appeared in polyacrylate crosspolymer-6. Polyacrylate crosspolymer-6 was the only raw material where a nitrile-containing compound appeared. Under accelerated storage, tetramethylbutanedinitrile increased up to a response/g at 10438 ± 65 after two months storage.

To sum up the results, the raw materials that contained butanal, pentanal, 3-methyl-1-butanol and butane nitrile are summarized in Table 14.

The concentration of butanal increased in several raw materials. Moreover, butanal increased significantly and above the ODT value in three raw materials: polyacrylate crosspolymer-6, squalane and vegetable oil. Therefore, the presence and increasing amount of butanal in topical skin formulation were especially related to the use of these raw materials.

Compared to butanal, the concentration of pentanal increased above ODT value at the end of accelerated storage in even more raw materials. The raw materials that increased most in pentanal concentration were: rice bran wax, polyacrylate crosspolymer-6, isostearyl isostearate, caprylic/capric triglyceride, butyrospermum parkii butter, vegetable oil and coconut oil.

The concentration of 3-methyl-1-butanol increased above ODT value in only one raw material, namely UV cinnamate, which increased to a concentration more than 100 times above ODT value.

Lastly, butane nitrile did not appear in any raw materials. However, tetramethylbutanedinitrile appeared in polyacrylate crosspolymer-6. The link between butane nitrile and tetramethylbutanedinitrile will be studied in more detail in the following section.

Table 14. Summary of butanal, pentanal, 3-methyl-1-butanol and butane nitrile present in raw materials. + = present, ++ = present above ODT value in raw material (only available for butanal, pentanal and 3-methyl-1-butanol), and - = absent.

Volatile compounds/ Raw material	Butanal	Pentanal	3-Methyl-1-butanol	Butane nitrile
Vegetable oil	++	++		
Glycerine	+	+	+	
PMEA	+	+		
UV triazine	+	+		
Lecithin	+	+		
UV benzoate	+	+		
Rice bran wax	+	++		
Polyacrylate crosspolymer-6	++	++		?
Isostearyl isostearate	+	++	+	
Caprylic/capric triglycerides (Tricaprylin and tricaprin)	+	++	+	
Squalane	++			
Butyrospermum parkii butter (Shea butter)		++	+	
Coconut oil		++		
AMEA		+	+	
UV cinnamate		+	++	
Glycerol			+	

5.3.3 Reactions leading to most important volatile compounds

In this section, the reactions leading to the formation of butanal, pentanal, 3-methyl-1-butanol and butane nitrile will be investigated. Throughout this investigation, possible reactions and the likelihood for them to occur under the storage conditions was examined.

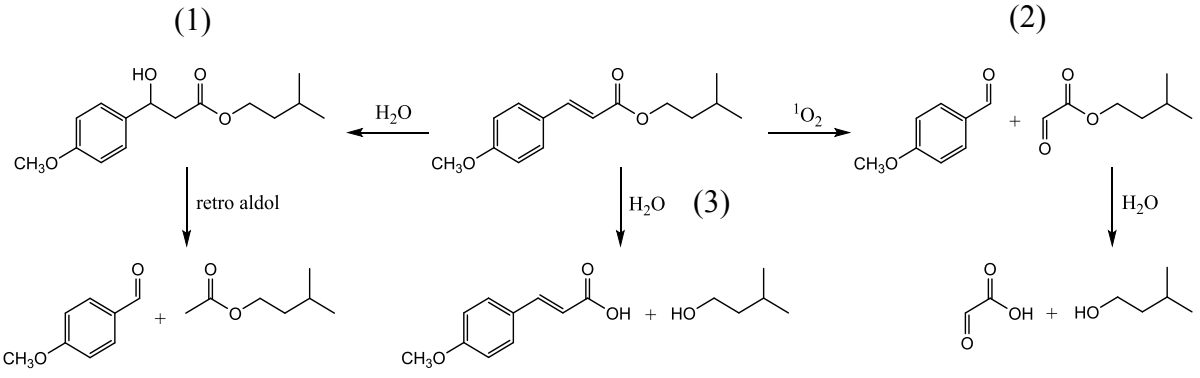
Butanal and pentanal were initially present or appeared under accelerated storage in several lipid-containing raw materials. Both, butanal and pentanal are well-known lipid oxidation products that most likely appeared due to autoxidation and/or photooxidation (section 3.1).

For 3-methyl-1-butanol, the high concentration in prototype skin cream formulation was clearly linked to the raw material UV cinnamate. The use of UV cinnamate resulted in a 3-methyl-1-butanol concentration above ODT value at 1926 ± 316 ng/g. Concentrations above ODT value was described by the trained sensory panel to give rise to an unpleasant odour (section 5.2). The route of reactions leading to 3-methyl-1-butanol is the key to find strategies to prevent it from affecting product odour.

In literature, two reaction routes were suggested to occur in a cinnamate-containing compound under photolytic conditions. Both reaction routes were reactions that occurred on the double bond located close to benzene. The double bond reactions occurred by addition of water followed by a retro-aldol reaction (1) or with singlet oxygen (2) (MacManus-Spencer *et al*., 2011). In our case, these reaction routes would lead to the following end-products from UV cinnamate: 4-methoxybenzaldehyde and isoamyl acetate from reaction route (1) and the corresponding aldehydes from reaction route (2) (Scheme 1).

Reaction route (1) was a possible reaction route. However reaction route (1) was very slow, probably too slow to be the main reaction route in our case (Widhalm *et al*., 2015).

In contrast to the slow reaction kinetics for reaction route (1), reaction route (2) was probably labile enough to hydrolyse completely at room temperature (DellaGreca *et al*., 2008; Murthy *et al*., 2009; Matsumoto *et al*., 1996; Brachais *et al*., 1999). In addition to the two reaction routes discussed above, a third reaction route was also so possible, namely direct hydrolysis of UV cinnamates. Hence, the direct hydrolysis of UV cinnamates would result in carboxylic acid and 3-methyl-1-butanol (3) (Scheme 1).



Scheme 1. Potential pathways for cleavage of isoamyl p-methoxycinnamate (UV cinnamate) (paper III).

Thus, multiple reaction routes were conceivable from UV cinnamate to 3-methyl-1-butanol. Moreover, the different by-products from the reaction routes might pinpoint the main reaction route leading to 3-methyl-1-butanol and were therefore assessed. Accordingly, the two by-products, 4-methoxybenzaldehyde and isoamyl acetate, can be used to distinguish between the two reaction routes (1) and (2). In contrast to the volatile by-product from reaction route (1) and (2), the by-product formed in reaction route (3) was not volatile enough to be detected in our method. Nevertheless, both by-products formed in (1) and (2) were present in the chromatograms from prototype skin cream formulation and UV cinnamates. However, the concentrations were much lower than that of 3-methyl-1-butanol. The lower amount of the by-products from reaction route (1) and (2) decreased the probability of these being the main reactions.

As a consequence, the last suggested reaction route may be the main reaction, namely, a direct hydrolysis of UV cinnamate leading to the formation of 3-methyl-1-butanol. However, the by-products from reaction route (3) were not volatile enough to be detected in our methods. An alternative way to assess the probability of direct hydrolysis was to calculate the amount formed due to direct hydrolysis based on kinetics reported in literature. Moreover, the half-life for hydrolysis of ethylcinnamate in water at pH 4.0 and room temperature was reported to be about 100 years (Rayne and Forest, 2016). However, hydrolysis is slower in a non-polar environment leading to a higher half-life. In our case, the 3-methyl-1-butanol concentration in UV cinnamate (Figure 30) only corresponds to approximately 0.2 ‰ (w/w). Moreover, the amount that can be formed after three months due to direct hydrolysis is 2.5 ‰ (w/w) in a polar environment. Consequently, direct hydrolysis of the ester-group in the UV cinnamate was suggested to be the main reaction route (3) leading to the formation of 3-methyl-1-butanol.

For butane nitrile, none of the raw materials contained any of the detected nitrile-containing compounds: butane, pentane, hexane or octane nitrile. However, the raw material polyacrylate crosspolymer-6 increased in the amount of one nitrile-containing compound, tetramethylbutanedinitrile. Tetramethylbutanedinitrile has been reported to be a by-product from plast or polymer production (Bevington, 1954; Krstina *et al*., 1993). This led to two possible explanations for the appearance of nitrile-containing compounds: (1) a migrant from the plast packaging material, and (2) a by-product from polymer production. Moreover, tetramethylbutanedinitrile can decompose into smaller nitrile-containing compounds (Figure 31).

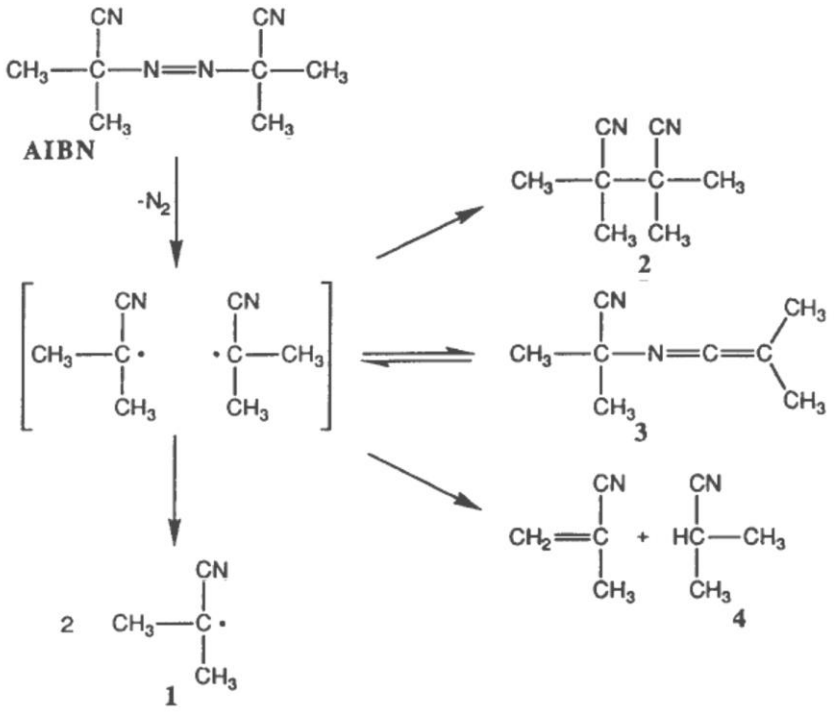


Figure 31. Azobisisobutyronitrile decomposes to form 1. 2-Cyanoprop-2-yl radicals, 2. Tetramethylbutanedinitrile, 3. Dimethyl-N-(2-cyanoprp-2-yl)ketenimine and 4. Isobutane nitrile. Adopted for Krstina *et al*. [28].

Option (1) that nitrile-containing compounds are migrants from the plastic packaging material. seemed unlikely as nitrile-containing compounds appeared in topical skin formulations both stored in plastic and glass packaging material.

Option (2) polymerisation was catalysed by initiators, among the initiators are azobisisobutyronitrile (AIBN). After initiating polymerisation, AIBN has been reported to decompose to 2-cyanoprop-2-yl radicals or/and tetramethylbutanedinitrile. Moreover, inseveral studies of the decomposition of AIBN, the appearance of isobutane nitrile has been reported. Isobutane nitrile is a secondary by-product from AIBN decomposition (Bevington, 1954; Krstina *et al*., 1993). However, the absence of isobutane nitrile in our chromatograms was obscure as the reaction route leading to isobutane nitrile was better resonans stabilised compared to the detected butane nitrile (Bennett *et al*., 1970). Therefore, no conclusion can be drawn for the appearance of the detected nitriles. More studies are needed to explain this mystery.

5.4 *Fucus vesiculosus* extract in facial moisturiser prototype (paper VI)

AOs are able to terminate lipid oxidation as described in chapter three. Moreover, AOs also function as antiaging agent. One source of multiple AOs is *Fucus vesiculosus*. *Fucus vesiculosus* extracts contain multiple AOs including carotenoids and phlorotannins. The

effect of adding *Fucus vesiculosus* extract on the physical and oxidative stability in facial moisturiser prototype was evaluated. Two *Fucus vesiculosus* extracts, AE and WE were examined in two concentrations 1 and 2 mg/g.

Physical stability

The physical stability was assessed by measuring changes in droplet size. The droplet size was measured as the surface area mean diameter that 90% of the droplets were smaller than (D (0.9)). An increasing D (0.9) is often related to instability in the emulsion due to interfacial interactions that can result in flocculation and coalescence (McClements, 2005). At storage with exposure to light, a tendency toward an increased droplet size was observed compared to storage in dark. Especially, AE2 increased in D (0.9) from 10.80 µm when storage at 20°C to 29.88 µm when storage at 20°C with exposure to light.
At high temperature, a significant increase in D (0.9) was also observed for AE2 and WE2. Moreover, an increasing instability occurred in facial moisturiser prototype that contained a high concentration of *Fucus vesiculosus*, WE2 and AE2. In AE2, the D (0.9) increased to 45.91 µm towards the end of the storage experiment at 40ºC. Moreover, syneresis was observed visually in the facial moisturiser prototype. For WE2 stored at high temperature, the D (0.9) also increased from 14.89 µm when storage at 20°C to 48.80 µm at 40°C.

Colour stability

The instability in the facial moisturiser prototype stored at 40ºC increased to such as a large extent that only samples stored up to 35 days were analysed. Moreover, the changes that occurred in the facial moisturiser prototype due to the pigments present can affect the product colour. The colour changes were measured based on the colour coordinates lightness (L*), redness (a*) and yellowness (b*). The changes were compared to the REF by calculating the Euclidean distance value (ΔE). Addition of *Fucus vesiculosus* in the facial moisturiser prototypes resulted in colour changes at day 0. The colour changes were larger with the highest concentration (Figure 32).

		ΔE at day 35
AE2	20°C	8.54
	20°C + Light	10.02
	40°C	15.79
AE1	20°C	6.91
	20°C + Light	6.91
	40°C	9.11
WE2	20°C	8.02
	20°C + Light	9.72
	40°C	15.66
WE1	20°C	6.48
	20°C + Light	7.16
	40°C	12.10
REF	20°C	7.57
	20°C + Light	7.16
	40°C	4.02

Figure 32. The colour changes observed visually in facial moisturiser prototype with or without *Fucus vesiculosus* extract (water or acetone). Abbreviations for topical skin formulations: reference (REF), water extract at 1 mg/g (WE1), water extract at 2 mg/g(WE2), acetone extract at 1 mg/g (AE1) and acetone extract at 2 mg/g(AE2) after 35 days of storage at 20ºC, at 20ºC with exposure to light and at 40ºC (paper VI).

Especially in the samples stored at 20°C with exposure to light and at 40ºC ΔE increased. These colour changes may be related to lipid oxidation of the pigments such as fucuxanthin and chlorophylls, in the samples containing extracts, however more thorough studies of the pigments are needed to confirm this.

Oxidative stability

Tocopherols which were added the facial moisturiser prototype can act as AOs that inhibit the formation of hydroperoxide. The inhibition of lipid oxidation occurs through tocopherols reacting with radicals to terminate the propagation (Frankel, 1998). Therefore, a decrease in the concentration of tocopherols can indicate oxidation has taking place. The tocopherols present in the facial moisturiser prototype originated from two raw materials: vitamin E and almond oil. The extract AE seemed to protect the tocopherols from oxidation at all three storage conditions. Even though the tocopherol concentration was higher, PV was not significantly lower than the REF for AE. The observed protection of the tocopherols may be related to synergistic effects between the tocopherols and phenolic compounds or pigments from the extracts (Farvin and Jacobsen, 2013; Wang *et al*., 2009).

The other extract, WE, only protected the tocopherols from oxidation at storage with exposure to light. In addition, WE also resulted in significant lower PV when exposed to light. The higher tocopherol content and lower PV can be related to the higher content of carotenoids in facial moisturiser prototype that contained WE. Carotenoids are well-known terminators of the propagation reactions caused by photooxidation (Stahl and Sies, 2003). In the beginning, PV was slightly higher in the facial moisturiser prototype that contained extracts. The extract contained iron and copper ions, which together with the high processing temperature at 70-75°C may initiate lipid oxidation. Moreover, lipid oxidation can be initiated by iron and copper ions in both the initial stage and during the decomposition to alkoxyl radicals (McClements and Decker, 2000; Let *et al*., 2007). Based on this, the presence of iron and copper trace metals in the extracts may be related to the slightly higher PV in the facial moisturiser prototype that contained extracts (Hermund *et al*., 2015; Honold *et al*., 2015). Table 15 below shows the tocopherol and hydroperoxide content prior to and after storage.

Table 15. The PV and tocopherol content in facial moisturiser prototype with or without F*ucus vesiculosus* extract (water or acetone). Abbreviations for topical skin formulations: reference (REF), water extract at 1 mg/g (WE1), water extract at 2 mg/g(WE2), acetone extract at 1 mg/g (AE1) and acetone extract at 2 mg/g(AE2) after 35/56 days of storage at 20ºC, at 20ºC with exposure to light and at 40ºC (paper VI). Significant differences on 0.05 level between the formulations within each column are noted.

	Peroxide value (meq O_2/kg oil)			Total tocopherols (mg/g)	
	Day 0	S.C.[1]	Day 35/56	Day 0	Day 35/56
RF	5.52 ±0.51 a	20°C	11.62 ± 0.31 a	8582	5259
		20°C + Light	18.93 ± 0.63 b		4120
		40°C	15.01 ± 0.78 a		5097
WE1	5.19±0.29 a	20°C	10.77 ± 1.25 ab	7941	4528
		20°C + Light	15.67 ± 1.29 a		3742
		40°C	14.31 ± 0.07 a		4812
WE2	6.58±0.69 b	20°C	17.67 ± 1.38 b	7855	4383
		20°C + Light	16.42 ± 1.42 ab		5808
		40°C	13.97 ± 0.19 a		4913
AE1	6.28±0.65 b	20°C	18.78 ± 0.71 b	7735	6246
		20°C + Light	23.97 ± 0.49 c		6024
		40°C	16.47 ± 1.73 a		5858
AE2	6.63±0.75 b	20°C	15.14 ± 0.95 b	8052	6369
		20°C + Light	18.89 ± 0.55 b		6563
		40°C	14.05 ± 1.05 a		7075

1. Storage condition (S.C.)

Moreover, *Fucus vesiculosus* extracts contain phlorotannins that have a good ferrous ion-chelating capacity. The ferrous ion-chelating capacity can reduce the decomposition rate of lipid hydroperoxide. A recently conducted study showed that polar highly polymerized phlorotannins were less efficient AOs than less polar and smaller oligomer phlorotannins

(Hermund *et al*., 2016). This may explain the higher antioxidative effect of AE in dark than WE since a nonpolar solvent such as acetone favours extraction of more amphiphilic phenolic compounds compared to the polar solvent water.

Several volatile compounds were identified from the headspace of the samples, especially aldehydes, pentanal, hexanal, heptanal, octanal, 2- heptenal and 2-octenal. Moreover, two alcohols 1-octen-3-ol and 2-ethyl-1-hexanol were also identified. As shown in chapter three (Figures 7 and 8), most of these volatile compounds originated from the autoxidation of linoleic acid, however also from oleic and a-linolenic acid. Oleic and a-linolenic acid were present in the facial moisturiser prototype (Belitz, 2009; Guillen, and Uriarte, 2012; Poyato *et al*., 2014). The good markers for lipid oxidation found in the first study in topical skin formulation, pentanal and heptanal, also showed greater differences among samples in this study than other volatile compounds (Table 16).

Table 16. The development of volatile compounds, pentanal and heptanal, in [ng/g] after 35/56 days of storage at 20ºC, at 20ºC with exposure to light and at 40ºC (paper VI). Abbreviations for topical skin formulations: reference (REF), water extract at 1 mg/g (WE1), water extract at 2 mg/g(WE2), acetone extract at 1 mg/g (AE1) and acetone extract at 2 mg/g(AE2) (paper VI). Significant differences on 0.05 level between the formulations within each column are noted.

Volatile	Storage	Day 1	Day 35/56	Oxidation rate (%)[1]
Pentanal (ng/g emulsion)				
REF	20°C	34.61 ± 1.81	62.9 ± 0.9 a	81.7 c
	20°C + Light	46.4 ± 1.35	113.5 ± 3.7 ab	144.6 b
	40°C	43.9 ± 4.94	593.1 ± 7.4 cd	1251.0 c
WE1	20°C	48.52 ± 5.6	59.0 ± 6.2 a	21.5 bc
	20°C + Light	57.32 ± 0.8	130.2 ± 8.9 b	127.1 b
	40°C	41.52 ± 2.69	569.9 ± 58.4 c	1272.6 c
WE2	20°C	75.29 ± 12.9	36.5 ± 20.2 a	-51.5 ab
	20°C + Light	59.89 ± 16.5	63.9 ± 7.7 a	6.7 a
	40°C	39.86 ± 12.3	552.8 ± 33.7 c	1286.9 c
AE1	20°C	51.29 ± 22.4	56.0 ± 2.2 a	9.1bc
	20°C + Light	56.57 ± 24.1	71.8 ± 15.5 a	26.9 a
	40°C	50.59 ± 3.39	351.2 ± 7.5 b	594.2 b
AE2	20°C	87.72 ± 8.73	17.2 ± 2.1 a	-80.4 a
	20°C + Light	71.61 ± 2.56	46.4 ± 33.2 a	-35.2 a
	40°C	81.16 ± 28.5	293.4 ± 23.6 a	261.5 a
Heptanal (ng/g emulsion)				
REF	20°C	18.61 ± 1.76	31.8 ± 1.2	70.9 b
	20°C + Light	23.33 ± 3.11	72.8 ± 5.7	211.9 b
	40°C	25.22 ± 2.43	269.1 ± 15.6 a	967.0 c
WE1	20°C	28.06 ± 2.18	37.7 ± 4.0	34.4 ab
	20°C + Light	28.7 ± 0.26	78.9 ± 3.8	174.9 b
	40°C	23.81 ± 2.93	257.3 ± 44.0 a	980.6 c
WE2	20°C	79.27 ± 1.35	70.4 ± 1.1	-11.2 a
	20°C + Light	71.89 ± 6.79	121.5 ± 5.5	69.0 a
	40°C	63.39 ± 7.35	321.4 ± 8.1 a	407.0 b
AE1	20°C	69.58 ± 5.74	78.7 ± 4.1	13.2 ab
	20°C + Light	70.11 ± 5.23	101.1 ± 9.7	44.2 a
	40°C	70.21 ± 2.49	1613.0 ± 102.0 b	2197.4 d
AE2	20°C	78.21 ± 4.26	65.1 ± 3.6	-16.7 a
	20°C + Light	77.51 ± 4.73	108.7 ± 12.1	40.2 a
	40°C	81.52 ± 3.26	220.6 ± 2.9 a	170.6 a

1. Oxidation rate=$((c_{end}-c_{start})/c_{start})*100\%$

Overall, the initial highest concentration of pentanal and heptanal at day 0 was measured in the facial moisturiser prototype containing extracts. This may be related to the extract containing pentanal and heptanal itself. Moreover, high concentrations of pentanal and heptanal were also found when the extracts were applied in fish-oil-enriched milk and mayonnaise (Hermund *et al*., 2015). The oxidation rate of pentanal and heptanal was significantly lower for storage at room temperature with and without exposure to light for both WE2 and AE2 compared to REF. However, the heptanal concentration at day 56 was higher both in WE and AE than in REF for storage at room temperature with and without exposure to light. At high temperature, only AE2 resulted in significantly lower oxidation rate and end concentration of pentanal and heptanal. The percent changes observed in the facial moisturiser prototypes are summarized in Table 17.

Table 17. Summary of the % changes occurring for PV, tocopherol content, pentanal, heptanal and colour in facial moisturiser prototype with or without F. vesiculosus extract (water or acetone). Abbreviations for topical skin formulations: reference (REF), water extract at 1 mg/g (WE1), water extract at 2 mg/g(WE2), acetone extract at 1 mg/g (AE1) and acetone extract at 2 mg/g(AE2) after 35/56 days of storage at 20°C, at 20°C with exposure to light and at 40°C (paper VI).

Δ (%)	Storage	PV	Tocopherols	Pentanal	Heptanal	Colour
REF	20°C	52.50	-63.19	81.7	70.9	7.57
	20°C + Light	70.84	-108.30	144.6	211.9	7.16
	40°C	63.22	-68.37	1251.0	967.0	4.02
WE1	20°C	51.81	-75.38	21.5	34.4	6.48
	20°C + Light	66.88	-112.21	127.1	174.9	7.16
	40°C	63.73	-65.02	1272.6	980.6	12.1
WE2	20°C	62.76	-79.22	-51.5	-11.2	8.02
	20°C + Light	59.93	-35.24	6.7	69.0	9.72
	40°C	52.90	-59.88	1286.9	407.0	15.66
AE1	20°C	66.56	-23.84	9.1	13.2	6.91
	20°C + Light	73.80	-28.40	26.9	44.2	6.91
	40°C	61.87	-32.04	594.2	2197.4	9.11
AE2	20°C	56.21	-26.42	-80.4	-16.7	8.54
	20°C + Light	64.90	-22.69	-35.2	40.2	10.02
	40°C	52.81	-13.81	261.5	170.6	15.79

Overall, exposure to light increased PV most in AE > RF > WE, suggesting a protective effect of the carotenoids in WE extracts. For pentanal and heptanal, the concentration was initially higher. Nevertheless, the oxidation rates were lower in the facial moisturiser prototypes with *Fucus vesiculosus* extracts. The oxidation rates were ranked in the following order: AE2 < WE2 < RF. At high temperature, AE2 had the highest oxidative stability by having the lowest PV, lowest concentration and lowest oxidation rate of pentanal and

heptanal, and lowest decline in tocopherol content. Yet, WE also resulted in lower PV and oxidation rate for pentanal compared to REF. Consequently, AE and WE were able to protect against thermo-oxidation, AE was the most effective. However, only WE protected against photooxidation.

5.5 Methods for rapid prediction of long-term oxidative stability by radical initiation (paper V)

In this chapter, the ability to predict oxidative stability by initiator addition was investigated. Currently in product development, product stability is tested for up to six month. This time-consuming stability test can be a bottleneck in product development. Consequently, approaches that can remove the bottleneck by predicting oxidative stability are desired. Ideally, prediction by initiator addition must shorten the oxidative stability test from six months to one month or shorter.

5.5.1 Initial screening for potential initiators using Oxygraph

An initial screening was conducted to select the effective concentration of the four initiators. The four initiators included two initiator systems and two synthetic radicals: $FeCl_3$/ascorbic acid and $FeCl_2/H_2O$; AAPH (water soluble) and AMVN (lipid soluble). A volume small volume such as 10 µl is desired to rule out the effect of solvent addition. AAPH and AMVN were intended to be evaluated from 0 mM to approximately 50 mM. A homogenous solution must be obtained because the radicals are injected into the electrochemical cell. However, a homogenous solution that had a high enough concentration of the radicals was not possible to prepare. Even though, both polar and non-polar solvents were tested. AMVN and AAPH were not possible to dissolve in high concentrations because powder residues started appearing in the bottom of the beaker. A homogenous solution was only possible to prepare with a high enough concentration to reach 10 mM and 5 mM for AAPH and AMVN, respectively. These low concentrations are five to ten times lower than the reported efficient concentrations (Matsumura *et al*., 2003; Mosca *et al*., 2010; Baron *et al*., 2006). Nevertheless, a prooxidative effect was observed even though the concentration was below the intended concentration.

In addition to the synthetic radicals, another approach to promote radical formation was to use an initiator system. Two initiator systems were included in this thesis: $FeCl_3$/ascorbic acid and $FeCl_2/H_2O_2$. The initiator system contained an iron and a Fenton oxidant to accelerate

lipid oxidation and degradation (Scheme 2). For $FeCl_2/H_2O_2$, a Fenton oxidant is added in addition to iron in order to produce highly reactive hydroxyl radicals.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$$

Scheme 2. Fenton reactions for H_2O_2. Adopted from Chevion (1988)

In contrast to the solvability problems with AAPH and AMVN, $FeCl_3$/ascorbic acid and $FeCl_2/H_2O_2$ were easy to dissolve. Hence, homogenous solutions were easily obtained with a high enough concentration to reach the target concentration in the electrochemical cell. Both $FeCl_3$/ascorbic acid and $FeCl_2/H_2O_2$ were proven effective in all three topical skin formulations (Figure 33).

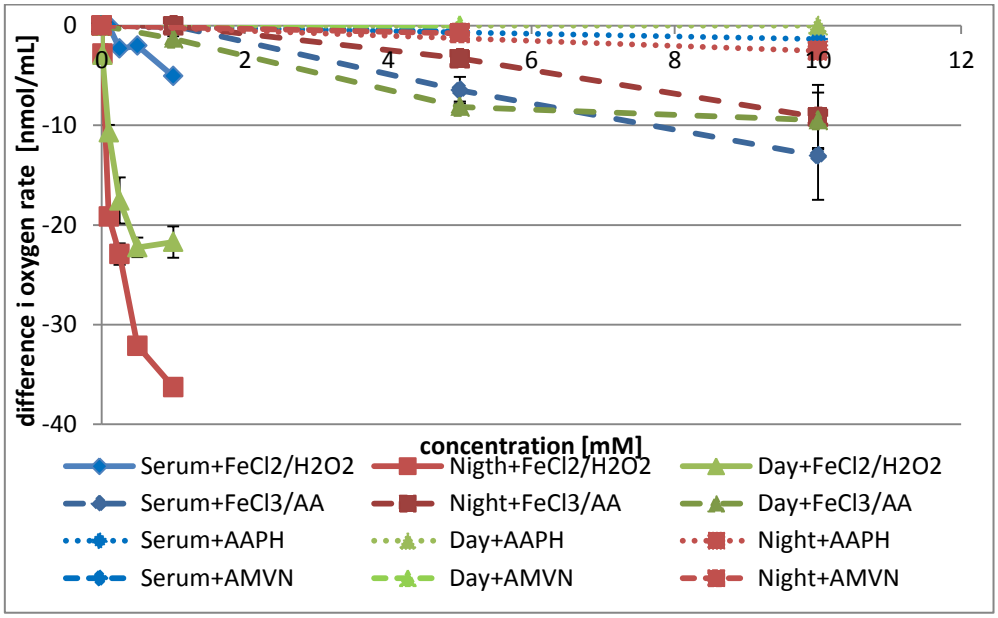


Figure 33. The difference in oxygen consumption rate [nmol/mL] due the initiator addition estimated using Oxygraph. Four initiators are used: AAPH, AMVN, $FeCl_3$/Ascorbic acid and $FeCl_2/H_2O_2$.

Hence, $FeCl_3$/ascorbic acid and $FeCl_2/H_2O_2$ significantly increased oxygen consumption in all three topical skin formulations even when added in low concentrations. The effective concentrations in all three topical skin formulations were possible to estimate. Accordingly, the effective concentrations were estimated to be 10 mM/25 mM for $FeCl_3$/ascorbic acid, and 1 mM/2 mM for $FeCl_2/H_2O_2$. Moreover, higher oxygen consumption was observed when $FeCl_2/H_2O_2$ was added than $FeCl_3$/ascorbic acid. Two studies in marine emulsions by Kristinova *et al*. (2014) may give a possible explanation to this observation. These studies showed increased oxygen consumption with increased iron concentration as observed in this study. However, they also observed that Fe^{2+} more efficient increased oxygen consumption rates compared to Fe^{3+}. The different oxygen consumption rate did the authors explained as a result of faster decomposition of LOOH by Fe^{2+} added as $FeCl_2/H_2O_2$ than Fe^{3+} added as $FeCl_3$/ascorbic acid. The faster decomposition may also be the case in our experiment.

5.5.2 Acceleration of lipid oxidation by initiator addition to topical skin formulation

In the initial screening using Oxygraph, all four radicals/initiator systems were able to initiate oxidation. However, the optimal concentration was not possible to estimate for AMVN and AAPH in topical skin formulations so the literature reported efficient concentration at 50 mM will be used.

However, the initiator system, $FeCl_3$/ascorbic acid, was not possible to apply in neat topical skin formulations. In neat topical skin formulations, some serious stability issues occurred since the topical skin formulation separated immediately and turned brown. Consequently, $FeCl_3$/ascorbic acid was deemed to be inappropriate.

For the remaining three initiators, the ability to accelerate lipid oxidation and degradation was tested in three topical skin formulations. The three topical skin formulations were selected to examine the initiators' ability to accelerate lipid oxidation in topical skin formulations with low and high lipid content: a prototype serum formulation (low), and prototype skin and night cream formulations (high).

The initiators effect on lipid oxidation was measured by PV and volatile compounds. The PV remained low in prototype serum formulation added AAPH and initiator systems at elevated temperature and with exposure to light. However, addition of AMVN increased PV noticeably. The same trends were observed in the prototype skin and night cream formulations (data not shown).

As mentioned earlier, it was the aim to be able to predict the oxidative stability after six months of storage within one month. Therefore, the oxidation rates of the volatile compounds were calculated for topical skin formulations after six months of storage and after one month of storage with initiators at 20°C (Table 18). Consequently at this stage, a good initiator must be able to initiate lipid oxidation in all the topical skin formulations to form the volatile compounds. The following three volatile compounds were included: butanal, pentanal and 3-methyl-1-butanol (only prototype skin cream formulation).

Table 18. Oxidation rate [%] of butanal, pentanal and 3-methyl-1-butanol (3-m-1-butanol) in the control stored for six months and topical skin formulations with initiators stored for one month.

Volatile/ Initiator	**Butanal**	**Pentanal**	**Butanal**	**Pentanal**	**Butanal**	**Pentanal**	**3-M-1-butanol**
	Serum formulation		*Night cream formulation*		*Skin cream formulation*		
Control	1985	1539	11132	6038	12707	7603	1912
AMVN	5177	1266	3838	1068	5200	939	-47
AAPH	7050	2328	5657	1375	11294	3383	580
$FeCl_2/H_2O_2$	39084	25577	39395	20237	54135	23577	5150

In prototype serum formulation, the oxidation rate for butanal increased slightly more when stored for one month with AMVN and AAPH added than in neat prototype serum formulation stored for six months. Especially, addition of the initiator system, $FeCl_2/H_2O_2$, increased the concentration markedly more.

For pentanal, the same pattern was observed for $FeCl_2/H_2O_2$. Hence, $FeCl_2/H_2O_2$ accelerated lipid oxidation effectively. However, the radical, AMVN, was not able to reach the same oxidation rate as the control. The lower effect of the radicals, AMVN and AAPH, may be related to the fact that lipid oxidation is dependent on a redox reaction. Hence, a redox reaction is an active part of the further decomposition of LOOH to secondary oxidation products. The decomposition products include radicals that further propagate lipid oxidation. The LOOH formed by AAPH and AMVN initiation does not include the redox reaction. Consequently, I suggest that the absent redox reaction leads to the lesser ability to propagate lipid oxidation.

In prototype night cream formulation, only the radical system $FeCl_2/H_2O_2$ was able to reach and exceed the oxidation rate of the control for both butanal and pentanal. Oxidation was accelerated more by the radical, AAPH, than AMVN.

As observed for the two other prototype formulations, $FeCl_2/H_2O_2$ was the best accelerator of lipid oxidation in skin cream formulation. In addition, $FeCl_2/H_2O_2$ was the only initiator which was able to accelerate degradation to 3-methyl-1-butanol. Moreover, radical addition of AAPH and AMVN increased the concentration slightly for butanal and pentanal. Nevertheless, the oxidation rate was not as high as for the control.

Comparison of the Oxygraph and storage experiment results revealed a clear gap in the conclusion between these two approaches. The Oxygraph may suggest that night cream formulation is less stable than serum formulation and skin cream formulation at room temperature with the addition of $FeCl_2/H_2O_2$. However, this result was not confirmed by the storage experiment. The storage experiment showed that skin cream formulation had the lowest stability followed by night cream formulation and serum formulation. Thus, the storage experiment led to a different ranking of the sample, the Oxygraph cannot be used as a fast approach to assess the oxidative stability in complex emulsion systems in this case skincare formulations. The Oxygraph´ s misleading result is properly related to the water addition because the water addition may have removed the matrix's effect on lipid oxidation. Consequently, a storage experiment cannot be replaced by Oxygraph to measure the oxidative stability.

At this point, it was clear that AAPH, AMVN and $FeCl_2/H_2O_2$ were able to accelerate lipid oxidation to some extent. However, the ability to accelerate lipid oxidation and degradation was one thing; another was the ability to reveal long term stability. Therefore, the ability to reveal unstable topical skin formulations faster was examined in the following section.

5.5.3 Ability to reveal products with low oxidative stability (papers VI and VII)

The ability to diclose products with low oxidative stability was investigated in a prototype skin cream formulation (containing a UV filter) and a facial moisturiser prototype both with and without AOs.

The physical stability was affected by AMVN and AAPH addition in a prototype skin cream formulation (containing a UV filter) and in a facial moisturiser prototype. However, to initiate lipid oxidation efficiently a concentration up to 50 mM may be needed. Consequently, the AAPH and AMVN concentrations were examined in concentrations of 50, 25 and 10 mM because poor physical stability can affect the extent of lipid oxidation. Moreover, the extent of lipid oxidation was measured by PV (only prototype skin cream formulation) and the secondary volatile oxidation product, pentanal.

As observed for prototype serum and night cream formulations in the previous section, PV remained low in prototype skin cream formulation added $FeCl_2/H_2O_2$ and AAPH. However, addition of AMVN increased PV most throughout the entire storage period (Table 19).

Table 19. Oxidation rate [%] of hydroperoxides in the control prototype skin cream formulations (Cream) with and without antioxidants (AO) stored for three months and the same creams with initiators added stored for one month.

Antioxidant/initiator	Oxidation rate [%]	
	Cream-AO	Cream+AO
Control (three months)	-100	-13
AMVN 10 mM	235	283
AMVN 25 mM	232	202
AMVN 50 mM	111	229
AAPH10 mM	57	19
AAPH25 mM	68	35
AAPH50 mM	87	91
$FeCl_2/H_2O_2$ 1 mM/2 mM	2	-8

The PV for prototype skin cream formulation with and without AOs both remained low and less than 2 meq/kg oil for three months. Accordingly, the higher PV in prototype skin cream formulation added AMVN was related to the addition of AMVN and may be physical instability. Moreover, the development of pentanal in prototype skin cream formulation

added AMVN was clearly affected by the physical instability. The physical instability may have resulted in a decrease of pentanal after 16 and 21 days of storage for formulation without and with AOs added AMVN, respectively (data not shown).
Compared to AMVN, addition of AAPH resulted in a significantly different pattern for pentanal. The pentanal concentration was significantly higher in prototype skin cream formulation without AOs. However, the initiator system, $FeCl_3/H_2O_2$, did not result in any significant difference between prototype skin cream formulation with and without AOs after 30 days of storage (Table 20).

Table 20. The oxidation rate [%] of pentanal in the control prototype skin cream formulations (cream) with and without antioxidants (AO) for three months and all formulations added initiators (AMVN, AAPH or $FeCl_3/H_2O_2$) for one month.

Antioxidant/initiator	Oxidation rate [%]	
	Cream-AO	Cream+AO
Control	383	387
AMVN 10 mM	580	426
AMVN 25 mM	889	405
AMVN 50 mM	488	392
AAPH 10 mM	1179	536
AAPH 25 mM	1522	810
AAPH 50 mM	1619	1313
$FeCl_3/H_2O_2$ 1 mM/2 mM	522	514

To summarise, addition of the initiators, AMVN and AAPH, resulted in a clear and concentration depending poorer oxidative stability of the formulation without AO. However, the initiator system, $FeCl_3/H_2O_2$, did not lead to any significant difference between the prototype skin cream formulations. At this point, the oxidative stability of prototype skin cream formulations predicted is ambiguous and contradictory. To clarify, neat prototype skin cream formulation with and without AOs were stored for three months at 40°C. After three months, the pentanal concentration was not significantly different upon AO addition. Moreover, the oxidation rate was almost the same at 387 % and 383 % in prototype skin cream formulation with and without AOs, respectively (Table 20). The absent AO effect may be related to antioxidative effects from the preservation system, which was EDTA. This possible AO effect was also mentioned in section 2.2 and reported in studies by Al-Adham (2000) and Patrone (2010). However, the preservation system was not removed from the prototype skin cream formulations because microbial growth can result in volatile

compounds. Volatile compounds formed due to microbial growth, in parallel, can complicate interpretation of the results (Kai *et al*., 2007).

Accordingly, $FeCl_3/H_2O_2$ was the only initiator that predicted the lack of significant difference between the prototype skin cream formulations. Because no significant difference was observed in the control with and without antioxidant, a new study was conducted, where three facial moisturiser prototypes with a larger difference in the oxidative stability were examined (Table 21).

Table 21. The oxidation rate [%] of pentanal in facial moisturiser prototype (REF) added *Fucus vesiculosus* water extract (WEI1), and added *Fucus vesiculosus* ethanol extract (EE1) for 42 days, and all formulations added initiators(AMVN, AAPH or $FeCl_3/H_2O_2$) for one month.

Antioxidant/initiator	Oxidation rate [%]		
	REF	WEI1	EE1
Control	1	-5	-22
AMVN 50 mM	-80	-75	-80
AAPH 50 mM	N/A	11	-41
$FeCl_3/H_2O_2$ 1 mM/2 mM	225	51	-55

The control facial moisturiser prototypes were stored at 20 °C for 42 days. After 42 days, the oxidation rates of pentanal in the control facial moisturiser prototypes were calculated. Based on the oxidation rates, the control facial moisturiser prototypes had the following significant ranking: REF>WEI1>EE1.

Subsequently, facial moisturiser prototypes added AMVN, AAPH and $FeCl_3/H_2O_2$ were stored at 20 °C for 30 days. After 30 days, the oxidation rate was calculated and compared to the control. It was observed that $FeCl_3/H_2O_2$ had the same ranking of the samples after 30 days as storage after 42 days without addition of initiator. Moreover, the initiator AAPH was only used in WEI1 and EE1 because of limited sample amount. In any case, AAPH was able to give the same ranking of these two samples as the control. However, the other radical, AMVN, did not have the same ranking of the samples as the control.

Based on these two experiments, the initiator system, $FeCl_3/H_2O_2$, was the most promising initiator system. Furthermore, the physical stability was not affected in the topical skin formulations upon addition of this initiator. In addition, only $FeCl_3/H_2O_2$ showed significant differences between the formulations when the control was significant.

Chapter 6: Conclusion and Perspectives

6.1 Conclusion

The main objective was to investigate lipid oxidation with special focus on volatiles in selected topical skin formulations. In the first study, pentanal and heptanal both increased and showed potential as markers for lipid oxidation in formulations both with high and low lipid content. Both pentanal and heptanal were selected as the best markers for showing difference between facial moisturiser prototypes with and without *Fucus vesiculosus* extracts. In another study of prototype skin cream formulation, the heptanal concentration was initially low and remained low in entire experiment even though pentanal increased. Accordingly, only pentanal could be used as a marker in this study. Therefore, it can be concluded that pentanal was a universal marker whereas heptanal only was a good marker in some topical skin formulations, namely prototype lip care formulation, prototype cleansing formulation and facial moisturiser prototype. Moreover, the conversion from primary to secondary oxidation product varied widely between product and storage conditions. Based on the different conversion rates, PV was demonstrated not to be a useful marker for the extent of lipid oxidation. The extent of lipid oxidation must be measured by volatile compounds using GC-MS.

The chemical analysis in combination with sensory evaluation showed that it was not possible to link a single compound to link to the sensory attributes. As hypothesised, the sensory evaluation showed that the matrix clearly and significantly increased the ODT value of volatile compounds above those in water. Moreover, the matrix effect led to different odour description of the volatile compounds compared to water.

In addition to the well-known lipid oxidation products which mainly increased in lipid-containing raw materials, butane nitrile (and other nitriles) increased in the topical skin formulations. Butane nitrile in high concentrations (above the level measured in this thesis) can cause skin irritation, therefore the raw material and route of reactions leading to its formation was of interest to discover. Throughout the raw material investigation, the hypothesised reaction between lipid oxidation products and amino groups present was found not to be the reaction leading to butane nitrile in the topical skin formulation. However, tetramethylbutanedinitrile appeared in polyacrylate crosspolymer-6 under accelerated storage. Tetramethylbutanedinitrile has been described to be a by-product from AIBN that was used

as initiator for production of polymers. AIBN decomposition has been reported to result in several other by-products including isobutane nitrile. Isobutane nitrile was an isomer of butane nitrile. The detection of butane nitrile instead of isobutane nitrile was surprising. More studies are needed to understand the route reactions leading to butane nitrile.

Another volatile compound, 3-methyl-1-butanol, was present in high concentrations, initially, and increased even further. The 3-methyl-1-butanol concentration was above ODT value in topical skin formulations. The raw material, UV cinnamate, contained 3-methyl-1-butanol in a high concentration initially and increased even further under accelerated storage. Several routes of reactions may lead to 3-methyl-1-butanol. However, based on the by-product in the reactions, the main reaction route was direct hydrolysis of the ester-group in the UV cinnamate. Accordingly, most volatile compounds were linked to reactions in raw material(s) as hypothesised. However, only an indication of a possible link between butane nitrile and a raw material was obtained.

To investigate, the hypothesised ability of *Fucus vesiculosus* to increase oxidative stability PV and concentration of volatile compounds were examined. Overall, the PV and volatile compounds increased less in facial moisturiser prototype when *Fucus vesiculosus* extracts, WE and AE, were added. According to PV, the WE extract protected best against photo-activated oxidation. The protective effect was most likely related to the carotenoids in the WE extract. However, the volatile compounds revealed that AE extract more efficiently reduced the decomposition from hydroperoxide to volatile compounds. At high temperature, the AE extract also had the lowest PV, concentration and oxidation rate of pentanal and heptanal, and decline in tocopherol content compared to WE and REF. Consequently, the lowest oxidation rates for pentanal and heptanal were obtained by AE extract compared to REF and WE extract.

The last part of this PhD focused on combating the bottle neck in product development caused by time-consuming stability tests. Several potential strategies to accelerate lipid oxidation were tested for one month. After one month, three initiators AAPH, AMVN and $FeCl_2/H_2O_2$ were able to maintain a physically stable emulsion and accelerate lipid oxidation to some extent. These three initiators´ ability to meet the outlined hypothesis were examined. Overall, the oxidative stability of prototype skin cream formulations with and without AOs was similar. $FeCl_3/H_2O_2$ was the only initiator that resulted in the same oxidation rate ranking

for pentanal as control (neat sample stored for longer time). Because, the oxidative stability was too close between the formulations to explore the ability to reveal poor oxidative stability, a second experiment was conducted. The second experiment was conducted in facial moisturiser prototype with *Fucus vesiculosus* extracts which had a clear difference in the oxidation rate for pentanal. $FeCl_3/H_2O_2$ addition resulted in the same ranking of the sample after 30 days as in the control experiment at 20 °C for 42 days. The initiator AAPH was only used in WEI1 and EE1 and showed the same pattern as the control. Consequently, $FeCl_3/H_2O_2$ clearly was the best initiator system that only showed differences between the formulations when the control increased significant. Moreover, $FeCl_3/H_2O_2$ was able to predict oxidative stability within one month.

6.2 Perspectives

Throughout the thesis, it has been demonstrated that lipid oxidation occurred in topical skin formulations. Moreover, two AO extracts from *Fucus vesiculosus* were evaluated as natural AO system in a facial moisturiser prototype. Addition of the extracts resulted in a higher initial concentration of volatile compounds than the REF. Nevertheless, *Fucus vesiculosus* extracts led to a significantly lower oxidation rate throughout the storage. The higher content of volatile compounds was probably related to oxidation of pigments under production because part of the production was done at 70+°C. A new study using a production method having more mild production conditions may result in a lower initial concentration of volatile compounds.

Topical skin formulations have a high number of raw materials which all can affect the development of volatile compounds. Volatile compounds which are not normally observed in emulsions were measured. Most of the volatile compounds that appeared were linked to lipid oxidation of lipid-containing raw materials and degradation of raw materials. However, the exact route of reactions leading to butane nitrile (and other nitrile-containing compounds) has not been confirmed. An exhaustive literature research resulted in a possible relationship between radicals used for production of polymer and plastics. Moreover, a new study that investigates the exact reactions leading to butane nitrile e.g. using Nuclear Magnetic Resonance (NMR) and isotope labelling must be conducted to confirm this suggestion.

Sensory evaluation was successfully applied to determine odour changes and ODT values for the volatile compounds which may affect product odour. Since, a single volatile compound could not be linked to the odour changes observed, the odour changes may occur as a result of a cocktail effect. A new study that investigates the cocktail effect between multiple volatile compounds may provide an explanation for the odour changes that occurred in the prototype cleansing formulations.

Unfortunately, in the first initiator study no significant difference between the two topical skin formulations in the pentanal oxidation rate was present. Therefore, a new study which included a facial moisturiser prototype having a large difference in the oxidative stability was conducted. In this study, the ability to differentiate a stable and unstable formulation using radical initiation could be examined. However, the study was small and short; therefore a new long-term study with more sampling points must be conducted to confirm the results.

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Appendix List

Appendix 1: Article I

J Am Oil Chem Soc (2017) 94:1287–1300
DOI 10.1007/s11746-017-3034-7

ORIGINAL PAPER

Investigation of Lipid Oxidation in High- and Low-Lipid-Containing Topical Skin Formulations

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Received: 20 May 2017 / Revised: 31 July 2017 / Accepted: 8 August 2017 / Published online: 22 August 2017

Abstract Lipid oxidation can impact the odour of skin care products during storage. A study was conducted to identify and monitor representative markers for lipid oxidation in skin care products over time. Four lip care formulations and three skin care formulations with different lipid contents were stored at various cosmetic industry-relevant conditions for 84 days. The skin care products were analysed for lipid hydroperoxides and secondary volatile oxidation products. A trained sensory panel performed an odour difference (triangle) test and odour-profiled the products to detect and describe odour changes during storage. Several potential markers for lipid oxidation were identified. In skin care formulations, peroxide value (PV) analysis was a useful marker for lipid oxidation if the product was exposed to light during storage, but no clear changes were observed for PV in samples stored under other conditions. Furthermore, concentrations of several secondary volatile oxidation products increased during storage, and the highest increase was observed for products exposed to light. Pentanal and heptanal were found to be reliable markers for secondary volatile oxidation products in the skin care formulations (especially during exposure to light), whereas in the lip care formulations the best candidates were pentanal (especially during exposure to light and iron), 2-methyl furan and 3-methyl-3-buten-2-one (especially during exposure to light, iron and high temperatures).

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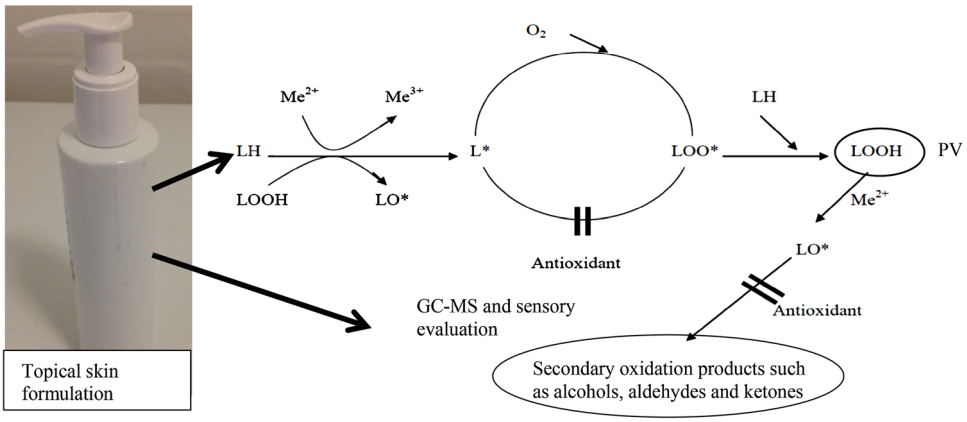
Graphical Abstract



Keywords Skin care emulsions · Volatile oxidation products · Peroxide value · GC–MS and sensory evaluation

Introduction

Lipid oxidation is well known for causing changes in odour and flavour and is one of the most important deteriorating processes for product quality. It is the secondary volatile oxidation products, especially aldehydes, ketones and alcohols that can be responsible for the development of off-odours even when present in low concentrations [1]. Most investigations on lipid oxidation have been performed in foods, feeds and model emulsion systems. However, to the knowledge of the authors, no reports are available in the literature on the assessment and measurement of the progress and effect of lipid oxidation in topical skin formulations during storage, despite lipids being key skin care ingredients. The most relevant peer-reviewed article on lipid oxidation in topical skin formulations that can be found in an international journal is a review by Khanum and Thevanayagam [2]. This article highlights the fact that oxidative stability is often overlooked. However, lipid oxidation can impact product quality and, potentially, safety of the formulations. Furthermore, good oxidative stability of cosmetic emulsions can maintain product efficacy of the formulations on-skin.

Since odour characteristics are important quality parameters for topical skin formulations, it is essential to control the odour of products for the entire shelf life. Numerous authors have investigated the effect of active ingredients on skin oxidation [3] or anti-aging [4], and manufacturers have investigated off-odour occurrence in topical skin formulations and masked them with masking or perfume agents to obtain consumer acceptance [5]. However, masking does not solve the underlying processes. Solving off-odour problems may be complex, but it can also be simple, e.g., changing a single raw material or using alternative packing materials to protect the product against lipid oxidation (e.g., by better protection from light). However, before solutions can be identified, it is necessary to investigate the reason(s) for and to what extent lipid oxidation occurs in topical skin formulations.

A pioneering study was designed to measure the progress of lipid oxidation and its effect on off-odour formation in topical skin formulations as a first objective. In order to achieve this, representative markers for lipid oxidation in topical skin formulations needed to be identified (a second objective). The study also investigated how lipid oxidation could be accelerated to more quickly expose the potential for off-odour formation in skin care products (a third objective). Three different acceleration methods were identified, as follows: increased temperature, light exposure, and the addition of iron sulphate solution. To support the experimentation highlighted above, different types of topical skin formulations (containing different types and concentrations of lipids) were prepared before being stored at various conditions. Two product types were investigated; lip care formulations as representative products for skin care products with a high fat content and skin care formulations as representative products for skin care products with a low fat content. The products were analysed over a 3-month period. To evaluate the progress of lipid oxidation, lipid hydroperoxides were determined by PV analysis and secondary volatile oxidation products were determined by dynamic headspace gas chromatography–mass spectrometry (GC–MS). Moreover, a trained sensory panel performed odour triangle (discriminative difference) tests and odour profiling on the products to detect odour changes during the period of storage.

Methods and Materials

Materials

Topical Lip Care Formulations

Lip care formulation 1, 2 and 3 had similar formulas except for the aroma/flavour compounds added. All lip care formulations contained 36% lipids with formulation 1 being fragrance-free, formulation 2 having a mint flavour and formulation 3 a creamy flavour. Lip care formulation 4 did not contain any aroma/flavour compounds and had a slightly higher oil content (approximately 39% lipids) than the other three formulations. In addition, lip care formulation 4 had higher shea butter and palm oil contents, and a lower glycerine content than lip care formulations 1, 2 and 3. The ingredients' (INCI) lists are in Table 1.

Skin Care Formulations

Three skin care formulations were used for this experiment: (1) a control containing proprietary lipid blend (skin care formulation 1), (2) skin care formulation 1 with added fruit oils but with reduced shea butter and caprylic/capric triglycerides content (skin care formulation 2), and (3) a skin care formulation similar to skin care formulation 2 but with reduced glycol content and added metal chelators caprylhydroxamic acid (CHA) (skin care formulation 3). The ingredients are listed in Table 2.

Table 1 Pertinent product constituents and approximate lipid contents for lip care formulation 1–4

Product	Lip care formulation 1–3	Lip care formulation 4
Product constituents pertinent to lipid oxidation investigation	Caprylic/capric triglycerides Butyrospermum parkii butter Olus oil Ethylhexyl salicylate Oryza sativa cera Butyl Methoxydibenzoylmethane Behenyl alcohol Capryloyl glycine Caprylyl glycol Ascorbyl palmitate Trisodium ethylenediamine disuccinate Tocopherols Hydrogenated lecithin PMEA Oryza sativa bran oil Squalene Phytoshingosine Ceramide-3 Aroma (only lip care formulation 2 and 3)	Butyrospermum parkii butter Olus oil Elaeis guineensis oil Oryza sativa cera Behenyl alcohol Oryza sativa bran oil Hydrogenated lecithin Capryloyl glycine Caprylyl glycol Tocopherols Trisodium eEthylenediamine disuccinate Ascorbyl palmitate Squalene Ceramide-3 Phytoshingosine PMEA
Lipid content	~36%	~39%

Table 2 Pertinent product constituents for skin care formulation 1–3

Product	Skin care formulation 1	Skin care formulation 2	Skin care formulation 3
Product constituents pertinent to lipid oxidation investigation	Caprylic/Capric Triglyceride Cocos nucifera oil Hydrogenated lecithin Caprylyl glycol Butyrospermum parkii butter Squalane Trisodium ethylenediamine disuccinate Ceramide-3	Caprylic/Capric Triglyceride Cocos nucifera oil Hydrogenated lecithin Olea Europa (Olive Oil) Caprylyl glycol Elaeis guineesis oil Squalane Vegetable oil PMEA Trisodium ethylenediamine disuccinate Ceramide-3	Caprylic/Capric Triglyceride Cocos nucifera oil Hydrogenated lecithin Olea Europa (Olive Oil) Caprylyl glycol Elaeis guineesis oil Squalane Vegetable oil Caprylhydroxamic Acid PMEA Trisodium ethylenediamine disuccinate Ceramide-3

Methods

Experimental Design

Four lip care formulations and three skin care formulations were stored in opaque packaging material for the following conditions:

(A) 2 °C in the dark for up to 3 months (control),
(B) 20 °C in the dark for up to 3 months,
(C) 20 °C exposed to artificial light (approximately 3500 lx) for up to 3 months,
(D) 40 °C in the dark for up to 3 months.
(E) 40 °C added 100 µmol/ng $FeSO_4$ stored in the dark for 56 days, and
(F) 50 °C in the dark for 2 weeks.

Samples were taken at day 0, 14, 28, 56 and 84 from the products stored at 2 °C (A). Samples were taken at day 14, 28, 56 and 84 from the products stored at 20 °C in light or darkness, and 40 °C in the dark (B, C and D). Only a single sample was taken from products stored at 40 °C with iron addition and 50 °C (E and F). These samples were taken at day 56 and 14, respectively. When samples were taken, the entire sample containers (one container for both PV and dynamic headspace GC–MS analyses) were moved from their respective storage conditions to storage at 2 °C in the dark until the analyses were conducted.

Oil Extraction Oil extraction was performed as described by Bligh and Dyer [6] but with a reduced amount of solvent as described by Iverson *et al*. [7]. In short, the lipids were extracted through the use of a homogenous mixture of 20 mL chloroform, 20 mL methanol and 15 mL water. The water soluble parts of the sample were separated from the lipid soluble parts of the sample by the addition of chloroform and methanol. Thereafter, the two-phased sample was centrifuged to fully separate. After phase separation, the chloroform in the chloroform and lipid phase was evaporated by purging with nitrogen at room temperature, and the subsequent oil contents could be determined gravimetrically. The method was used to extract neutral lipids, polar lipids and free fatty acids from the samples. The lipid extract was used as the starting material for PV analysis.

Peroxide Value

The primary oxidation products were measured by PV using the IDF method [8] and quantified by colorimetric determination of iron thiocyanate. The spectrophotometer UV mini 1240 (Shimadzu, Duisburg, Germany) was adjusted to 500 nm and reset to detect chloroform/methanol (7:3) solvent as zero.

Secondary Volatile Oxidation Products by Dynamic Headspace GC–MS

The dynamic headspace collection was performed as described by Rørbæk [9] with the following modifications: Volatiles were collected from 3 g of the sample mixed with 30 mg of internal standard solution (30 µg/g of 4-methyl-1-pentanol in rapeseed oil), 15 mL water and 5 g of Synperonic antifoam solution (8 g/L). The volatiles were collected on Tenax® tubes (Gerstel, GmbH & Co. KG) for 30 min at 45 °C with a nitrogen flow of 150 mL/min. Water was removed by a nitrogen flow at 50 mL/min for 15 min after collection. The collected volatiles were desorbed from the Tenax® tubes by using an automatic thermal desorber at 220 °C (ATD-400, PerkinElmer, Norwalk, CT, USA) connected to an Agilent 5890 IIA model (Palo Alto, CA, USA) GC equipped with a MS HP 5972 mass selective detector. The settings for the MS were electron ionization mode, 70 eV, and mass to charge ratio scan between 30 and 250. The chromatographic separation of volatile compounds was performed on a DB1701 column (30 m × ID 0.25 mm × 0.5 µm film thickness, J &W Scientific, Folsom, CA, USA) using a helium gas flow (1.3 mL/min).

The GC oven was programmed by the following protocol: initial temperature of 45 °C for 5 min, then increasing with 5 °C/min to 90 °C and then with 7 °C/min to 220 °C, where it was held for 4 min (Total run time 36.57 min).

The volatile compounds were identified individually by the MS-library Wiley 138 K (John Wiley and Sons, Hewlett-Packard) and quantified by comparison with an external standards calibration curve. The volatile compounds, which were present in the topical skin formulations during storage, were quantified. The external standards used for calibration curves in the study were 2-ethylfuran, 2-methyl furan, 2-methyl-3-buten-2-one, 1-penten-3-one, 1-butanol, pentanal, butane nitrile, 3-methyl-1-butanol, 2-pentenal, 1-pentanol, 2-hexanone, hexanal, pentane nitrile, 1-hexanol, 2-heptanone, heptanal, hexane nitrile, 1-heptanol, octanal, 2-ethyl-1-hexanol, 1-octanol and octane nitrile dissolved in ethanol.

The standard solution was diluted in ethanol to obtain concentrations from 10 to 1000 ng/µL of the volatiles. These were directly injected into Tenax® tubes. The ethanol was evaporated using nitrogen flow at 50 mL/min for 5 min. Thereafter, standards were analysed on GC–MS using the same conditions as for the samples.

Odour Evaluation by Human Sensory Panel

Sensory odour evaluation was conducted on samples after 8 weeks storage at 2 °C (A), 20 °C (B), 20 °C +light (C) and 40 °C (D) and samples after 2 weeks storage at 50 °C (F).

Sensory Lab Design The test room fulfilled the ISO (and Nordic) standards and guidelines for the design and construction of sensory assessment rooms as per the following standards: ISO 8589, 1988; ISO 8586-1, 1993; ISO 11035, 1994; NMKL Procedure No. 6, 1998.

Sensory Odour Assessors All panel members passed aptitude tests to both detect and effectively communicate any differences. According to ISO 5496 (2006), ISO 8586-1 (1993), ISO 8586-2 (1994), ISO 3972 (1991), ISO/CD 13300-1 (2002) and ISO/CD 13300-2 (2002), the tests include training in detection and recognition of (tastes and) odours, sensitivity, ranking and/or triangle tests of basic tastes, odour, texture and appearance tests as well as scale and product training. The number of assessors in the panel was 10–12 (ISO 4120, 2004; ISO 5495, 2005).

Sensory Odour Evaluation

Odour Triangle Tests The objective of the triangle tests was to assess the effect of the storage treatments compared to a sample stored at 5 °C (reference). Samples were prepared in the following way: 2 mg/cm^2 of sample was spread onto a neutral surface in a plastic cup with lid and given a random three-digit number and presented at room temperature. This procedure was used for both product types. Formulations stored under all conditions, except those with iron addition (E), were included in triangle tests.

The assessors were trained in handling the samples and how to odour assess them in a standardized way.

For each sample code, a minimum of 30 triangle tests were served in random order in such a way that all six possible sequences of the test sample and the reference sample were used (ISO 4120). In each session, each assessor received 12 sets of samples. The assessors were instructed in evaluating the samples in the given order. Data was collected using the FIZZ Network (Version 2.0, Biosystems, France).

Sensory Odour Profiling of Skin Care Formulations

The samples were prepared as described for the triangle tests.

There were two phases in sensory odour profiling: an attribute-determination/training session and an analysis session: the first session developed relevant attributes to describe the odour of the samples. The second session then scored the products vs. these pre-determined attributes. The panel used a scale from 0 to 15 for describing the intensity of the attributes using different samples ensuring that all experimental conditions that could be foreseen were represented. Thereby, it was ensured that the assessors were able to use the whole scale for each attribute and that all relevant attributes were trained. At each session, panelists were served ten samples in random order.

The sensory attributes that were selected for profiling are described in Table 3.

Statistics

Statistics on PV and Volatile Oxidation Products Data

The volatiles ($n = 3$) and PV ($n = 2$) data were analysed statistically using Graph Pad prism version 6 (Graph Pad, La Jolla, USA). A two-way analysis of variance followed by a Bonferroni multiple comparison test was employed to evaluate significant changes during storage. The significance level was 0.05. The significant differences are denoted in Figs. 1 and 2 with volatile compounds.

Table 3 Attributes used in the sensory profiling of skin care formulation

Profiling attributes	Description
Soap flake	Soap flake without any additions
Carbamide cream	Carbamide lotion 10%, without perfume. Ingredients: Aqua, glycerine, urea, parafilm liquidum, propylene glycerol, cetearyl alcohol, PEG-40 hydrogenated castor oil, sodium ceteranyl sulphate, citric acid, sodium gluconate, dimethicone, caprylyl glucol, phenoxyethanol
Lanoline	Fresh wool (unprocessed)
Sour	Sour dishcloth/sour sock
Stale	Stale, musty, cardboard, dusty
Sourish	Acidic, acetic acid, citric acid
Caramel	Caramel
Peppermint	Peppermint
Sweet	Sweet
Almond peel	Peel from almonds

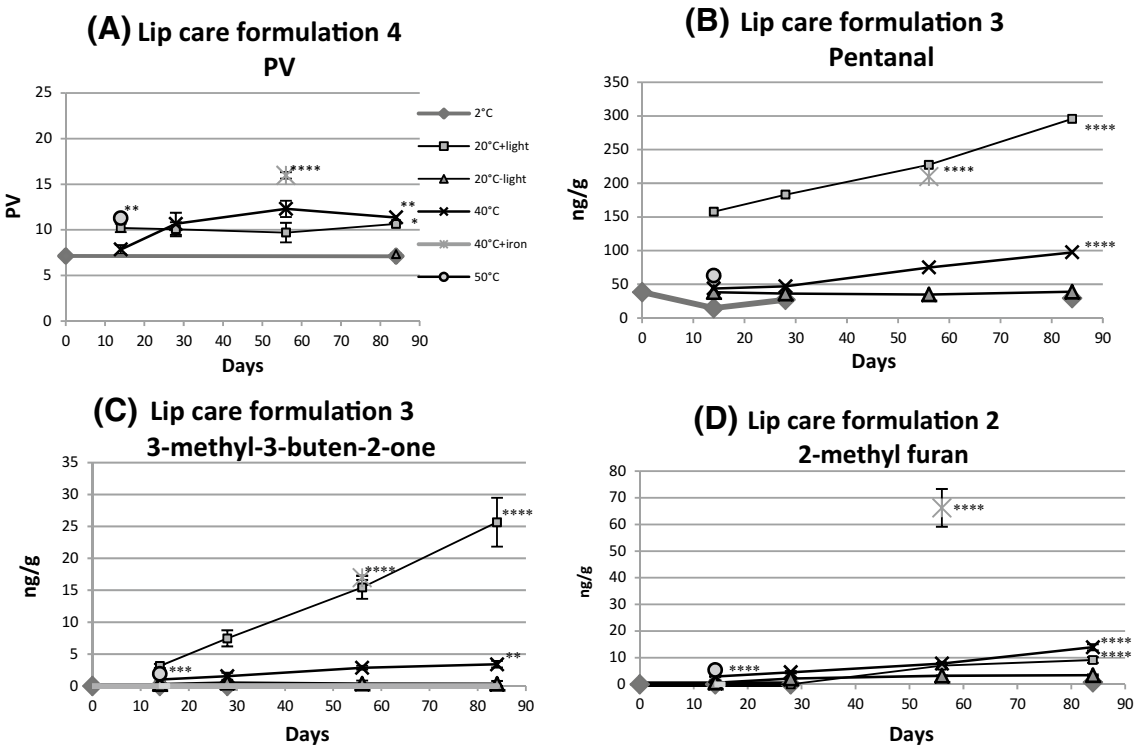


Fig. 1 The progress of lipid oxidation in lip care prototype formulations measured by **a** PV [meq/kg oil] in lip care formulation 4, and the development of three secondary volatile oxidation products [ng/g], **b** Pentanal in lip care formulation 3, **c** 3-methyl-3-buten-2-one in lip care formulation 3 and **d** 2-methyl furan in lip care formulation 2. The *stars* on the graphs show the significant levels compared to the control stored at 2 °C. Samples were taken after 0 and 84 days storage at 2 °C; after 84 days storage at 20 °C without light exposure; after 14, 28, 56 and 84 days storage at 20 °C with exposure to light and 40 °C without light exposure; after 56 days storage at 40 °C and added iron and after 14 days storage at 50 °C for graph A. Samples were taken after 0, 14, 28, 56 and 84 days storage at 2 °C; after 14, 28, 56 and 84 days storage at 20 °C in dark, 20 °C in light and 40 °C; after 56 days storage at 40°C and added iron and after 14 days storage at 50 °C for graphs **b**, **c** and **d**. The level of significance is only marked for the last sampling point compared to reference (2 °C); $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ and $****P \leq 0.0001$

Sensory Odour Data

The sensory profiling data were analysed using Panel Check V1.4.0. For analysis of the performance of the assessors, a two-way analyse of variance was used. Data from the triangle tests were analysed with one-way analysis of variance (Tables 4, 5) and principal component analysis.

Partial Least Squares Regression

All data (PV, volatiles and sensory profiling) from the skin care formulation tests were analysed using Unscrambler version 10.3 (Camo, Oslo, Norway). The partial least squares regression (PLS2) model was built on the average of the measured data and full cross validation was used to validate the model. Chemical data (PV and volatiles) were used as X-data and sensory attributes were used as Y-data. Auto scaling 1/standard deviation was applied.

Results and Discussion

Lip Care Formulations

Peroxide Value

Generally, the PV was higher in lip care formulation 4 than 1, 2 and 3, and, therefore, only PV data for formulation 4 is shown (Fig. 1a). Even in formulation 4, the PV did not increase significantly when stored at 20 °C in the dark. The PV increased slightly, but significantly, when exposed to light and higher temperatures (40 or 50 °C) (Fig. 1a). Thus, initially, the PV was 7 meq/kg and increased to approximately 12 meq/kg when exposed to light or high temperatures. The addition of iron accelerated lipid oxidation slightly more- as PV increased to 16 meq/kg under those conditions.

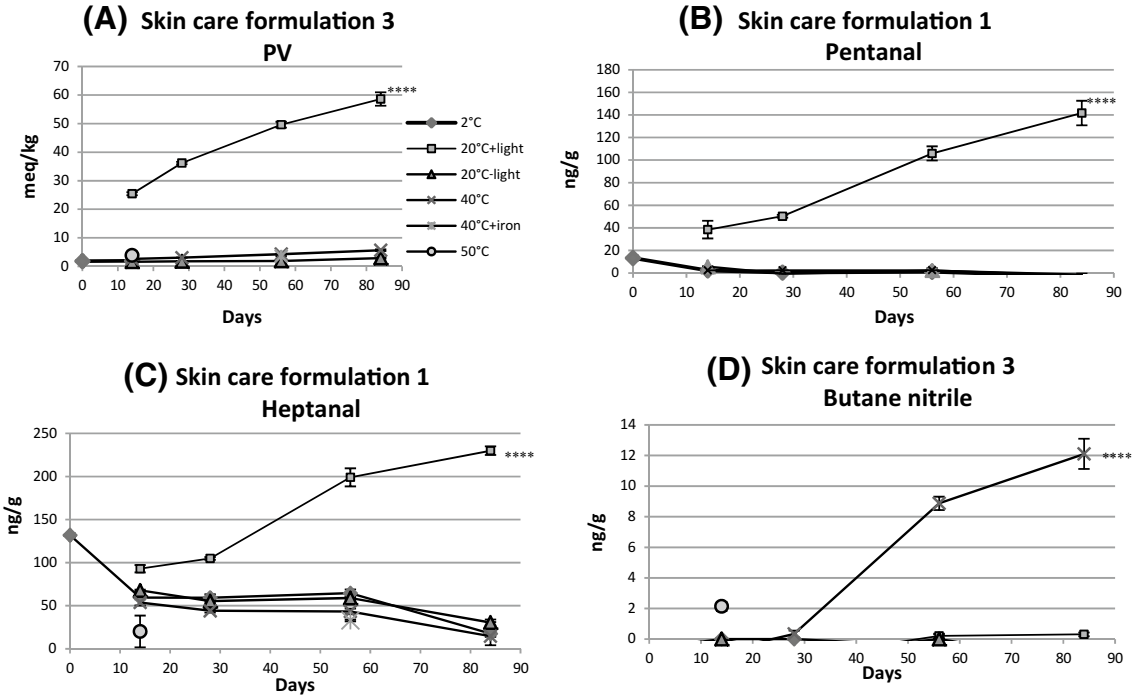


Fig. 2 The progress of lipid oxidation measured by **a** PV [meq/kg oil] in skin care formulation 3 and two secondary volatile oxidation[ng/g] and one other volatile compound [ng/g]; **b** pentanal in skin care formulation 1, **c** Heptanal in skin care formulation 1 and **d** Butane nitrile in skin care formulation 3. The stars on the graphs show the significant levels compared with control stored at 2 °C after 84 days storage. Samples were taken after 0 and 84 days storage at 2 °C; after 14, 28, 56 and 84 days storage at 20 °C with exposure to light; 20 and 40 °C without light exposure; after 56 days storage at 40 °C and added iron without light exposure, and after 14 days storage at 50 °C for graph A. Samples were taken after 0, 14, 28, 56 and 84 days storage at 2 °C; after 14, 28, 56 and 84 days storage at 20°C without exposure to light, 20°C with exposure to light with exposure to light and 40 °C; after 56 days storage at 40 °C and added iron and after 14 days storage at 50 °C for graphs **b**, **c** and **d**. The level of significance is only marked for the last sampling point compared to reference (2 °C); $^{**}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ and $^{****}P \leq 0.0001$

Volatiles

Several secondary volatile oxidation products were identified and quantified. The volatiles that increased in concentration during storage can be divided in two groups having two different patterns.

The first pattern was observed for the volatiles pentanal, 3-methyl-3-buten-2-one, heptanal and 2-pentenal. These volatiles increased most in the concentration when exposed to light, as exemplified by pentanal in Fig. 1b, 3-methyl-3-buten-2-one in Fig. 1c. Pentanal reached concentrations of 344, 378, 300 and 336 ng/g and 3-methyl-3-buten-2-one up to 37, 52, 26 and 21 ng/g in formulations 1–4 when exposed to light, respectively. Pentanal also increased in all four formulations when stored at higher temperatures and when iron was added. The volatile 3-methyl-3-buten-2-one was affected by high temperature to a lower extent, but also increased significantly with the addition of iron. Heptanal and 2-pentenal had patterns similar to pentanal.

The second pattern was observed for the volatiles 2-methylfuran, 2-ethyl-1-hexanol, 1-octanol, octanal and 2-heptanone. The concentrations of these volatiles slightly increased when exposed to light and higher temperatures as exemplified by 2-methylfuran (Fig. 1d). However, these volatiles increased markedly more when iron was added compared with the volatiles following the first pattern. The addition of iron thus increased 2-methylfuran to 47, 68, 51 and 150 ng/g in formulations 1–4, respectively.

Lipid oxidation can be significantly affected by raw material selection in the finished product. Therefore, ingredient differences can often explain differences in oxidative stability results between the formulations. In lip care formulations, formulation 4 had a higher shea butter and palm oil content, and lower glycerin content than lip care formulations 1, 2 and 3. Shea butter and palm oil contain 48 and 51% of unsaturated fatty acid, respectively. This difference both increases lipid content and degree of unsaturation [10]. This higher lipid content and more unsaturated profile may explain the higher PV level. Even though the PV was higher, the overall concentration of volatile compounds was not higher for lip care formulation 4.

Table 4 Number of triangle tests and right answers together with the significance levels

Triangle tests								
Name	Storage condition	2 Weeks storage			8 Weeks storage			
		No triangles	Right answers	Significance level	No triangles	Right answers	Significance level	
Lip care formulation 1	50 °C	30	13	0.166	No			
Lip care formulation 1	40 °C	30	7	0.9162	No	30	11	0.4152 No
Lip care formulation 1	20 °C-light	30	15	0.0435	*	30	14	0.0898 No
Lip care formulation 1	20 °C-dark	30	13	0.166	No	30	11	0.4152 No
Lip care formulation 2	50 °C	30	15	0.0435	*			
Lip care formulation 2	40 °C	30	12	0.2761	No	30	10	0.5683 No
Lip care formulation 2	20 °C-light	30	10	0.5683	No	30	15	0.0435 *
Lip care formulation 2	20 °C-dark	30	7	0.9162	No	30	11	0.4152 No
Lip care formulation 3	50 °C	30	8	0.8332	No			
Lip care formulation 3	40 °C	30	5	0.9878	No	30	9	0.714 No
Lip care formulation 3	20 °C-light	30	13	0.166	No	30	9	0.714 No
Lip care formulation 3	20 °C-dark	30	7	0.9162	No	30	13	0.166 No
Lip care formulation 4	50 °C	36	15	0.1872	No			
Lip care formulation 4	40 °C	36	13	0.4225	No	33	11	0.5651 No
Lip care formulation 4	20 °C-light	36	11	0.6967	No	33	11	0.5651 No
Lip care formulation 4	20 °C-dark	36	8	0.9486	No	33	6	0.9833 No

Results of triangle tests for lip care formulations stored at 20, 40 and 50 °C, respectively

The level of significance is * $P \leq 0.05$

Table 5 Number of triangle tests and right answers together with the significance levels

Triangle test								
Name	Storage condition	2 Weeks storage			8 Weeks storage			
		No triangles	Right answers	Significance level	No triangles	Right answers	Significance level	
Skin care formulation 1	50 °C	30	11	0.4152 No				
Skin care formulation 1	40 °C	30			30	15	0.0435 *	
Skin care formulation 1	20 °C-light	30			30	18	0.0025 **	
Skin care formulation 1	20 °C-dark	30			30	14	0.0898 No	
Skin care formulation 2	50 °C	30	17	0.0072 **				
Skin care formulation 2	40 °C	30			30	17	0.0072 **	
Skin care formulation 2	20 °C-light	30			30	16	0.0188 *	
Skin care formulation 2	20 °C-dark	30			30	9	0.714 No	
Skin care formulation 3	50 °C	30	20	0.0002 ***				
Skin care formulation 3	40 °C	30			30	20	0.0002 ***	
Skin care formulation 3	20 °C-light	30			30	22	<0.0001 ***	
Skin care formulation 3	20 °C-dark	30			30	13	0.2761 No	

Results of triangle tests for skin care formulations stored at 20, 40 and 50 °C, respectively

The level of significance is * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$

Triangle (Odour Difference) Test

To evaluate whether the increasing concentration of these secondary volatile oxidation products had a detectable effect on the odour of the lip care formulations, a sensory triangle test was performed (Table 4).

Exposure to light seemed to have a larger effect on odour changes in formulations 1 and 2. However, no significant

effect of light was observed for formulations 3 and 4 in triangle testing. Lip care formulation 2 was also significantly affected by storage at 50 °C. Since the only difference between formulations 2 and 3 was the aroma/flavour addition compared with formulation 1, it is interesting that a significant difference was observed between formulations 1, 2 and 3—potentially attributable to the presence of aroma/flavour compounds and their different constitutions.

Several secondary volatile oxidation products increased in concentration for lip care formulations 1 and 2. Namely, 3-methyl-3-buten-2-one and the aldehydes (pentanal, 2-pentenal, hexanal and heptanal) when compared with lip care formulation 4. This pattern for the volatile compounds for lip care formulations 1 and 2 is in agreement with the triangle test, where the odour changed more for formulations 1 and 2 than lip care formulation 4. Exposure to light seemed to have an especially larger effect on 3-methyl-3-buten-2-one and the aldehydes. This was confirmed by the triangle test where odour changed most in lip care formulations 1 and 2. No significant effect from light was observed for lip care formulations 3 and 4 in the triangle test. As only aroma addition was changed in lip care formulation 3 compared with 1 and 2, it was unexpected that a significant difference would be observed between lip care formulations 1, 2 and 3.This result may suggest a masking effect of the aroma used in lip care formulation 3. However, more studies are needed to confirm this finding.

Skin Care Formulations

Peroxide Value

The result from the PV analysis showed that exposure to light increased the PV in skin care formulation 3 (Fig. 2a), indicating a potentially increased susceptibility to lipid oxidation. When exposed to light, the PV increased to approximately 7, 15 and 60 meq/kg in skin care formulations 1–3, respectively.

All other storage conditions did not significantly increase the PV compared with storage at 2 °C.

Volatiles

In general, the number and concentration of volatile oxidation products were lower in the skin care formulations than in the lip care formulations. Mainly, three aldehydes increased during storage: pentanal, hexanal and heptanal, and they all exhibited a similar trend. Some alcohols were also detected in low concentrations (1-butanol, 1-pentanol, 1-hexanol, 1-heptanol, 2-ethyl-1-hexanol and 1-octanol). These alcohols will not be discussed further as no significant increase in their concentration was observed during storage.

Both formulations 1 and 2 had an increase in the concentration of pentanal when exposed to light (from 13 up to 142 and 133 ng/g, respectively) (Fig. 2b). However, formulation 3 did not show the same increase in the concentration of pentanal when exposed to light.

Similar to pentanal, only hexanal and heptanal increased in formulations 1 and 2 when exposed to light (from 132 up to 230 and 157 ng/g, respectively) (heptanal is shown in Fig. 2c). Under all other storage conditions, except with exposure to light, the concentration of pentanal, heptanal and hexanal followed the trend of the control sample stored at 2 °C. Moreover, at the end of the storage period, formulation 3 increased to 100 ng/g of 2-heptanone when exposed to light. This was not observed for formulations 1 and 2 (data not shown).

A new volatile compound, butane nitrile, which has not been previously reported in lipid oxidation literature, was found in this study. We decided to include this volatile compound in our study to investigate its effect on odour formation. This will be addressed in the section entitled Partial least square regression (PLSR) analysis. However, where it originates from is unknown and requires further study. The concentration of butane nitrile in formulation 3 increased when stored at 50 °C (Fig. 2d). However, the concentration of butane nitrile in formulations 1 and 2 did not increase during storage. It was detectable but below the quantification limit. The same pattern was observed for pentane and hexane nitrile (data not shown).

In the skin care formulations, there was no observed difference in oxidation pattern between skin care formulations 1 and 2, despite the fact that skin care formulation 2 contained reduced shea butter and caprylic/capric triglycerides content. Hence, the removal of shea butter and caprylic/capric triglycerides did not decrease lipid oxidation. Lastly, skin care formulation 3 was similar to skin care formulation 2 but with reduced glycol content and added metal chelators; CHA. Skin care formulation 3 decomposed to a lower extent for volatile oxidation products than the other skin care formulations, probably due to the presence of the metal chelators EDDS and CHA [11, 12]. Metal chelators have, in other studies, been shown to prevent oxidation [13]. The decreased decomposition of primary oxidation products to secondary volatile oxidation products may explain why the high initial PV did not result in higher concentrations of volatile aldehydes for skin care formulation 3.

Triangle Test

Sensory triangle tests were used to evaluate if the increasing concentration of the volatile compounds had a detectable effect on the odour of skin care formulations (Table 5). In contrast to the lip care formulations, several of the skin care formulations were significantly affected by the storage

conditions. Significant differences were observed for all skin care formulations when stored at 20 °C + light, 40 and 50 °C, with the exception of formulation 1 at 50 °C. The significance level was highest for formulation 3.

Sensory Profiling

A sensory profiling study was performed in order to explore the significant odour changes occurring in the skin care formulations during storage (Fig. 3). In general, most changes were observed for formulations 1 and 2 exposed to light and formulation 3 at high temperatures. The highest intensities of odour were observed for "soap flake" and "carbamide". Formulations 1 and 2 stored at 20 °C + light had a markedly higher intensity of several descriptors than formulation 3 and also vs. those stored at 2 and 20 °C in the dark. In particular, the intensity of "soap flake" and "carbamide" was higher for formulation 1 and 2 than for formulation 3. Formulation 1 also had a higher intensity of "lanoline" whereas formulation 2 had a higher intensity of "sourish" than formulation 3. When stored at 20 °C in the dark or light (Fig. 3a), formulation 3 had the highest odour intensity of "caramel" and "sweet" (only 20 + light).

During storage at 40 °C and 50 °C (Fig. 3b), the odour profile for skin care formulation 3 changed to have the highest intensity for "soap flake", "sourish", "sweet" and "almond peel", both when comparing with storage at a lower temperature and when comparing with formulations 1 and 2. Formulation 2 developed the highest intensity of "carbamide" at 40 and 50 °C.

Partial Least Square Regression

A PLSR was performed to explore the link between increasing secondary volatile compounds after 14 and 56 days of storage and odour profile (Fig. 4). Two interesting observations were made, as highlighted in Fig. 4: (A) Butane nitrile is located close to the appearance of "sourish" and "peppermint" odours for both data from day 14 and 56. Therefore, it is likely that they are related. However, butane nitrile after 14 days is also located close to other attributes, so it may also influence other odours. (B) On day 56, pentanal is located close to "carbamide". However, the same is not observed on day 14. There is a tendency toward a relation between these variables. To explore the effect of butane nitrile on odour, 100 ng/g was added in a freshly prepared skin care formulation 4 to conduct a simple evaluation of its effect on odour changes. The addition of butane nitrile to the skin care formulation was above the concentration observed during storage (0–12 ng/g). However, the butane nitrile addition only resulted in a slight increase in "bitterness" and did not lead to other noticeable changes in the odour of the skin care formulation. Butane nitrile increased during storage at higher temperatures (50 °C) Butane nitrile has been described as having a "bitter almond-like odour" [14, 15]. Significant odour changes were observed when skin care formulation 3 was stored at higher temperatures, and these were not clearly related to other volatile compounds, and; therefore, butane nitrile was included in this study. Butane nitrile was related to sourish and peppermint odour in PLSR; however, an evaluation showed that it did not cause the odour change observed in skin care formulation 3. This finding may suggest that (1) butane nitrile did not cause the "sourish" or "peppermint" odour, or (2) it may be a combination of several volatiles which gave rise to the increasing "sourish" or "peppermint" intensity.

To investigate the effect of pentanal on odour changes in skin care formulation 4, a pentanal standard was added at a concentration of 150 ng/g to formulation 4, which was just above the highest concentration found in the samples (0–142 ng/g). The odour of the product changed to more "pungent", "apple" and "cheese like" upon the addition of pentanal as described by [16]. Again, it was not exactly the same odour as the "carbamide" detected in the product during storage.

The odour changes may be due to a combination of several volatile compounds.

In general, most changes were observed for formulations 1 and 2 when exposed to light. For formulation 3, changes were observed at high temperatures. Even though PLSR links volatile compounds to odours, it was not possible to confirm that a single volatile compound caused the observed changes.

Another study on fish oil enriched milk investigated the effect of combinations of volatile compounds and found that markers for metallic odour were due to a combination of 2,6-(E,Z)-nonadienal and 1-penten-3-one [17]. Therefore, the odour changes observed during this study in the skin care formulations could have been caused by a combination of several volatile compounds.

The concentrations of various volatiles in samples were determined by calibration curves based on standards dissolved in ethanol and added directly on the Tenax tube. Therefore, the concentration in the samples may be higher than determined as direct standard addition on the tube did not include the matrix-effect on the release of volatile compounds. GSK Toxicology group has assessed the human safety impact of the volatiles included in this report. At the determined levels these substances do not raise any toxicological concern, neither locally or systemically.

Universal Markers for Lipid Oxidation

One of the goals of the study was to investigate the progress of lipid oxidation in topical skin products. PV was found

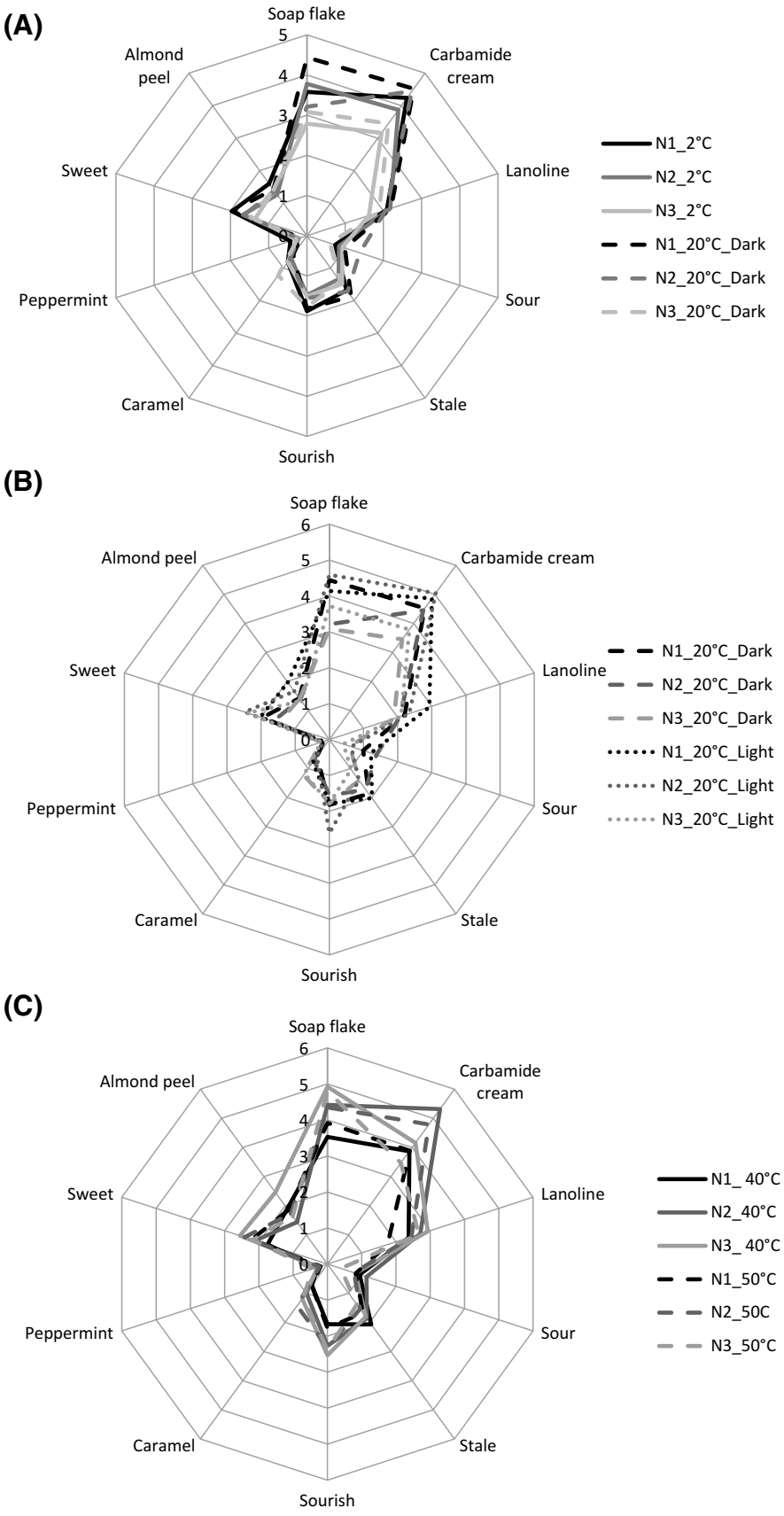


Fig. 3 Spider plots of profiling intensity of attributes: **a** intensity of skin care formulations *1* (N1), *2* (N2) and *3* (N3) after 8 weeks at 2°C, 20°C in the dark, **b** intensity of skin care formulations *1* (N1), *2* (N2) and *3* (N3) after 8 weeks 20°C in the dark and light, and **c** intensity of skin care formulations *1* (N1), *2* (N2) and *3* (N3) after 8 weeks at 40 °C and after 2 weeks at 50 °C

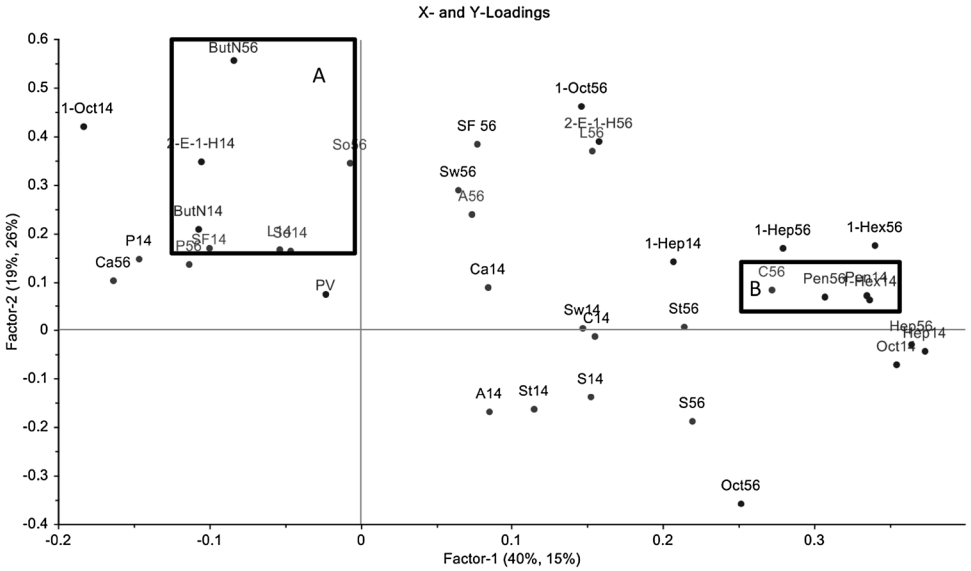


Fig. 4 *Partial least squares* regression where all data were included. Data from day 14 are marked 14, and volatile compounds are 56 on day 56. Abbreviations used in the plot for sensory attributes; Lanoline (*L*), Sour (*S*), Sourish (*So*), Soap flake (*SF*), Peppermint (*P*), Caramel (*Ca*), Sweet (*Sw*), Almond peel (*A*), Carbamide cream (*C*) and Stale (*St*). Abbreviations used in the plot for volatile compounds; 2-ethyl-1-hexanol (*2-E-1-H*), 1-octanol (*1-Oct*), Butane nitrile (*ButN*),1-heptanol (*1-Hep*), 1-hexanol (*1-Hex*), pentanal (Pen), Heptanal (*Hep*) and Octanal (*Oct*)

to be a more useful marker for lipid oxidation in skin care formulations than for lip care formulations. During storage at 20 °C, a slight increase in PV was observed in skin care formulations. However, the hydroperoxide concentration alone did not provide a sufficient view on the progress of lipid oxidation. Therefore, it must be combined with measurements of secondary volatile oxidation products both to investigate if a fast conversion from primary to secondary volatile oxidation products conceals the progress of lipid oxidation and to identify the specific volatile compounds, which are increasing. Several scientists have applied this approach, and useful results have been obtained by using GC–MS for measuring secondary volatile oxidation products [9, 18]. In the present study, this approach was also demonstrated to be useful as several volatile oxidation products increased during storage in both lip and skin care formulations at the different storage conditions.

Several aldehydes increased during storage; pentanal, 2-pentenal and heptanal in lip care formulations, and pentanal, hexanal and heptanal in skin care formulations. All four aldehydes could be useful markers for lipid oxidation in the formulations. Pentanal and heptanal were especially reliable markers for the development of secondary volatile lipid oxidation products for both lip care formulations and skin care formulations, although, they increased most when exposed to light. The use of pentanal, hexanal and heptanal as a marker for secondary volatile oxidation products seems logical as several authors have linked these compounds to oxidation of n-6 polyunsaturated fatty acids, which were also present in the lipids used in this study [1, 19–21]. Furthermore, other authors have used pentanal and hexanal as markers for the occurrence of secondary volatile oxidation products in emulsions [19, 20].

In the lip care formulations, 2-methyl furan and 3-methyl-3-buten-2-one developed during "iron acceleration" and they seemed to be useful markers for stability. However, 2-methyl furan is not often used as a marker for lipid oxidation, but it has been suggested to be a by-product from Maillard-type reactions by several authors [22–24].

In general, the sensory evaluation showed that odour changes most in skin care formulations and only slightly in lip care formulations. This finding may at least partly be related to the higher viscosity of lip care formulations. An ability to reduce lipid oxidation has been observed and linked to high viscosity by other authors [25, 26]. It is, therefore, hypothesised that viscosity of the product can affect the diffusion of oxygen and pro-oxidants from the water phase to oil phase and thereby inhibit oxidation in lip care formulations. Furthermore, high viscosity may lower the ability of volatiles to escape.

Evaluation of Accelerated Stability Methods

The third goal of this study was to investigate different methods to accelerate lipid oxidation. As mentioned, three different acceleration methods were explored: increased temperature, light exposure, and the addition of iron.

For the Tested Skin Care Formulations

Increased temperature (20, 40 and 50 °C) did not have any clear effect on PV and formation of volatile oxidation products, but it gave rise to formation of butane, pentane and hexane nitriles in formulation 3. Moreover, storage at

increased temperature affected sensory properties compared to storage at 2 °C.

Light exposure had a more pronounced effect vs. temperature on PV and formation of pentanal, hexanal and heptanal, so this approach may be a helpful tool to perform accelerated studies.

Iron addition did not significantly accelerate oxidation and, therefore, is deemed ineffective as a means of accelerating oxidation stability in the selected skin care formulations. The absence of lipid oxidation from this method may be related to an incomplete dissolution of iron in the skin care formulation or the presence of iron chelating ingredients. Other studies have successfully used $FeSO_4$ to initiate lipid oxidation in simple oil-in-water emulsions [27]. More studies are needed to explore the reason for the absent initiating effect of iron in this study.

For the tested lip care formulations

Increased temperature (40 and 50 °C) had a clear effect on PV and rate of formation of the volatiles 2-methylfuran, 2-ethyl-1-hexanol, 1-octanol, octanal and 2-heptanone in lip care formulations, so it can be used to perform accelerated studies in this type of product.

Light exposure had a more pronounced effect vs. temperature increase on PV and formation of particularly pentanal, 3-methyl-3-buten-2-one, heptanal and 2-pentenal. Hence, exposure to light more efficiently accelerates lipid oxidation than increasing the temperature in this product.

Iron addition was associated with a slight increase in PV, and formation of 2-methylfuran, 2-ethyl-1-hexanol, 1-octanol, octanal and 2-heptanone. Iron addition showed a greater effect on lip care formulations compared with skin care formulations. Therefore, it may be considered an effective accelerator of lipid oxidation in certain lip care formulations.

Elevated temperatures and especially exposure to light had a clear effect in both lip care formulations and skin care formulations. Therefore, temperature and light variation may be appropriate conditions to perform accelerated studies of both types of products.

Conclusion

Lipid oxidation was found to occur in all formulation types, but the level of detected oxidation products were generally higher in skin care formulations than in lip care formulations. The extent of oxidation was detected by PV for the primary oxidation products and dynamic headspace GC–MS for the secondary volatile oxidation products.

Overall, the samples exposed to light had larger increases in PV than the samples from other storage conditions where only minor increases were observed. Therefore, light may be considered an effective inducer of lipid oxidation (products should be protected from light to prevent lipid oxidation).

Based on the results obtained, several potential markers (indicators) for detecting secondary volatile oxidation products have been identified. For lip care formulations, they are: pentanal, 2-pentenal, 2-methylfuran, 3-methyl-3-buten-2-one, 2-ethyl-1-hexanol, 1-octanol, 2-heptanone, heptanal and octanal. For skin care formulations, they are: pentanal, hexanal, heptanal and 2-heptanone. Testing all of these analytes in an odour stability study would be extremely time-consuming. The detection of two (well-known) secondary volatile oxidation products was common across both product families and was indicative of the detected lipid oxidation which occurred during storage; it is thus recommended to use pentanal and heptanal as overall markers instead of all of the aforementioned volatiles.

In addition, three volatile compounds were detected (butane, pentane and hexane nitrile) which increased during storage; these species have not been reported in lipid oxidation studies to date. More studies are needed to explore the origin and effect of these nitriles.

Several acceleration methods were evaluated for predictive odour stability. The most effective methods for inducing lipid oxidation were exposure to light and elevated temperatures. The addition of iron was not able increase lipid oxidation in skin care formulations, but may be able to do so following method optimisation for both lip care and skin care formulations.

For sensory odour testing, the triangle tests showed that odour changed during storage for both types of formulations and sensory profiling described the specific changes. However, it was not possible to link a single volatile compound to a specific odour change. The odour changes are likely to be related to an increase and combination of several volatile compounds, but more studies are needed to confirm this.

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Appendix 2: Article II

European Journal of Lipid Science and Technology

Odour Detection Threshold Determination of Volatile Compounds in Topical Skin Formulations

Journal:	*European Journal of Lipid Science and Technology*
Manuscript ID	ejlt.201700231.R1
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	09-Nov-2017
Complete List of Authors:	Thomsen, Birgitte; Technical University of Denmark, National Food Institute, Division of Industrial Food Research Hyldig, Grethe; Technical University of Denmark, National Food Institute, Division of Industrial Food Research Taylor, Richard; GlaxoSmithKline, New Product Development, Skin Health Blenkiron, Peter; GlaxoSmithKline, New Product Development, Skin Health Jacobsen, Charlotte; Technical University of Denmark, National Food Institute, Division of Industrial Food Research
Keywords:	lipid oxidation, skin care products, odour detection threshold values, volatile oxidation products, sensory evaluation
Additional Keywords (select from list):	

Odour Detection Threshold Determination of Volatile Compounds in Topical Skin Formulations

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Abstract

Several studies have shown that lipid oxidation can occur in topical skin formulations, but the impact of volatile compounds on off-odour has not yet been explored. In this study, lipid oxidation was investigated in prototype skin care formulations. Firstly, lipid oxidation volatile compounds were identified which increased during storage. The results showed that the concentration of six volatile compounds increased above previously reported odour detection threshold values in water. These volatile compounds were selected for odour detection threshold value determination and also odour description by a trained sensory panel. In one case, the odour detection threshold value was 50 times higher (less detectable) in skin care products than in water, whereas for other volatile compounds the odour detection threshold value was only 1.5 times higher. The odour description of the volatile compounds was, in most cases, different from that reported in literature. The observed differences are hypothesised to be due to a masking effect of the base odour of the skin care product(s), a volatile-retaining power of the base matrix and to a cocktail effect of the combined odours from different volatile oxidation products.

Introduction

For cosmetics, product odour is known to impact consumer acceptance, product usage experience, efficacy perception and re-purchase intent. In daily life, some consumers decide whether they like a skin care product or not based on odour [1, 2].

Since the sensory attributes of topical skin products are vitally important to consumers, product development can be hugely challenging when including natural ingredients as key attributes such as odour, colour and product stability need to be assured [3]. A stable product not only maintains physical stability but also oxidative stability. Oxidation is considered to be one of the most important factors responsible for off-odour generation in lipid-containing foods and topical skin formulations. Off-odours, which may be caused by lipid oxidation, can spoil a product [4]. Antioxidants are often used to prevent/control lipid oxidation; however, some of the most efficient antioxidants, which ensure oxidative stability, have been restricted due to their toxicological profiles [5, 6].

It is important to measure how the product performs during its entire shelf life based on scientific and reproducible methodologies. Even though several highly sensitive analytical methods are available, no analytical tool can substitute the human nose. Therefore, a trained sensory panel must be used to investigate the sensory properties of topical skin formulations [7].

By using a trained odour sensory panel, it is possible to study the impact of specific volatiles including volatile lipid oxidation products on off-odour formation. It is also possible to determine the odour detection threshold (ODT) of these compounds.

Lipid oxidation has been studied widely in model-emulsions and foods, and these studies have contributed to an understanding of the analytes that are useful for indicating the progress of lipid oxidation in various products [8]. The number of studies in topical skin formulations is limited, but much of the understanding from emulsion studies can be reapplied to skin care emulsions.

Existing studies have assessed oxidative stability in skin care emulsions and simple model emulsions. In these studies, the oxidative stability was assessed by peroxide value (PV), anisidine value (AV), thiobarbituric acid reactive substances (TBARS) and colour changes [9, 10, 11]. These methods are all unspecific methods. In an earlier study we applied a combination of PV, gas chromatography–mass spectrometry (GC-MS) analysis and sensory evaluation to measure lipid oxidation during storage [12, 13]. We identified several markers for lipid oxidation. Particularly, pentanal was found to be an excellent marker (indicator) of lipid oxidation topical skin formulations with both high and low lipid content. Whilst measuring the development of specific volatile compounds is important, it is only half the story. Understanding the ODT levels for the selected

volatiles (in specific matrices) completes the understanding. However, ODT levels have not been studied in topical skin formulations to date.

Therefore, the purpose of this study was to determine ODT - with a trained sensory panel - for the selected volatile compounds which increased during storage or had a high concentration in two different topical skin care formulations in order to 1) obtain more exact ODT for the selected volatiles, 2) link results obtained by GC-MS to potential effects on odour properties, and 3) understand whether ODT obtained in water can be used to predict ODT in more complex systems such as topical skin care formulations.

Material and methods

Chemicals for the Bligh and Dyer method of extraction of lipids and PV determination as well as volatile standards (butanal, pentanal, 3-methyl-1-butanol, 2-ethyl furan, 2-pentyl furan and 1-heptanol) used for identification were obtained from Sigma–Aldrich.

Formulation Prototypes

Skin Cream Formulation Prototype (SCFP) and Cleansing Formulation Prototype (CFP) contain a blend of lipids. The lipids included in SCFP and CFP are listed in table 1 together with the approximate lipid contents, below.

Storage conditions

SCFP and CFP prototypes were stored for 6 months at 5ºC, 20ºC, 20ºC + light (app 3500 lux) and 40ºC, and for 2 weeks at 50ºC. Samples were taken after 0, ½, 1, 2, 3 and 6 months.

After sampling, all of the samples were stored at 5ºC until analysis.

Oil extraction Methodology

The Bligh and Dyer method [14] was applied using a reduced amount of solvent as described by Iverson et al. [15]. In brief, a homogenous mixture of 20 ml of chloroform, 20 ml of methanol and 15 ml of water was applied for lipid extraction from 5 g of topical skin formulation. By adding methanol followed by chloroform, water, methanol and water-soluble parts of the sample were separated from the lipid-soluble part. Centrifugation was then applied to complete phase separation. Thereafter, the chloroform in the lipid phase was evaporated and the oil content could be determined gravimetrically. The lipid extract was used for PV analysis.

Peroxide value

PV was measured on 2 g of Bligh and Dyer extract using the IDF method [16] and quantified by colorimetric determination of iron thiocyanate. It was measured on a UV mini 1240 spectrophotometer (Shimadzu, Duisburg, Germany) at 500 nm and reset to detect chloroform/methanol (7:3) solvent as zero.

Automatic dynamic headspace collection

Collection of volatile oxidation products from 1 g of topical skin formulation was performed by automatic dynamic headspace collection, transferred by thermal desorption unit/CIS [17] with the following modifications: Samples were incubated for 4 min in a 10 ml vial at a temperature of 45°C. Thereafter, the volatile compounds were collected by purging nitrogen at 50 ml/min through the headspace of the vial for 20 min. Water was evaporated by nitrogen at 30 ml/min for 44 min. The volatiles were transferred to a GC 6890N Series (Agilent Technologies, Santa Clara, USA) and analysed by MS 5973 inert mass-selective detector (Agilent Technologies, Santa Clara, USA). The settings for the MS were: EI mode, 70 eV, mass to charge ratio (*m/z*) scan between 30 and 250. A DB1701 column (30m x ID 0.25mm x 0.5µm film thickness, J&W Scientific, Folsom, CA, USA) using helium gas flow (1.3 ml/min) was used for separation.

The GC temperature program was as follows: initial 45°C for 5 min, then increasing with 5°C/min to 90°C and then with 7°C/min to 220°C and held for 4 min (Total run time 36,57 min).

The volatile compounds were identified individually by the MS-library Wiley 138K (John Wiley and Sons, Hewlett-Packard) and quantified by comparison with an external standards calibration curve. Volatile compounds that were present in the skin care products during storage were quantified. The external standards that were used for the calibration curves in the study were butanal, pentanal, 3-methyl-1-butanol, 2-ethyl furan, 2-pentyl furan and 1-heptanol.

Standard addition (spiking)

The ODT values were both determined in SCFP and CFP. Hence, the lipid content highly influence the ODT value. A pre-experiment was conducted using an expert panel to find the six volatile compounds detected by GC-MS in SCFP and CFP, which affected the odour the most. The expert panel estimated an ODT value for each volatile compound individually for SCFP and CFP. These ODT were subsequently used for selecting the concentrations of the volatile compounds for assessment by the trained sensory panel. The six volatile standards selected for odour evaluation in the SCFP and CFP were: 2-ethyl furan, 2-pentyl furan, butanal, pentanal, 1-heptanol and 3-methyl-

1-butanol. The standards for each of the selected volatile compounds were added to the SCFP and CFP in six concentrations. The lowest concentration (Conc. 1 in Table 2 and 3) was close to the ODT value of the compound in water [18-28], thereafter the concentration was increased gradually just above the estimated ODT value determined in SCFP and CFP during a pre-experiment using an expert panel. The selected concentrations for ODT determination are shown in Table 2 and 3 for SCFP and CFP, respectively.

Sensory evaluation

The sensory lab and test room used were compliant with national standards (ISO 8589, 1988; ISO 8586-1, 1993; ISO 11035, 1994; NMKL Procedure No. 6, 1998).

The determination of the ODT was obtained by a 3-alternative forced-choice (3AFC) according to ASTME-679, with 8-10 tested and trained assessors. The procedure for sample analysis and the statistical analysis was carried out as described in ASTME-679 in which panellists were exposed to increasing odour intensity starting at a level below odour detection limit, and then the level was gradually increased until the panellist reported that they could detect a change. During the last session, the assessors were asked to set up a set of sensory attributes that described the odour for the six volatiles. In each session, each assessor received 8-10 services. The assessors were instructed to evaluate the samples in the given order. Data were collected using FIZZ Network (Version 2.0, Biosystems, France).

Results and discussion

Skin Cream Formulation Prototype

PV was used to measure the primary oxidation product, lipid hydroperoxides. PV were below 1 meq/kg oil at day 0 and remained below 2 meq/kg during storage at 5°C, 20°C and 40°C in darkness (Figure 1), as expected due to the low lipid content of the formulation. However, storage at 20°C with exposure to light increased the formation of lipid hydroperoxides, as observed in other studies [12, 13]. This pattern was also observed for the volatile aldehydes for which exposure to light resulted in an increased concentration of the aldehydes butanal and pentanal (Figures 1B and 1C). The maximum concentrations of butanal and pentanal were marginally above the lowest ODT value reported in the literature for water, namely 9 ng/g [18-21] and 12 ng/g [19, 20, 21, 23, 24]. Therefore, it is likely that these compounds impact odour changes in the product.

In general, concentrations of 2-ethyl furan and 2-pentyl furan increased during storage at 40°C, but were stable at 5°C and 20°C. However, exposure to light seemed to initiate a further reaction or decomposition of 2-ethyl furan and 2-pentyl furan (Figures 1D and 1E). The concentration of these volatiles in SCFP stored at 40°C increased after 6 months above the ODT in water determined to be 2.3 ng/g [25, 26] and 6 ng/g [18, 27], respectively.

The concentration of 3-methyl-1-butanol was higher than for the other volatiles from the beginning of the storage period and increased further during storage at 5°C, 20°C and 40°C (Figure 1F). The concentration of 3-methyl-1-butanol was almost 10 times greater than the ODT value in water reported in literature at 250- 300 ng/g [19, 27, 28]. At this high concentration, 3-methyl-1-butanol was excepted to dominate product odour from the beginning of storage.

The last volatile compound included was 1-heptanol (Figure 1G). The concentration was low at the start of the experiment, but during storage it increased to 15 ng/g at 40°C. This was above the odour treshold value in water at 3 ng/g [19, 21].

The concentration of secondary lipid oxidation products increased most when stored at higher temperatures and exposed to light. The concentration surpassed the literature-reported ODT values observed for these volatiles in water but not in oil. Therefore, these volatile compounds may affect the odour of the product. To further evaluate the odours of the individual volatiles in the two products, a sensory odour evaluation was performed by a trained panel.

Cleansing Formulation Prototype

PV were below 1 meq/kg at day 0 and remained below 3 meq/kg during storage at 5°C, 20°C and 40°C in darkness (Figure 2A), as expected due to the low lipid content of the formulation. However, storage at 20°C with exposure to light increased the formation of lipid hydroperoxides significantly to approximately 20 meq/kg (Figure 2A). Similar observations have been reported earlier [12, 13] in skin care emulsions. The only secondary volatile oxidation product to increase significantly during storage was butanal, which increased to concentrations above the lowest ODT value reported in literature (for butanal dissolved in water (9 ng/g) [18-21]. In contrast to the pattern observed for PV, GC-MS results showed an increase in butanal, particularly at 40°C (Figure 1B). Despite low lipid hydroperoxide levels, an increase in butanal was observed during storage. This is hypothesised to be due to a faster conversion from primary to secondary oxidation products at elevated temperatures [20].

Since the amounts of pentanal, 2-ethylfuran, 2-pentylfuran and 1-heptanol were below their threshold values in water and/or they did not increase significantly during storage, data for the other volatile compounds are shown in supplementary material S1.

Sensory odour description and odour detection threshold value of volatile compounds

The sensory panel at DTU Food assessed (via a free text field) the odour of the volatile standards added to the two different topical skin formulations (Table 4). The odour of the volatile compounds in "concentration 6" (see table 2) differed slightly for some volatile compounds from SCFP to CFP. SCFP and CFP were assessed individually because the lipid content can affect ODTs and the odour descriptors. The similarities and differences between ODTs and odour descriptors due to formulation type were examined.

The trained sensory panel described 3-methyl-1-butanol as a sharp odour of cleaning agent in the SCFP, which was an unpleasant odour. It was sharper in the SCFP than the CFP. However, 3-methyl-1-butanol gave rise to descriptors of chemical and medicinal odours in both products. This is quite different from the odour description in the literature, where it is described as balsamic, whiskey, malt or burnt [29, 30, 31, 32].

The volatile standard of 1-heptanol provided a sweet coconut odour in both products, as well as other, more different descriptors. These descriptions are also different from reports in the literature where the odour of this compound is described as mushroom [29].

2-ethyl furan addition provided a sweet vanilla odour in both products, but also a stearin odour in the SCFP. The description in literature is not the same, although stearin may be slightly related to rubber and burnt, but not to pungent [27, 31].

Interestingly, the 2-pentyl furan addition resulted in two markedly different odours. In the SCFP, it provided a soil, moss and mushroom odour, whereas in CFP it provided a perfumed, soap flake and liquorice odour. These descriptions were similar with the description in literature where it is described as grassy, liquorice, green, bean and butter [27, 29, 31, 33].

Butanal gave rise to a cheese-like sour odour in both products. It is slightly different from the description in literature where it is described as pungent and green [29]. But the description of the sourness was quite different for the products. In the SCFP, it was an unpleasant sourness described as sour dishcloth and baby regurgitation. In the CFP, it was described as a pleasant citrus sourness.

Pentanal gave rise to a green base odour but with two different side odours in SCFP and CFP. The side odours were flower in SCFP and acidic milk in CFP. This is quite different from literature where it is described as almond, malt, strawberry, fruit and tomato [27, 29]. Since several of the volatile compounds provided a different odour compared with the literature description of the pure volatile compounds, it can be concluded that the matrix/solvent has a large impact on the odour effects of volatile compounds. This was also concluded by Costa *et al.* [34].

Because the odour profiles of the volatile compounds added to the skin care products were often different from literature, the same may be the case for the ODT values. This difference may be related to an initial content of volatile compounds in both products, but may also be due to a matrix effect on the release of the volatile compounds. It is also possible that the volatiles may have a cocktail effect, which will result in a completely different odour than any of the individual volatiles.

Odour detection threshold value determined in Skin Cream Formulation and Cleansing Formulation Prototypes

The detected volatile compounds could also be present in both products before they were spiked with each volatile compound. Therefore, the exact volatile concentration could be greater than the amounts being dosed in. For that reason, the exact volatile concentrations were determined by dynamic headspace GC-MS. The ODT values for the volatile compounds are shown in Table 5.

Since 3-methyl-1-butanol was present in high concentration from the beginning of the storage period in the SCFP, it is not surprising that the exact amount measured by GC-MS was higher than the added amount. Based on the exact ODT value of 1926 ng/g, it is possible that the concentration increase during storage could affect the product odour, particularly if exposed to light (3-methyl-1-butanol in the SCFP ranged from 1940 ng/g up to 2465 ng/g).

In CFP, the ODT value was lower (approximately 5x) than SCFP, which is expected because the viscosity and product odour intensity of the CFP was lower than that of the SCFP. Since the maximum concentration of 3-methyl-1-butanol observed during storage was 200 ng/g in the CFP, it will most likely not affect the product odour as an individual compound during 6 months of storage. However, a cocktail effect cannot be discounted.

The ODT value in the SCFP was almost 10 times higher than in water (250-300 ng/g), whereas the ODT value in CFP was only approximately 1.5 times higher. This highlights the importance of determining the ODT value in specific matrices and not to apply those for water or oil.

The ODT values for 1-heptanol were the same in the SCFP and the CFP (overlapping standard deviations), it was 155 and 170 ng/g in the SCFP and CFP, respectively. This value was more than 50 times higher than the ODT value for water at 3 ng/g – again – this highlights the importance of using ODT values determined in similar matrices. These ODT values were higher than the amount found during storage. Therefore, 1-heptanol was most likely not a contributory factor to off-odour generation as an individual compound. The same was the case for 2-ethyl furan, 2-pentyl furan, butanal and pentanal. The higher ODT values vs. in a water matrix may be related to a masking effect of the base odour of the product.

As mentioned earlier, ODT values for the volatile compounds only include the individual effect and not the cocktail effect. Other scientists have found that the cocktail effect also contributed to odour changes despite the fact that ODT values of the individual compounds were not exceeded [35, 36]. More studies are needed to investigate the cocktail effect of all the volatile compounds.

GSK Toxicology group has assessed the human safety impact of the volatiles included in this report. At the determined levels these substances do not raise any toxicological concern, neither locally or systemically.

Conclusion

This investigation has shown that the matrix in which the selected volatiles are assessed can significantly impact the ODT limit i.e. the ODT results for the volatiles in the topical skin emulsions evaluated are different from the values reported in literature for water or oil matrices. For example, butanal increased in the CFP above the ODT value for a water matrix. However, when tested, it was significantly below the ODT in the CFP itself.

Overall, the ODT values for the identified volatiles in the formulations tested were higher than those for water, which was expected. However, it was surprising that 1-heptanol had a 50 times higher ODT value in the formulations than in a water matrix. This was hypothesised to be related to a masking effect of the base odour of the products and/or reaction with other compounds, which thereby increase the ODT value. Hence, this study illustrates that it can be expected that ODT values will be substantially higher in complex emulsion systems including both cosmetic and food emulsions than in water. However, the exact increase in ODT value compared to water depends on the matrix´s retention of the volatile compounds or masking effects. For example, the ODT value for 3-methyl-1-butanol was almost 50 times higher in SCFP compared to CFP. All leading to the

conclusion that ODT of volatile compounds in complex products cannot be predicted using ODT value determined in water but they must be determined in the specific emulsion system. The storage condition, which led to the highest concentration of primary oxidation products (as measured by PV) was 20°C + light.

For secondary oxidation products the conditions of (1) 20°C + light and (2) elevated temperature accelerated the formation of several of the measured volatiles.

At elevated temperatures it is hypothesised that primary oxidation products convert rapidly to secondary oxidation products and therefore primary oxidation products are not detected in the same relative abundance with elevated temperatures.

3-methyl-1-butanol was the most impactful volatile on odour in both products. Therefore, it may be important to limit its formation and/or impact. This volatile was present initially and may therefore originate from one of the ingredients used. More studies are needed to identify the raw material(s) responsible for its presence.

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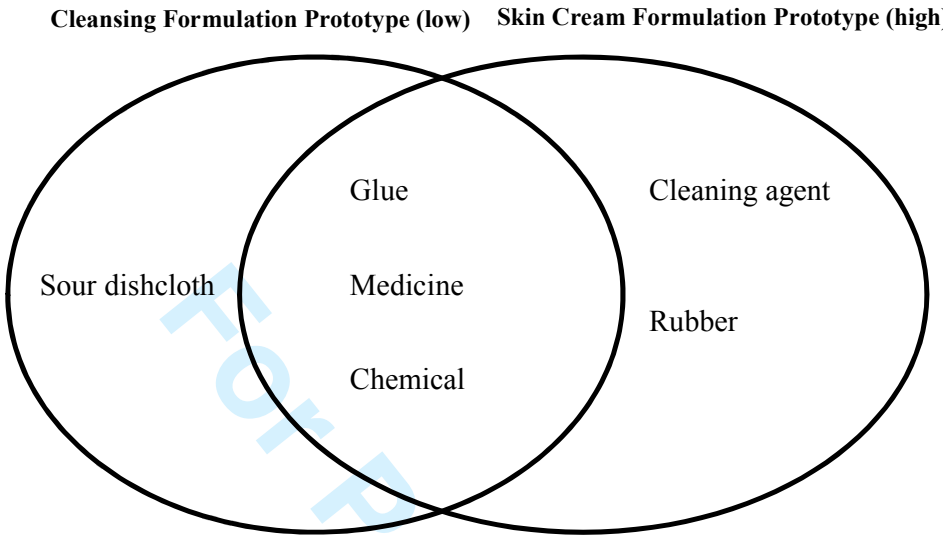
Graphical abstract: Overview of the different odour descriptors of the same volatile (3-methyl-1-butanol) when found in topical skin formulations with different lipid contents.



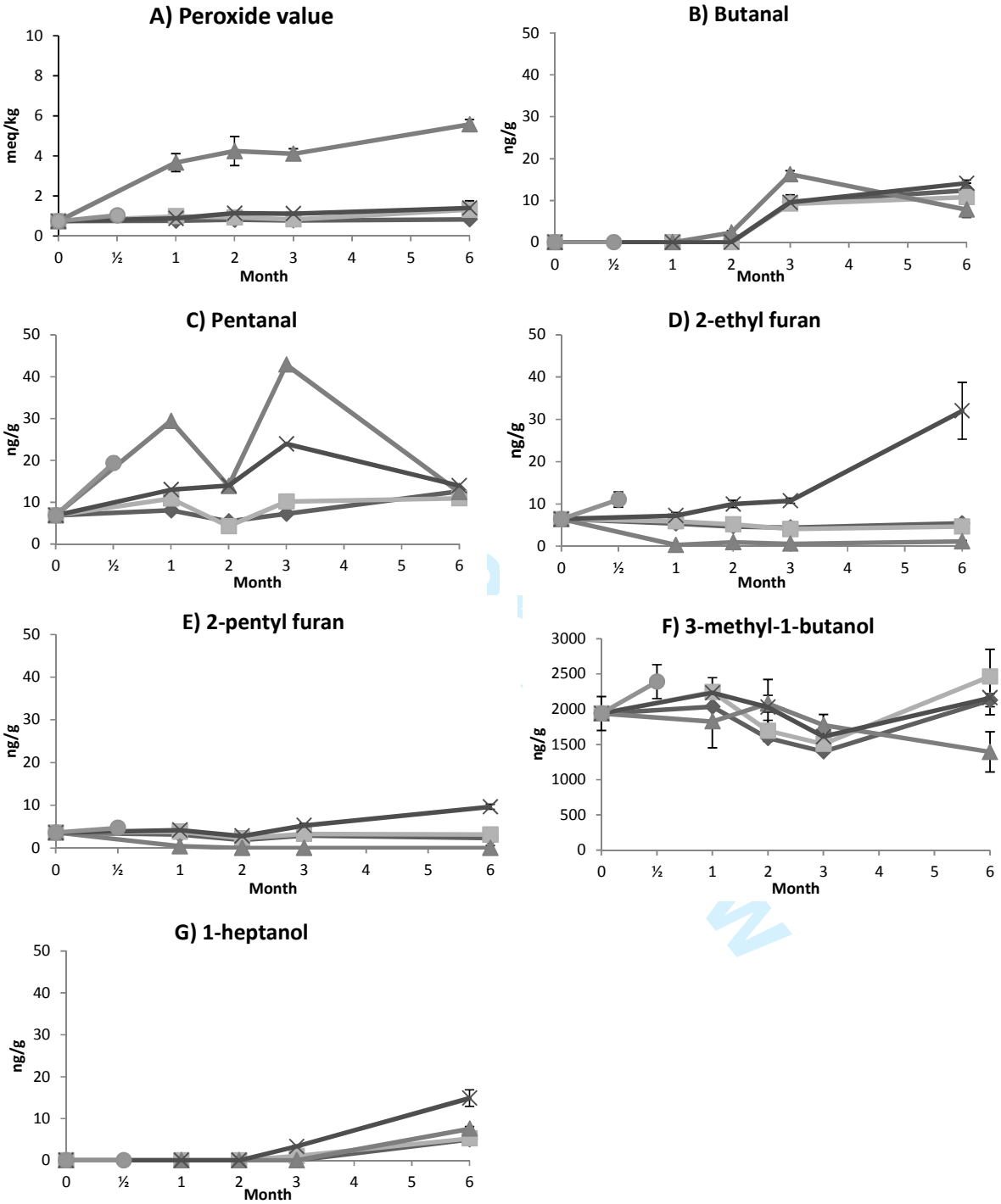


Figure 1. The progress of development of lipid oxidation products during 6 months of storage in Skin Cream Formulation Prototype under various storage conditions. The storage conditions are marked in the following way; 5°C (◆), 20°C (■), 20 + light (▲), 40°C (×) and 50°C (●). The development of A) peroxide value [meq/kg], B) butanal [ng/g], C) pentanal [ng/g], D) 2-ethyl furan [ng/g], E) 2-pentyl furan [ng/g], F) 3-methyl-1-butanol during storage [ng/g], and G) 1-heptanol [ng/g] during storage.

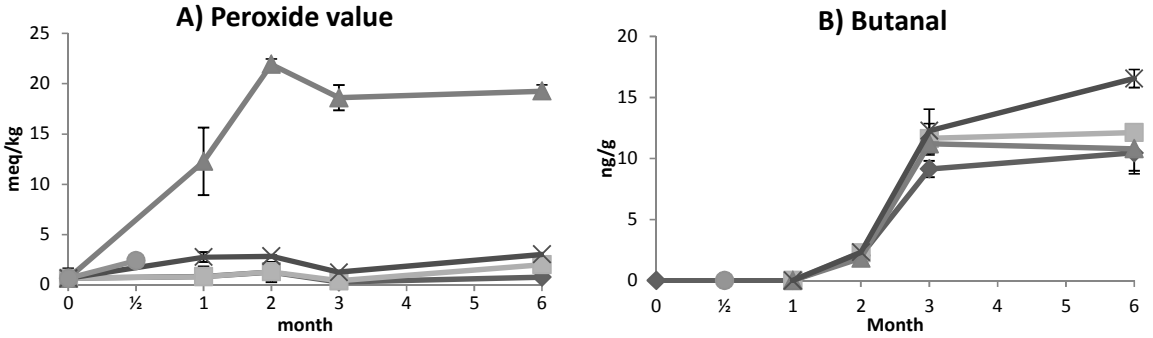


Figure 2. The progress of lipid oxidation in Cleansing Formulation Prototype during 6 months storage at various storage conditions. The storage conditions are marked in the following way; 5°C (◆), 20°C (■), 20 + light (▲), 40°C (×) and 50°C (●). The development of A) peroxide value [meq/kg], and B) butanal [ng/g] during storage.

Table 1. The lipid blends included and approximate lipid contents for Skin Cream Formulation and Cleansing Formulation Prototypes.

Product	Skin Cream Formulation Prototype	Cleansing Formulation Prototype
A blend of the following lipids	Butyrospermum Parkii Butter, Isostearyl Isostearate, Caprylic/Capric Triglyceride, Oryza Sativa Cera and Squalane	Caprylic/Capric Triglyceride, Cocos Nucifera Oil, Olea Europaea Fruit Oil, Elaeis Guineensis Oil, Olus Oil and Squalane
Total Lipid Content	~15% (N.B. any organic UV filters not included)	~10%

Table 2. Concentrations (Conc.) applied to determine the odour detection threshold value in the Skin Cream Formulation Prototype for 2-ethyl furan, 2-pentyl furan, butanal, pentanal, 1-heptanol and 3-methyl-1-butanol. Conc. 1 is close to odour detection threshold value in water. The concentration is gradually increased in conc. 2-5 until the estimated odour detection threshold value in skin cream formulation prototype determined by an expert panel has been reached. Conc. 6 is above the estimated odour detection threshold value in skin cream formulation prototype.

Concentration selected for Skin Cream Formulation Prototype						
Volatile standard/ Conc. [ng/g]	**Conc. 1**	**Conc. 2**	**Conc. 3**	**Conc. 4**	**Conc. 5**	**Conc. 6**
2-ethyl furan	10	20	30	40	55	70
2-pentyl furan	10	30	50	65	80	100
Butanal	10	25	40	55	65	80
Pentanal	10	30	50	65	80	100
1-heptanol	10	25	40	55	65	80
3-methyl-1-butanol	200	240	290	320	360	400

Table 3. Concentrations (Conc.) applied to determine the odour detection threshold value in the Cleansing Formulation Prototype for 2-ethyl furan, 2-pentyl furan, butanal, pentanal, 1-heptanol and 3-methyl-1-butanol. Conc. 1 is close to odour detection threshold value in water. The concentration is gradually increased in conc. 2-5 until the estimated odour detection threshold value in cleansing formulation prototype determined by an expert panel has been reached. Conc. 6 is above the estimated odour detection threshold value in cleansing formulation prototype.

Concentration selected for Cleansing Formulation Prototype						
Volatile standard/ Conc. [ng/g]	**Conc. 1**	**Conc. 2**	**Conc. 3**	**Conc. 4**	**Conc. 5**	**Conc. 6**
2-ethyl furan	10	20	30	40	55	70
2-pentyl furan	10	25	40	55	65	80
Butanal	20	60	100	130	160	200
Pentanal	10	25	40	55	65	80
1-heptanol	10	25	40	55	65	80
3-methyl-1-butanol	200	240	290	320	360	400

Table 4. Odour description by the trained sensory panel of the six volatile standards when added to Skin Cream Formulation and Cleansing Formulation Prototypes.

Volatile standard	Odour in Skin Cream Formulation Prototype	Odour in Cleansing Formulation Prototype
3-methyl-1-butanol	Glue, rubber, chemical, medicine, cleaning agent and toilet cleaner	Medicine, glue, sour dishcloth and chemical
1-heptanol	Perfume, lime / citrus juice, sweet and coconut	Coconut, dried banana, caramel and condensed milk
2-ethyl- furan	Stearin, white chocolate and artificial vanilla	Creme anglaise, artificial vanilla, white chocolate and caramel
2-pentyl-furan	Mushroom, moss and soil	Soap flakes, liquorice and perfume
Butanal	Parmesan, sour dishcloth and baby regurgitation	Parmesan, sour and sickly sweet
Pentanal	Green and milk acidic	Flower, green and (willow) bark

Table 1. Odour detection threshold values for the Skin Cream Formulation and the Cleansing Formulation Prototypes.

Volatile	Skin Cream Formulation Prototype		Cleansing Formulation Prototype	
	ODT avg (added ng/g)	GC-MS (total ng/g)	ODT avg (added ng/g)	GC-MS (total ng/g)
3-methyl-1-butanol	315.65	1926 ± 316	203.1	394 ± 17
1-heptanol	56.05	155 ± 24	47.9	170 ± 23
2-ethylfuran	59.35	125 ± 15	52.65	75 ± 9
2-pentylfuran	77.6	79± 13	53.85	292 ± 41
Butanal	46.2	72 ± 3	112.55	130 ± 10
Pentanal	45.75	87 ± 5	19.9	100 ± 6

Odour detection threshold (ODT) is added amount of standard. GC-MS is a measured actual value by GC-MS (including both initial content and added amount).

Appendix 3: Article III

Investigation of lipid oxidation in the raw materials of a topical skin formulation

Journal:	*Journal of the American Oil Chemists Society*
Manuscript ID	JAOCS-17-0298.R2
Manuscript Type:	Original Article
Date Submitted by the Author:	07-Nov-2017
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Keywords:	Oxidative Stability < Food and Feed Science / Nutrition and Health, Autoxidation < Lipid Chemistry / Lipid Analysis, Lipids

Investigation of lipid oxidation in the raw materials of a topical skin formulation: A topical skin formulation containing a high lipid content.

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Keywords

Oxidative Stability, Nutrition and Health, Autoxidation, Lipid Chemistry, Lipid Analysis and Lipids

Abstract

Several studies have demonstrated that lipid oxidation often occurs in topical skin formulations which can affect product odour (both positively and negatively). Furthermore, odour detection threshold values and odour descriptors of identified volatile oxidation products in cleansing and skin cream formulation prototypes were recently determined by a trained sensory panel at the Technical University of Denmark in the Division of Food Technology. In this study, we investigated lipid oxidation in a prototype skin cream formulation as well as in selected cosmetic skin care raw materials. Lipid oxidation was measured regularly over a six-month period for the product and over a three-month period for the raw materials by headspace gas chromatography–mass spectrometry. The volatile compound present in the highest initial concentration, and which increased most during storage, was 3-methyl-1-butanol (medicinal, chemical/cleaning agent odour), and its formation was linked to the raw material isoamyl p-methoxycinnamate. The odour character of the product after storage was assessed and informally deemed acceptable for consumer usage and typical of topical dermocosmetic products. A potential pathway for its formation was also identified. In addition, the concentrations of several well-known lipid oxidation products increased during storage and were suggested to originate primarily from rice bran wax, which oxidized more readily than other raw materials due to its unsaturated nature.

Introduction

Several studies have shown that lipid oxidation often occurs in topical skin care formulations containing unsaturated lipids and that lipid oxidation products can affect product quality (1–6) (i.e. odour (4–6) and colour (2)), potentially impacting product both positively and/or negatively.

Earlier studies have shown that raw materials were at least partly responsible for volatile compounds present in simple emulsions immediately after their production (7,8). Since topical skin care formulations are often emulsions, knowledge obtained from studies on simple emulsions can provide some understanding of the mechanisms behind the formation of volatile compounds in topical products. However, the composition of topical skin care formulations is far more complex than that of simple emulsion systems and so are the oxidation mechanisms. In order to determine whether/which raw materials are responsible for volatile compounds present in freshly produced topical skin formulations, several factors must be considered: volatiles introduced by raw materials, production method (e.g. temperature and other processing conditions as well as exposure to oxygen and light), and the mechanisms leading to the formation of volatile compounds (7–12). Volatile lipid oxidation products can also be formed during storage as a result of interactions between raw materials, production method and storage conditions. Temperature and exposure to oxygen and light during storage are factors that can influence the rate of lipid oxidation after production.

Other studies have investigated the effect of impurities in raw materials on oxidative stability in finished food products and model emulsions, as summarised in a review by Waraho *et al.* (12), who concluded that the oxidative stability of the finished product was linked to the quality of the raw materials. Since some raw materials used in foods are common with cosmetics, studies performed on raw materials for food can be used as guidance for cosmetics.

In a study on raw materials for personal care products, the impact of the production method on the quality of myristyl myristate, a skin conditioning and opacifying agent, was explored (13). The purity of myristyl myristate products varied from 80.1% to 97.5% between manufacturers. Furthermore, the oxidative status of the myristyl myristate products measured by peroxide value (PV) fluctuated from <0.1 to 6.0 meq/kg depending on the manufacturer and product grade. In addition, the colour, acid value (0.2 - 0.8 mg/g), hydroxyl value (1.6 - 14.0 mg/g) and saponification value (128 – 134 mg/g) also varied widely between the production methods used (13).

Two other studies investigated the oxidative stability of skin creams with new active ingredients, and both studies showed significant changes in physical and oxidative stability as well as odour properties as a result of the addition of extracts from Icelandic brown algae *Fucus vesiculosus* (2,14). This highlights the importance of securing each raw material´s quality, stability and an understanding of raw material interactions.

The aim of this study was to explore lipid oxidation in selected raw materials and in a topical skin formulation containing high levels of lipids. A second aim was to correlate any raw material oxidation

with the finished product oxidation to identify any culpable agents. In addition, we aimed to understand the mechanism leading to the formation of any identified volatile compounds.

Materials

Prototype Skin Cream Formulation (PSCF)

The prototype skin cream formulation was produced by GlaxoSmithKline (Brentford, United Kingdom) and contained several raw materials including rice bran wax, glycerine, isostearyl isostearate, palmitic acid monoethanolamine (PMEA). The prototype skin cream formulation contained approximately 29 % of lipid.

Raw materials

Separately to the aforementioned prototype product, individual (cosmetic-industry-relevant) raw materials were assessed for lipid oxidation potential:

- Rice bran wax (Koster Keunen, Bladel, Netherlands),
- Glycerine (Croda Europe Ltd, East Yorkshire, England),
- Isostearyl isostearate (Croda Europe Ltd, East Yorkshire, England),
- Palmitic Acid Monoethanolamine (PMEA; Jan Dekker, Wormerveer, Netherlands),
- Isoamyl p-methoxycinnamate (UV cinnamate) (Symrise AG, Holzminden, Germany),
- Bis-ethylhexyloxyphenol methoxyphenyl triazine (UV triazine) (BASF SE, Ludwigshafen, Germany),
- Hexyl 2-(1-(diethylaminohydroxyphenyl)methanoyl)benzoate (UV benzoate) (BASF SE, Ludwigshafen, Germany).

Methods

Storage conditions

PSCF was stored for 6 months at 5ºC, 20ºC and 40ºC without exposure to light and at 20ºC with exposure to light and for 2 weeks at 50ºC. Samples were taken after 0, ½, 1, 2, 3 and 6 months.

Raw materials were stored at 40ºC for 3 months; samples were taken after 0, 1, 2 and 3 months of storage.

The samples were stored in closed 40 ml opaque bottles. Samples were stored in individual bottles, to be withdrawn at each time point for each analysis. After sampling, all samples were stored at 5ºC until analysis.

Oil extraction methodology

Oil was extracted from 5 g of PSCF and UV cinnamate with the Bligh and Dyer method (15) (n = 2). However, a reduced amount of solvent was applied as described by Iverson *et al*. (16). In brief, lipids were extracted by the use of a homogenous mixture of 20 ml of chloroform, 20 ml of methanol and 15 ml of water. The water soluble parts were separated from the lipid soluble parts by a subsequent addition of chloroform and methanol. Phase separation was completed by centrifugation. After phase separation was completed, chloroform in the chloroform and lipid phase was evaporated, and the oil content could then be determined gravimetrically. The lipid extract was used as the starting material for analysis of PV and determination of fatty acid composition.

Determination of Peroxide Value

PV was measured using the IDF method (17) and quantified by colorimetric determination of iron thiocyanate spectrophotometrically at 500 nm by UV mini 1240 (Shimadzu, Duisburg, Germany) (n = 2). The spectrophotometer was reset to detect chloroform/methanol (7:3) solvent as zero.

Quantification of volatile compounds

Extraction of volatile compounds, GC-MS analyses and quantification were done automatically as described by Thomsen *et al.* (18) with the following modification of the sample preparation, collection and water evaporation (Table 1). These modifications were done in order to extract volatile compounds from all matrices, to avoid contamination of the tube by powders and to remove water residues.

Briefly, volatile compounds were collected from 1 g of sample in a 10 mL vial (n = 3). The automation sequence was: incubation for 4 min at a temperature of 60 °C or 45 °C (see Table 1). The sample was agitated at 300 rpm (agitator on time: 10 s, agitator off time: 1 s). Thereafter, purging with nitrogen at 50 ml/min through the headspace of the vial was started for 20 min. The volatile compounds were trapped on tubes containing Tenax GR 300 (Gerstel GmbH & Co. KG., Mülheim an der Ruhr, Germany). Water residues were removed from the tubes with a 50 mL/min purge flow (see Table 1). Then the volatile compounds were desorbed from tubes in the thermal desorption unit (initial temp 40 °C, then 720 °C/min to 280 °C kept there for 5 min) to the GC. The volatile compounds were analysed

on a GC-MS model: HP 6890 - HP 5973 (Agilent Technologies, USA). Chromatographic separation was performed on a DB1701 column (30 m × ID 0.25 mm × 0.5 µm film thickness, J&W Scientific, Folsom, CA, USA) using helium gas flow (1.3 mL/min) in the GC. The MS settings were: 70 eV, electron ionization mode, mass to charge ratio (m/z) scan between 30 and 250. The GC temperature-program was as follows: initial 45°C, 5°C/min until 90°C, 4°C/min to 220°C and held for 4 min.

Fatty acid methyl esters (FAME)

Fatty acid compositions in oil and Bligh and Dyer extracts were determined as described by Safafar *et al*. (19) (n = 2). In brief, 1 g of Bligh and Dyer extract or 0.3 g of oil were weighed in test tubes. The chloroform was evaporated from Bligh and Dyer extract with nitrogen. Then, internal standard 23:0 was added to the oil and extracted together with heptane with BHT, toluene and borontrifluoride in methanol. Samples were mixed and methylated in a microwave oven (Microwave 3000 SOLV, Anton Paar, Ashland, VA, USA) and then cooled down. Saturated NaCl and heptane with BHT were added and thereafter phase separation occurred. The upper phase of the sample was transferred into 1 mL vials and analysed by Agilent 7890A gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) with a DB-WAX fused silica capillary column (10 m×0.1 mm, 0.1 µm; Agilent Technologies, Palo Alto, CA, USA), helium as carrier gas and a flame ionization detector. The GC temperature program: initial 160 °C, 10.6 °C/min until 200°C and held for 0.3 min, 10.6°C/min to 220°C and held for 1 min, and 10.6°C/min to 240°C and held for 3.8 min. Fatty acids were identified by comparing their retention time to that of authentic standards. Fatty acids were expressed as % fatty acid of total fatty acids from C8-C24.

pH determination

The pH was measured using a Metrohm 827 pH meter (Metrohm, Herisau, Switzerland).

Description of difference scale

An expert panel of 3 scientists conducted a fast industry standard method to assess the odour changes. In this method, the sample odour was graded versus a reference sample stored at 5°C. The samples were ranked from one to five based on a scale description of difference (DOD) between sample and reference sample (Table 2). All samples ranked three or less were deemed within product range.

Statistical analysis

A two-way analysis of variance and a Bonferroni multiple comparison test were employed to evaluate significant changes in Figure 1 and 2. The significance level was 0.05. The statistical analysis was conducted using Graph Pad Prism version 6 (Graph Pad, La Jolla, USA).

Results and discussion

Lipid oxidation in PSCF: PV and volatile analysis

PV was used as a measurement of the primary oxidation products, lipid hydroperoxides. PV was initially 0.62 ± 0.01 meq/kg and remained below 0.65 meq/kg during the 6 months of storage at 5°C, 20°C and 40°C (data not shown). When exposed to light during storage, the PV increased slightly to 1.44 ± 0.17 meq/kg. According to PV, lipid oxidation only occurred to a low extent. However, a low PV does not necessarily imply that no oxidation has occurred; it may be related to rapid conversion of lipid hydroperoxides to secondary volatile oxidation products. It is therefore also advisable to assay for secondary lipid oxidation products.

The assay for secondary volatile oxidation products, via dynamic headspace GC-MS analysis, confirmed that the low PV was due to a fast conversion to aldehydes and alcohols. The concentration for the following volatile aldehydes increased significantly during storage (Figure 1): butanal, 3-methylbutanal, pentanal, hexanal, benzaldehyde and octanal. Butanal, pentanal, hexanal and octanal are all well-known lipid oxidation products. 3-methylbutanal and benzaldehyde have been suggested to originate from non-enzymatic browning reactions (20–22). Butanal, 3-methylbutanal, pentanal and hexanal increased to a greater extent during storage at 20°C and 40°C without exposure to light and at 20°C with exposure to light than at 5°C (Figure 1A-D). Unexpectedly, benzaldehyde and octanal increased most during storage at 20°C without exposure to light followed by 20°C with exposure to light.

In an earlier study, we determined odour detection threshold values for lipid oxidation products, which is the concentration at which the volatile compounds start to affect product odour. However, these were only determined for the volatile compounds that increased during storage in a PSCF. In general, we found that odour detection threshold values in PSCF were above 70 ng/g (5,6). Therefore, volatile compounds present in concentrations below 70 ng/g were not considered to affect product odour when present alone (3-methylbutanal and octanal) in the current study.

The odour detection threshold value determined for butanal was 72 ± 3 ng/g (5,6). In the present study, the concentration was above this level after 3 months storage at 20°C, 20°C with exposure to light or

40°C, and after 6 months at 5°C (Figure 1A). Butanal odour in PSCF has been described as parmesan and sour dishcloth (5,6).

The odour detection threshold value for pentanal (87 ± 5ng/g) was slightly higher compared with butanal (5,6). The concentration was above this level after 3 months at 20°C with exposure to light (at 92 ng/g) or 40°C (at 104 ng/g), and after 6 months at 20°C or 5°C (Figure 1C). Pentanal odour in PSCF has been described as green and milk acidic (5,6). The odour detection threshold value for hexanal has not been determined in PSCFs. Based on the odour detection threshold values obtained for butanal and pentanal, it is estimated to be above 90 ng/g. Hexanal concentrations were above this level after 6 months of storage at all storage conditions. In literature, its odour has been described as fatty, green and fresh (23,24). In addition to aldehydes, a few alcohols and ketones increased as well (Figure 2).

The concentration of 3-methyl-1-butanol was significantly above its odour detection threshold value of 1926 ± 316 ng/g after 6 months of storage. Odour detection threshold values have not been determined for the ketones. However, none of the ketones increased to concentrations above 70 ng/g. Therefore, it is assumed that these ketones did not affect product odour. In a previous study, 3-methyl-1-butanol was described with the odour of glue, rubber, chemical, medicine, cleaning agent (5,6). An expert panel of 3 scientists conducted a DOD sensory evaluation to assess the odour changes, PSCF increased in intensity of chemical and cleaning agent, and scored 3 on the DOD scale after 6 months storage with exposure to light and at 40°C. Since many volatile compounds were present from the beginning of the storage period, they may originate directly from raw materials. Selected raw materials were explored to link volatile compounds in PSCF to those present in raw materials.

Lipid oxidation in selected raw materials

One of the primary functions of a cream is to moisturise and protect the skin so they often contain high levels of lipids, but unsaturated lipids can oxidize and form volatile compounds. Several volatile compounds were present initially in the lipid ingredients and more were generated during accelerated storage at 40°C in the following ingredients: rice bran wax and glycerine (Figure 3A and 3B). PSCF also contained D-panthenol, which was very stable during accelerated storage. Thus, benzaldehyde was the only volatile aldehyde that could be detected and this was not possible until 3 months of storage when 139±9 ng/g was detected (data not shown).

Initially, some raw materials (rice bran wax and glycerine) contained several aldehydes and thus contributed to the initial concentration of all 10 volatile compounds detected in PSCF. Two raw

materials, rice bran wax and glycerine, contained butanal and contributed to the presence of this volatile compound in the freshly produced PSCF. Furthermore, rice bran wax contained 1-pentanol at 262 ng/g and 2-pentanone at 6 ng/g after accelerated storage. Therefore, it is likely that these two raw materials contributed to the development of 1-pentanol and 2-pentanone in PSCF. Moreover, the initial content of pentanal, 3-methylbutanal, 2-hexanone and hexanal in PSCF originated partly from rice bran wax and glycerine. The last aldehyde, benzaldehyde, may originate from D-panthenol (data not shown), rice bran wax and glycerine.

Only low concentrations of volatile compounds were present in glycerine compared with rice bran wax. Glycerine can oxidize to aldehydes such as glyceraldehyde in presence of metal ions and elevated temperature. Overall, 11 different oxidation products that have a three carbon structure have been identified for glycerine. However, the oxidation products can react with other molecules to form compounds with more than three carbons. One proposed mechanism is a reaction between glyceraldehyde and glycerine to form glycerine acetate described by Jungermann and Sonntag (25). Another possibility is simple polymerisation. The purity of glycerine was 99.5%. Moreover, the impurities may also contribute to the volatile compounds developing during accelerated storage.

Rice bran wax (mostly wax esters) mainly contained saturated fatty acids (86%; 16:0,18:0, 20:0, 22:0 and 24:0), in addition to monounsaturated (6.5%; 18:1 n-9) and polyunsaturated fatty acids (3%; 16:3 n-4, 18:2 n-6, 20:3 n-6 and 20:4 n-6). Despite a low concentration of polyunsaturated fatty acids, rice bran wax had significantly higher concentrations of most volatile compounds detected than glyerine because polyunsaturated fatty acids were highly susceptible to auto-oxidation. Auto-oxidation of polyunsaturated fatty acids gives rise to formation of primary oxidation product which can decompose further to secondary oxidation products. One of most likely decomposition pathways is scission. Scission (either α or β) results in a complex mixture of secondary oxdation products inclunding the measured alcohols, ketones and aldehydes (21,22).

The following two raw materials, PMEA and isostearyl isostearate, work as skin conditioners in PSCF. Initially, only hexanal, butanal and pentanal were present in PMEA and isostearyl isostearate (Figure 4), and they may thus partly be responsible for the initial presence of hexanal in PSCF.

Several volatile compounds appeared in the raw materials during the 3 months of storage, but some of these volatile compounds only appeared in PMEA and isostearyl isostearate (2-heptanone, heptanal and nonanal) (Figure 4). However, all 10 volatile compounds that increased during storage in PSCF also appeared and increased in isostearyl isostearate and PMEA, namely butanal, 3-methylbutanal (only isostearyl isostearate), pentanal, 2-pentanone, 1-pentanol (only isostearyl isostearate), 3-methyl-

1-butanol (only isostearyl isostearate), hexanal, 2-hexanone, octanal and benzaldehyde (only PMEA). Therefore, it is likely that PMEA and isostearyl isostearate contributed to the increase observed in PSCF of most of the volatiles. The structure of both PMEA and isostearyl isostearate did not indicate a clear reactive group/site, which can result in the observed volatile compounds. More studies are therefore needed to understand where they originate from. Their presence may be related to impurities present in the raw materials.

The last raw materials included in this study were UV filters. These raw materials were produced with the purpose of being reactive towards pro-oxidants. Three UV filters were investigated: UV benzoate, UV triazine and UV cinnamate. Initially, only a small amount of octanal was present in UV benzoate, and UV triazine did not contain any known oxidation products (Figure 5).

After 3 months of accelerated storage, aldehydes predominantly formed in UV benzoate and UV triazine. Some of the volatile compounds that appeared during the 3 months of storage were not present in PSCF (heptanal and nonanal). UV benzoate and UV triazine generated butanal, 3-methylbutanal (only UV benzoate), pentanal, hexanal, 2-hexanone, octanal and benzaldehyde after 3 months storage.

In contrast to the other two UV filters, UV cinnamate contained substantial amounts of 3-methyl-1-butanol initially, and the concentration of this compound increased further during storage (Figure 5). In addition, UV cinnamate also generated 3-methylbutanal during storage. After three months of accelerated storage, octanal 28 ng/g, pentanal 42 ng/g and benzaldehyde 495 ng/g appeared as well (Figure 5C). Although several aldehydes occurred after three months of storage, their concentrations were low in UV filters compared with the concentrations in humectant, skin texture modifying and skin conditioning raw materials. Therefore, UV benzoate and UV triazine were not explored further. However, the high concentration of 3-methyl-1-butanol generated by UV cinnamate would be expected to impact a finished product odour. A trained sensory panel described 3-methyl-1-butanol as glue, rubber, chemical, medicine and cleaning agent (5,6). Therefore, it is important to understand the route of reactions leading to 3-methyl-1-butanol in order to identify ways to control it.

MacManus-Spencer *et al.* (26) have previously investigated the degradation of octyl p-methoxycinnamate under photolytic conditions and identified 4-methoxybenzaldehyde and 2-ethylhexanol among the products. Two cleavage routes were considered in their work where the alkene in the UV-filter either reacted with water followed by a retro-aldol reaction or a reaction occurred with singlet oxygen to form the aldehydes through an unstable dioxetane (26). The same pathways can be envisioned in our case where UV cinnamate either would form 4-methoxybenzaldehyde and isoamyl

acetate by reaction with water or undergo a cleavage with singlet oxygen to give the corresponding aldehydes (Scheme 1). The addition of water to cinnamates followed by a retro-aldol reaction is a known biosynthetic pathway in the synthesis of plant benzoic acids from cinnamates (27). As a result, it should also be a feasible chemical route although the transformation is probably very slow. The cleavage of olefins by singlet oxygen is well-known (28–30) and the formed isoamyl ester of glyoxylic acid is presumably labile enough to hydrolyse completely under the storage conditions (31). Finally, direct hydrolysis of UV cinnamate to the carboxylic acid and 3-methyl-1-butanol should also be included in the considerations (Scheme 1).

In addition to 3-methyl-1-butanol the degradation of UV cinnamate may thus also form 4-methoxybenzaldehyde and isoamyl acetate which can be used to distinguish between the different pathways. The pH of UV cinnamate was 4.23 initially and decreased slightly to 4.01 after 3 months storage at 40°C. Inspection of the chromatograms from UV cinnamate did indeed reveal the presence of both 4-methoxybenzaldehyde and isoamyl acetate. The retention time was 31.887 for 4-methoxybenzaldehyde and 14.024 for isoamyl acetate and both signals were confirmed by external standards. Notably, the acetate of the alcohol was not detected in the earlier work by MacManus-Spencer *et al.* (26). 4-Methoxybenzaldehyde and isoamyl acetate were both present in UV cinnamate from the beginning of the storage period and their amounts increased further during storage. Although, the two by-products have not been quantified by the use of calibration curves, they appear to be formed in somewhat equal amounts and certainly to a much lesser degree than 3-methyl-1-butanol, which is the main by-product. As a result, 4-methoxybenzaldehyde and 3-methyl-1-butanol cannot be formed by the oxidative cleavage with singlet oxygen since this would give rise to similar amounts of both compounds. Instead, it is very likely that 4-methoxybenzaldehyde and isoamyl acetate are formed by the addition of water and a retro-aldol reaction.

This leaves the direct hydrolysis of the ester as the main pathway for the formation of 3-methyl-1-butanol. It is known that esters can hydrolyse under near neutral conditions, but the reaction is very slow. For ethyl cinnamate the half-life for hydrolysis in water at pH 4.0 and 25 °C is estimated to be about 100 years (32). This number will be higher for UV cinnamate in the present case since the hydrolysis is slower in a non-polar environment. However, the amount of 3-methyl-1-butanol released in Figure 5 only corresponds to about 0.2‰ (w/w) after 3 months storage at 40°C. Therefore it is hypothesised that this is a result of a very slow direct hydrolysis of the ester in the UV-filter under the near neutral conditions.

Linking volatiles in PSCF with those in raw materials

The volatile compounds present in PSCF and raw materials are summarized in Table 3. In brief, the increase observed in butanal in PSCF during storage may mainly originate from isostearyl isostearate, for which the concentration was above odour detection threshold value from the beginning of the storage. However, butanal also developed in rice bran wax, glycerine, PMEA, UV triazine and UV benzoate during storage.

The formation of 3-methylbutanal was related to several raw materials, namely, rice bran wax, glycerine, isostearyl isostearate, UV cinnamate and UV benzoate. The concentration of pentanal increased in all raw materials and the concentration was above odour detection threshold value in rice bran wax, isostearyl isostearate and PMEA after 3 months of storage. Hexanal increased significantly in the PSCF during storage and also increased to high concentrations in rice bran wax and PMEA (more than 150 ng/g). In addition, hexanal was present in glycerine, isostearyl isostearate, UV triazine and UV benzoate in low concentrations (less than 70 ng/g). Benzaldehyde mainly increased in PSCF at 20°C with exposure to light to 112 ng/g after 6 months' storage. It was possible to relate benzaldehyde to all raw materials except isostearyl isostearate. The last aldehyde octanal appeared in all raw materials except glycerine during accelerated storage. Particularly the concentration of octanal increased in rice bran wax. The alcohol 1-pentanol marginally increased in a few materials, rice bran wax and isostearyl isostearate. In contrast, to the low concentration of 1-pentanol and 3-methyl-1-butanol in isostearyl isostearate, 3-methyl-1-butanol was present in high concentration in PSCF from the beginning and throughout the storage period. The RMs shown to generate 3-methyl-1-butanol during storage were UV cinnamate, glycerine and isostearyl isostearate. Lastly, the two ketones, 2-pentanone and 2-hexanone, were present in both PSCF and several raw materials but only in low concentrations.

GSK Toxicology group (2017) has assessed the human safety impact of the volatiles included in this report. At the determined levels these substances do not raise any toxicological concern, neither locally or systemically (33).

Conclusion

This study explored lipid oxidation and oxidative degradation in a topical skin formulation (PSCF) containing high levels of lipid. Some secondary volatile oxidation products were present initially and more were generated during the 6 months of storage. Most notably, 3-methyl-1-butanol was present in a high concentration initially and it increased further during storage. Since the concentration of 3-

methyl-1-butanol was higher than the odour detection threshold value after six months of storage, it was expected to affect product odour after long term storage, generating an increase in the medicinal, chemical/cleaning agent-type odour character. This product was therefore assessed for odour changes (informally vs. a 5°C control sample) and deemed acceptable and typical of a dermocosmetic product, highlighting again the importance of considering the combination effect (of other volatiles present) and the product base odour when interpreting the impact of any lipid oxidation on product odour.

Selected raw materials were explored in order to link volatile compounds affecting the quality in the topical skin formulation to raw material(s). The UV cinnamate filter developed high levels of 3-methyl-1-butanol during storage so was identified as a material to control. A potential pathway leading to 3-methyl-1-butanol was proposed.

Furthermore, well-known lipid oxidation products and non-enzymatic browning products were suggested to originate from rice bran wax in particular because of its unsaturated nature. It was surprising that volatile lipid oxidation products occurred in PMEA and isostearyl isostearate, as these raw materials did not contain reactive sites for oxidation. More studies are needed to explore why volatile compounds appeared.

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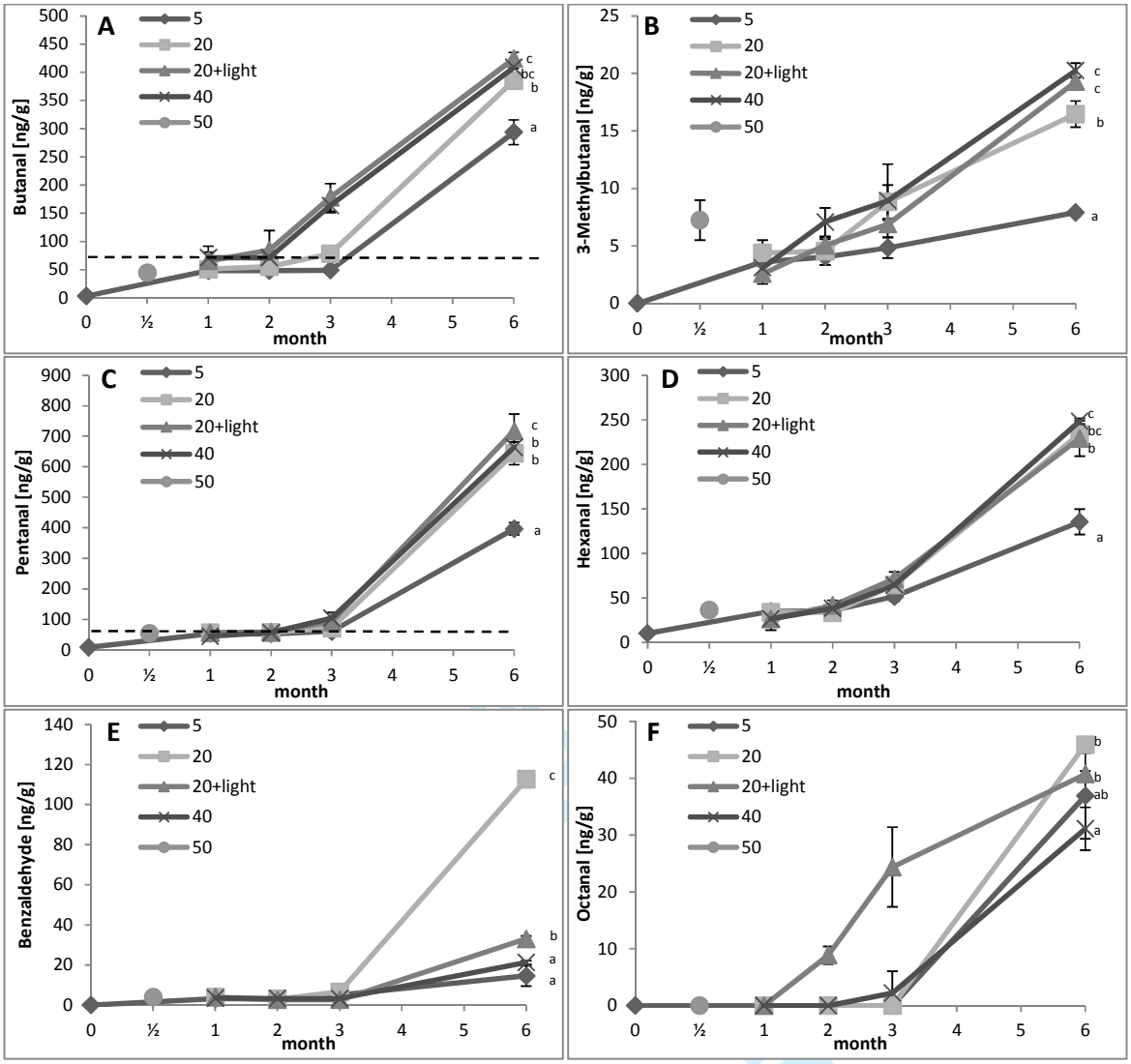


Figure 1. Aldehydes increasing in PSCF during 6 months of storage at 5°C (◆), 20°C (■), 20°C with exposure to light (▲), 40°C (×) and 50°C (●). The dotted line indicates the odor detection threshold value (butanal and pentanal). The development of A) butanal, B) 3-methylbutanal, C) pentanal, D) hexanal, E) benzaldehyde and F) octanal during storage [ng/g]. Results are presented as average +/- SD and N=3. Significance differences at 0.05 level are only marked for the last sampling point.

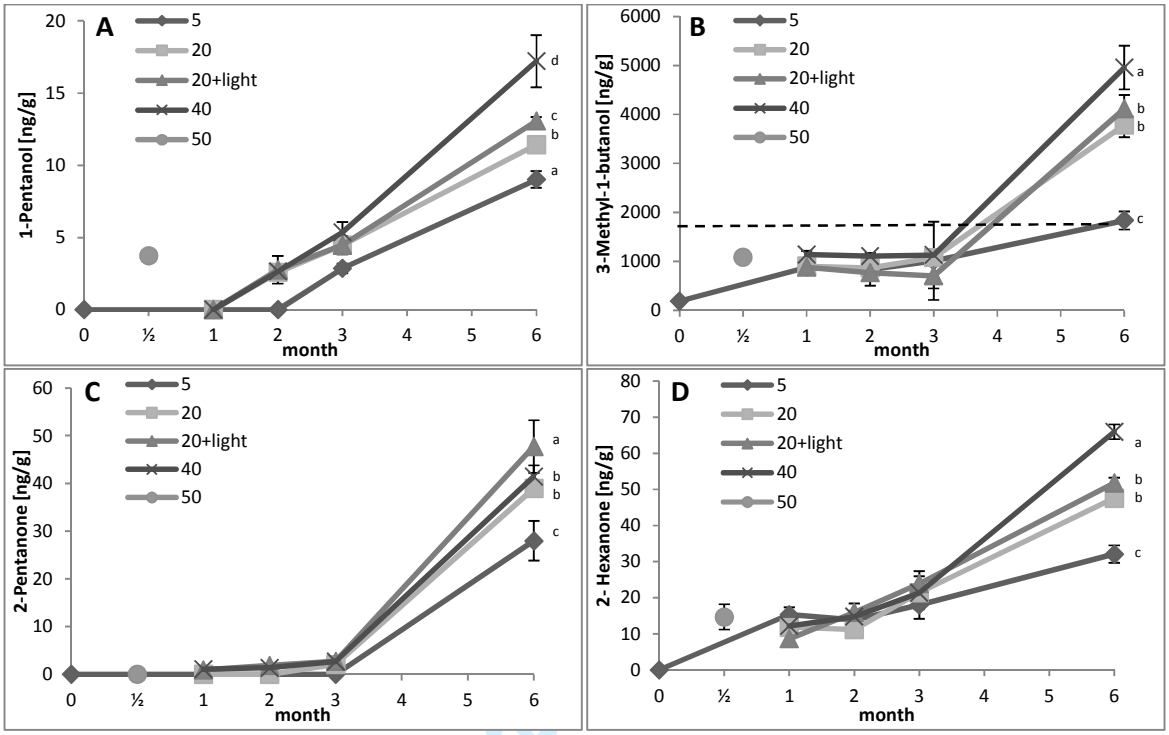


Figure 1. Alcohols and ketones increasing in the PSCF during the 6 months of storage at 5°C (◆), 20°C (■), 20°C with exposure to light (▲), 40°C (×) and 50°C (●). The dotted line is added for the exact threshold value (3-methyl-1-butanol). The development of A) 1-pentanol, B) 3-methyl-1-butanol, C) 2-pentanone, and D) 2-hexanone during storage [ng/g]. Results are presented as average +/- SD and N=3. Significance differences at 0.05 level are only marked for the last sampling point.

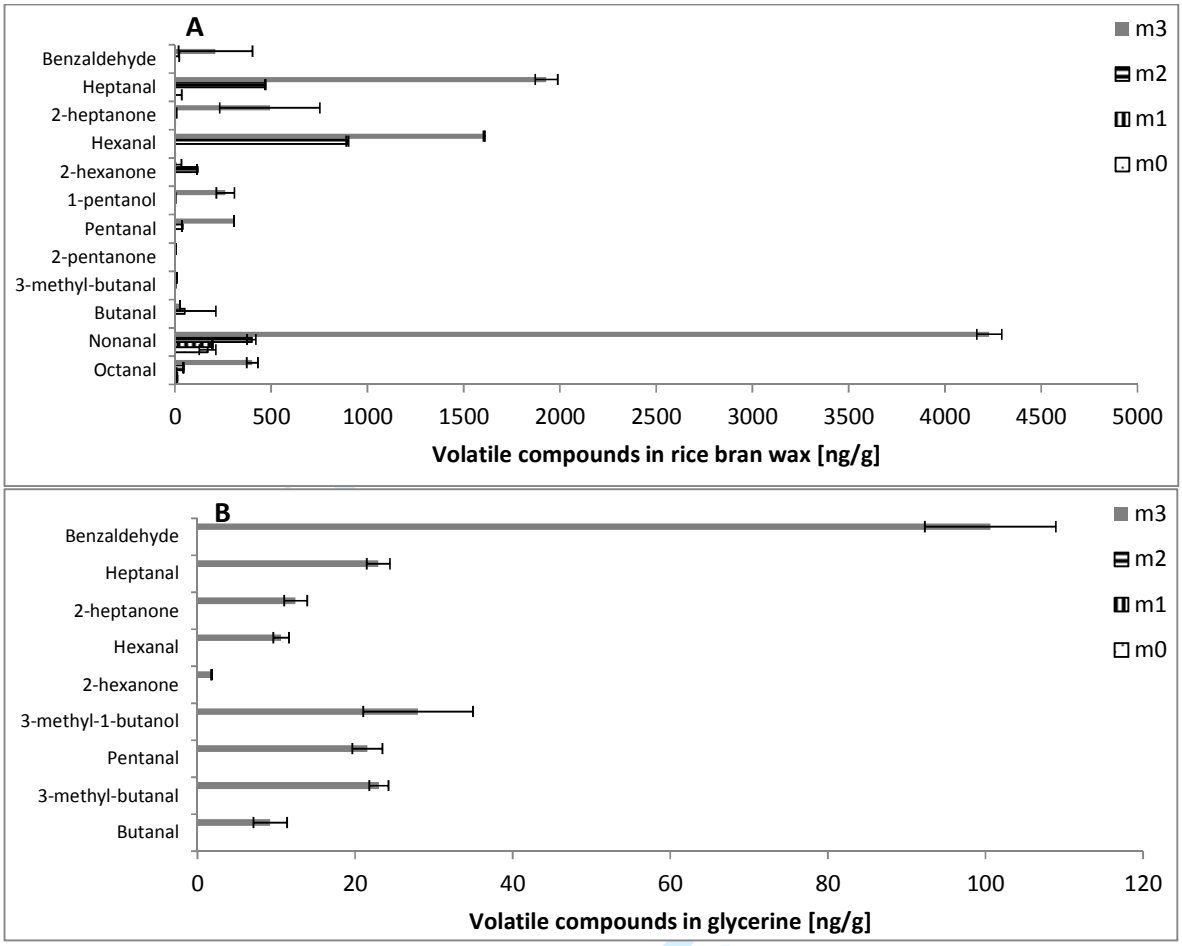


Figure 3. Volatile compounds [ng/g] present in raw materials during the 3-month storage at 40ºC. A) rice bran wax and B) glycerine. Results are presented as average +/- SD and N=3.

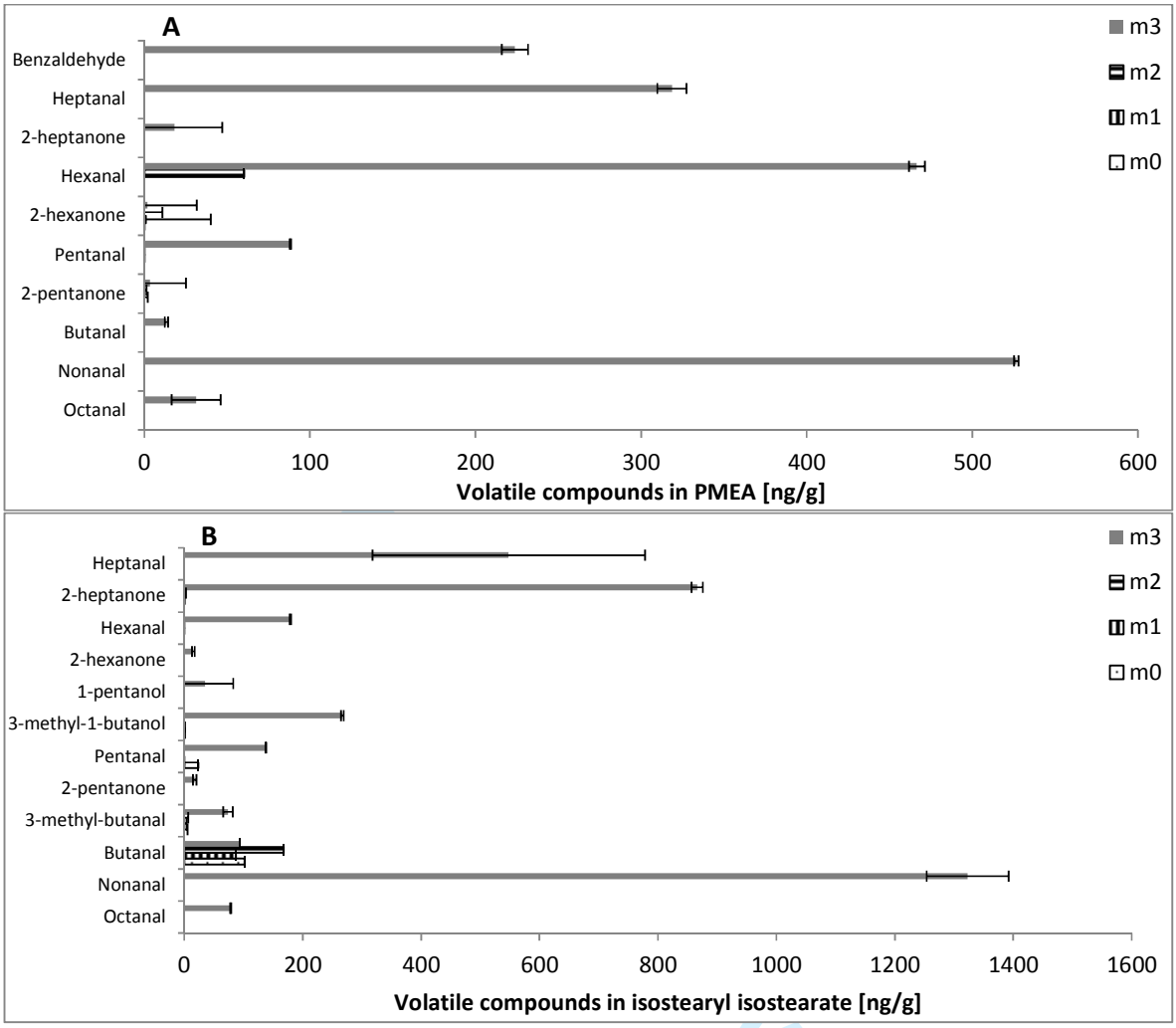


Figure 4. Volatile compounds [ng/g] present in skin texture modifying and skin conditioning raw materials during the 3-month storage at 40ºC. A) PMEA and B) isostearyl isostearate. Results are presented as average +/- SD and N=3.

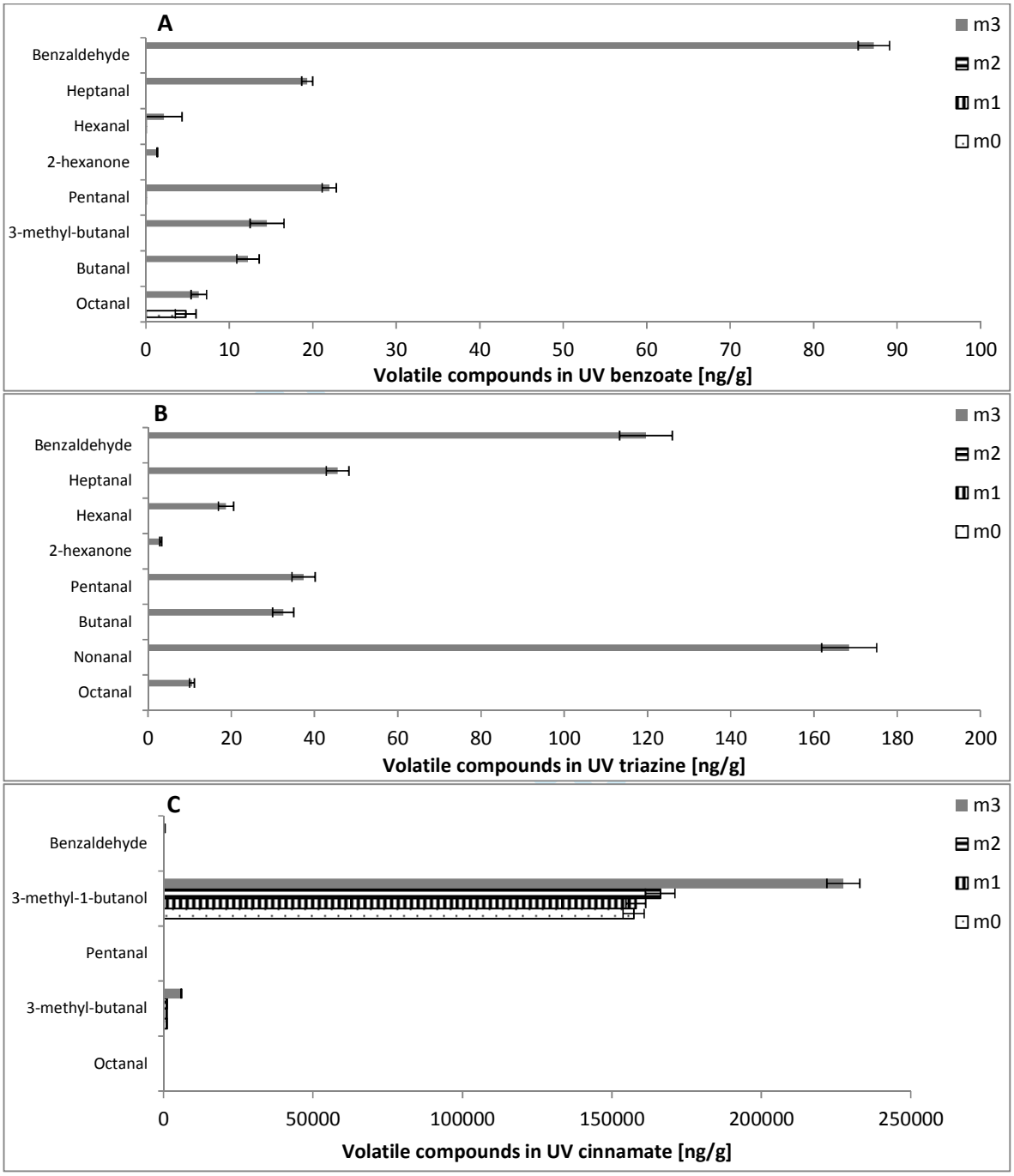
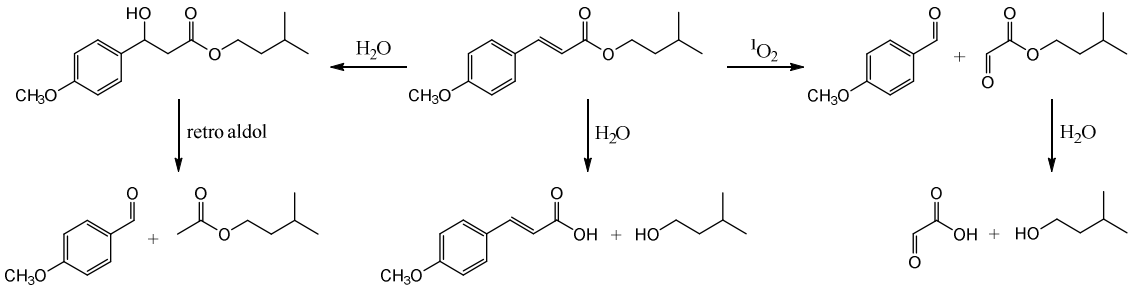


Figure 5. Volatile compounds [ng/g] present in UV filter raw materials during the 3 months of storage at 40ºC. A) UV benzoate, B) UV triazine, and C) UV cinnamate. Results are presented as average +/- SD and N=3.



Scheme 1. Potential pathways for cleavage of UV cinnamate.

Table 1. Sample preparation, collection conditions and water evaporation applied for collection of volatile compounds from PSCF and raw materials.

Samples	Preparation	Collection	Evaporation
PSCF	1 g sample. Incubation at 45°C for 5 min.	50 mL/min at 45°C for 10 min	50 ml/min at 25°C for 22 min.
Rice bran wax Glycerine Isostearyl isostearate UV cinnamate	1 g sample. Incubation at 60°C for 4 min.	50 mL/min at 60°C for 20 min	-
PMEA UV triazine UV benzoate	1 g of sample and water were mixed (1:1). Incubation at 45°C for 5 min.	50 mL/min at 45°C for 10 min	50 ml/min at 25°C for 22 min.

Table 2. Description of difference scale.

DOD Scale	Description of Difference
1	No differences in character or intensity noted
2	Reasonably sure difference exists, though difference may be too subtle to accurately describe
3	Definite difference, can describe difference with reasonable surety
4	Product or material out of expected range. Moderate or large intensity differences or ANY character differences.
5	Outside normal range. Large intensity and/or character differences.

Note: DOD = Degree of Difference

Table 3. Summary of volatile compounds present in both PSCF and raw materials. + = present, ++ = present above threshold value (5,6) in raw material (only available for butanal, pentanal and 3-methyl-1-butanol), and - = absent.

Volatile compounds/ Raw material	Butanal	3-methylbutanal	Pentanal	Hexanal	Benzaldehyde	Octanal	1-pentanol	3-methyl-1-butanol	2-pentanone	2-hexanone
Rice bran wax	+	+	++	+	+	+	+	-	+	+
Glycerine	+	+	+	+	+	-	-	+	-	+
Isostearyl isostearate	++	+	++	+	-	+	+	+	+	+
PMEA	+	-	++	+	+	+	-	-	+	+
UV cinnamate	-	+	+	-	+	+	-	++	-	-
UV triazine	+	-	+	+	+	+	-	-	-	+
UV benzoate	+	+	+	+	+	+	-	-	-	+

Appendix 4: Article IV

Investigation of lipid oxidation and degradation products in raw materials: Low fat topical skin care formulations

Journal:	*Journal of the American Oil Chemists Society*
Manuscript ID	JAOCS-17-0638
Manuscript Type:	Original Article
Date Submitted by the Author:	01-Dec-2017
Complete List of Authors:	Thomsen, Birgitte; Technical University of Denmark, National Food Institute Taylor, Richard ; GlaxoSmithKline, New Product Development, Skin Health Hyldig, Grethe; Technical University of Denmark, National Food Institute Blenkiron, Peter ; GlaxoSmithKline, New Product Development, Skin Health Jacobsen, Charlotte; Technical University of Denmark, National Food Institute
Keywords:	Oxidative Stability < Food and Feed Science / Nutrition and Health, Emulsions/Colloids < Lipid Chemistry / Lipid Analysis, Polymers/coatings < Biobased Products, Autoxidation < Lipid Chemistry / Lipid Analysis

Investigation of lipid oxidation and degradation products in raw materials responsible for the presence and development of volatile compounds during storage: Low fat topical skin care formulations

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Abstract

Topical skin formulations with a lipid content below 15% were placed on accelerated stability testing for 6 months. The volatile lipid oxidation compounds formed during this storage were compared to those formed in the raw materials during three months of accelerated stability storage. The volatile compounds were collected by dynamic headspace and analysed by GC-MS.

It was possible to link eight out of nine volatile compounds detected during storage of topical skin formulations to the raw materials. In addtion, a possible link between the appearance of butane nitrile and the decomposition of an initiator used for polymer production was observed. The polymer may originate from texture modifiers added to the topical skin formulation or from plastics used for packaging of topical skin formulations.

Furthermore, six well-known lipid oxidation and non-enzymatic browning products were suggested to originate from the two raw materials, tricaprylin/tricaprin and coconut oil.

Introduction

In earlier studies, it was clearly demonstrated that lipid oxidation can occur in topical skin formulations with either high or low lipid content and that their sensory quality can be affected by lipid oxidation as changes in both odour and colour were observed [1-6]. It is tempting to assume that the extent of lipid oxidation will decrease with decreasing lipid content. However, this may not always be the case in "real" topical skin formulations because factors other than the absolute lipid content may be more important. Similar to other emulsions, topical skin formulations consist of three phases: the lipid and aqueous phase and an interface. Lipid oxidation is affected by partitioning and diffusion of anti-oxidants and pro-oxidants in all three phases and this can significantly influence lipid oxidation. Physical factors such as viscosity and droplet size may affect diffusion of pro-oxidants and, thereby, increase or decrease lipid oxidation [7].

Topical skin care formulations are complex systems consisting of many different ingredients, which may affect lipid oxidation positively or negatively. In addition, interactions between ingredients could influence lipid oxidation as also observed for simple emulsions [8]. However, only a limited number of studies on this topic are available in the literature. An earlier study of the skin conditioning raw material, coconut oil, showed that its peroxide value (PV) increased from approximately 2 to 65 meq/kg during 42 days of storage at 60 °C [9]. PV measures the primary oxidation products, which are odourless. Hence, PV cannot be used to asses odour changes in raw materials as a result of lipid oxidation. Odour changes are caused by secondary volatile oxidation products which were not measured in the above mentioned study.

Data on the quality of other raw materials used in topical skin care formulations are available in the literature [3, 9, 10]. However, the quality may vary widely depending on manufacturing process. This was shown in a more recent study, which compared the quality of coconut oils produced in India [10]. The authors concluded that the quality varied widely depending on crop quality and production method. They analysed three types of coconut oil; virgin coconut oil from wet mature coconut, and refined, bleached, and deodorized and unrefined coconut oil prepared from copra. Furthermore, they measured oxidative status by PV, which fluctuated from 0.0 to 2.7 meq/kg depending on manufacturer. In addition, the colour (0.00 – 2.7 Lovibond unit), free fatty acid (0.01 – 2.02 %), polyunsaturated fatty acid (0.13 – 1.57 %), monounsaturated fatty acid (3.31 – 5.23 %), saponification value (239.9 – 260.2) also varied widely between the production methods. The conclusion was that virgin coconut oil had the best quality independent of manufacturer [10].

In an earlier study, we investigated lipid oxidation in raw materials used for production of a topical skin formulation with a high lipid content. We found that the most dominating volatile compounds clearly orginated from specific raw materials [1].

Several studies have explored the effect of lipid content on the oxidative stability in oil-in-water emulsions. One of these studies investigated the effect of pH and emulsifier type in two emulsions with a lipid concentration of 5 % and 70 %, respectively [11, 12]. In general, the study showed that the oxidative stability of the 5 % lipid emulsion was lower than the oxidative stability of the 70 % lipid emulsion. The lower oxidative stability was independent of emulsifier type. The findings were surprising as more oxygen can be dissolved in oil than in water. It was suggested that the accessibility to direct interaction of lipid hydroperoxides with the prooxidant metal oils in the aqueous phase was lower in the emulsions with high lipid content [11, 12, 13]. Aligned with our previous studies [11, 12], another study compared the oxidative stability of 10 % vs 30 % oil-in-water emulsions [13]. The oxidative stability was assessed by PV and anisidine value (AV) of the emulsions. The PV and AV were significantly affected by the lipid content, the 10 % emulsions had significantly higher amounts of hydroperoxides and aldehydes after 15 days storage compared to the 30 % emulsions. Overall, a decrease in the lipid content resulted in increased lipid oxidation [13].

Before we are able to understand interactions between the raw materials in topical skin formulations, the oxidative status of the raw materials must be explored. The hypothesis of this study is therefore that oxidation occurs to a higher extent in low fat than in high fat topical skin care formulations and that it is possible to link the volatile compounds to the raw material(s) used in the formulations.

The purpose of this study was thus to measure lipid oxidation and oxidative degradation products in topical skin formulations with a lipid content below 15 % as well as in selected raw materials. A second aim was to investigate the link between volatile compounds affecting quality in topical skin formulations to the presence and formation of the same volatile compounds in raw material(s). In addition, we aimed at obtaining an understanding of the mechanism leading to the formation of certain volatile compounds. We used refined coconut oil and medium chain tricaprylin and tricaprin lipid sources in the formulations.

Lipid oxidation was accelerated by increasing temperature or light exposure to more quickly generate potential volatile compounds.

Materials

Prototype skin formulations

The topical skin care formulations used in this study were produced by GlaxoSmithKline (Brentford, United Kingdom). Two types of topical skin formulations were included in this study due to their different purposes and therefore different raw materials; 1) a prototype cleansing formulation (PCF) that contains rinsing agents to clean the skin. 2) a prototype serum formulation (PSF) that has a higher concentration of performance ingredients than other topical skin formulations with a low lipid content. It is used for targeting specific skin care concerns. The PCF contained several raw materials including glycerine, tricaprylin and tricaprin, coconut oil, lecithin and polyacrylate crosspolymer-6. The PSF contained several raw materials including glycerine and lecithin. Manufacturing protocols for the formulations are proprietary information.

Raw materials

- Tricaprylin and tricaprin having the commercial name "Caprylic/capric triglycerides" (BASF SE, Ludwigshafen, Germany),
- Glycerine (Croda Europe Ltd, East Yorkshire, England),
- Coconut oil (Henry Lamotte oils, Bremen, Germany),
- Lecithin (Lipoid, Ludwigshafen, Germany),
- Polyacrylate crosspolymer-6 (Seppic, Paris, France)

Methods

Storage experiment

PCF and PSF were stored for six months at 5 ºC, 20 ºC, and 20 ºC with exposure to light, 40 ºC, and for 2 weeks at 50 ºC with sampling points after 0, ½ (only 50 ºC), 1, 2, 3 and 6 months.

Raw materials were stored at 40 ºC for 3 months. Samples were taken each month (0, 1, 2 and 3 months).

The samples were stored at 5 ºC until PV and GC-MS analysis.

Oil extraction

Oil was extracted from PCF and PSF with Bligh and Dyer method using a reduced amount of solvent [14, 15]. The method is described in more details in [1]. In brief, the water soluble parts were separated from the lipid soluble parts by addition of chloroform, water and methanol followed by centrifugation. The lipid phase was used as starting material for PV analysis and determination of fatty acid composition.

Peroxide Value

PV was determined spectrophotometrically at 500 nm using the IDF method [16].

Quantification of volatile compounds

Purge and trap on PCF

Extraction of volatile compounds and GC-MS analyses were performed as described by Thomsen et al. [17] for emulsions. In brief, the volatile compounds were released by continuously disturbing the equilibrium between the sample and headspace by purging nitrogen directly through the sample. The volatile compounds released from the sample were absorbed on a tube containing Tenax GR. After collection, the Tenax tube was manually inserted into an automatic thermal desorption unit (ATD 400, Perkin Elmer, Norwalk, CT, USA), which transferred the volatile compounds from the Tenax tube to a focusing cold trap (-30 ^{o}C). Thereafter, the volatile compounds were transferred to the GC (Agilent 5890 IIA model Palo Alto, CA, USA) equipped with a DB1701 column (30 m × ID 0.25 mm × 0.5 µm film thickness, J&W Scientific, Folsom, CA, USA) using helium gas flow (1.3 mL/ min). The GC was connected to MS HP 5972 (Palo Alto, CA, USA) for analysis.

TDU/DHS on PSF and raw materials

Extraction of volatile compounds and GC-MS analyses were performed automatically using thermal desorption unit/dynamic headspace (DTU/DHS) as described by Thomsen et al. [17] with the following modifications for sample preparation, collection and water evaporation (Table 1). The extraction modification was performed in order to avoid contamination of the system and to remove water residues. Briefly, volatile compounds were automatically collected by purging the headspace (and not through the sample) followed by trapping the volatile compounds on the adsorbent tube using the Gerstel Tenax GR 300 tubes in a dynamic headspace station (Gerstel GmbH & Co. KG., Mülheim an der Ruhr, Germany). Then, the absorbent tube was automatically transferred by a thermal desorption unit/CIS (Gerstel GmbH & Co. KG., Mülheim an der Ruhr, Germany) into the

GC 6890N Series –MS 5973 inert mass-selective detector (Agilent Technologies, Santa Clara, USA).

GC temperature program and MS settings for both purge and trap, and DTU/DHS

GC temperature-program: initial 45 °C for 5 min, 5 °/min til 90 °C, 4 °C/min to 220 °C and held for 4 min. The MS settings: electron ionization mode, 70 eV, mass to charge ratio (m/z) scan between 30 and 250.

Fatty acid methyl esters (FAME)

Fatty acid compositions of coconut oil, tricaprylin and tricaprin were determined in accordance with the method by Safafar *et al*. [18]. The analysis was conducted on 0.3 g of oil. Then, to the oil was added 100 µl internal standard 23:0 together with 200 µl heptane with BHT, 100 µl toluene and 1ml borontrifluoride in methanol. Samples and reagents were mixed and methylation was performed in a microwave oven at 100 °C for 5 min (Microwave 3000 SOLV, Anton Paar, Ashland, VA, USA) and the methylated sample was cooled down to room temperature. Then, to the methylated sample was added 1ml saturated NaCl and 0.7 ml heptane with BHT. Phase separation occurred, and the lipid/heptane phase of the methylated sample was analysed with Agilent 7890A GC (Agilent Technologies, Palo Alto, CA, USA) equipped with a DB-WAX fused silica capillary column (10 m×0.1 mm, 0.1 µm; Agilent Technologies, Palo Alto, CA, USA), using helium as carrier gas and a flame ionization detector. The individual fatty acids were identified by matching their retention times to those of authentic standards. The result was expressed as area % of total fatty acids having a chain length between C8-C24, however the values reported below C14 are estimations. Only individual fatty acids present above 0.5 % was included.

Statistics analysis

A two-way analysis of variance followed by a Bonferroni multiple comparison test was employed to evaluate significant changes in PV (duplicates) and volatile oxidation products (triplicates) during storage. The calculation was conducted using Graph pad prism version 6 (graph pad, La Jolla, USA).

Results and discussion

Lipid oxidation in products

In PCF, PV was low initially and remained below 1 meq/kg during six months at 5 °C, 20 °C, 40 °C and during two weeks at 50 °C (Figure 1A). It was not surprising that exposure to light increased PV significantly to 20 meq/kg after two months of storage. This pattern was observed in other studies [2, 4, 6]. GC-MS analysis of the volatile compounds confirmed that lipid oxidation only occurred to a limited extent. Several volatile compounds were present in low concentrations (below odour detection threshold (ODT) value in water) and did not show any clear pattern during six months (data not shown). This was the case for 2-ethyl furan, pentanal,1-penten-3-ol, 3-methyl-1-butanol, hexanal, 1-hexanol, heptanal, 1-heptanol, 2-ethyl-1-hexanol, 1-octanol, nonanal and decanal.

Even though lipid oxidation only occurred to a low extent, the concentrations of six volatile compounds increased during storage namely butanal, butanenitrile, 1-pentanol (Figure 1B-D), pentanenitrile, hexanenitrile and octanenitrile (data not shown). Butanal was not present initially and increased only slightly but significant during six months storage to 11-12.1 ng/g at 5 °C, 20 °C and 20 °C with exposure to light (Figure 1B), respectively. When exposed to 40 °C a significantly higher concentration was obtained after six months (16.5 ng/g). 1-Pentanol increased significantly to approximately 7 ng/g at all conditions after 6 months storage (Figure 1D). Butanal and 1-pentanol are well-known lipid oxidation products [19, 20, 21]. In an earlier study, we determined ODT values for volatile oxidation products, which increased during storage in a prototype cleansing formulation. In general, we found that ODT values in a prototype cleansing formulation were above 70 ng/g [6]. Therefore, butanal and 1-pentanol most likely did not affect product odour as individual compounds, but they may contribute to a cocktail effect when present together with other volatile compounds.

Butanenitrile, pentanenitrile, hexanenitrile and octanenitrile were reported to be present in topical skin formulations [2] but the mechanism of their formation during storage was not explained. Butanenitrile has been described as having a bitter almond-like odour [22, 23]. The ODT value in water was 32 ng/g for butanenitrile [24]. The concentration of butanenitrile, pentanenitrile, hexanenitrile and octanenitrile increased significantly during storage at 20 °C with exposure to light and 40 °C. However, the concentrations were below 70 ng/g, they are not expected to affect odour as individual compounds.

Similar to PCF, PSF was also selected as a representative of topical skin formulations with low lipid content. PSF contains a higher number of skin conditioning raw materials compared to PCF.

For PSF, initially PV was 0.25 meq/kg and it remained below 0.3 meq/kg at 5 °C. It increased significantly after 6 months storage to 1.0 meq/kg, 1.3 meq/kg and 2.5 meq/kg at the storage conditions 20 °C, 20 °C with exposure to light and 40 °C, respectively (Figure 2A). Again, the low PV may be related to a fast conversion of hydroperoxides to secondary volatile oxidation products. After 3 months storage, most volatile oxidation compounds seemed to be formed almost simultaneously with the peroxides indicating that there was a lag period before oxidation took off after 2 months storage (Figure 2). Concentrations of several aldehydes increased in PSF during storage: butanal, pentanal, hexanal and benzaldehyde. Butanal, pentanal and hexanal are all well-known lipid oxidation products, whereas benzaldehyde has been suggested to arise from non-enzymatic browning reactions [19, 20, 25]. In addition to the aldehydes, the concentration of two ketones (2-pentanone and 2-hexanone) and one alcohol (1-pentanol) increased as well.

For the two short-chained aldehydes, butanal and pentanal, concentrations were initially low but increased significantly during storage at all conditions. After 6 months of storage, their concentrations increased significantly and above the ODT values at 130±10 ng/g and 100±6 ng/g for butanal and pentanal obtained in topical skin formulations with a low lipid content [6]. The ODT values were exceeded for PSF stored at 20 °C (only for pentanal), 20 °C with exposure to light and 40 °C. For butanal, the concentration increased to 154 ng/g and 141 ng/g in PSF stored during 6 months at 20 °C with exposure to light and 40 °C, respectively (Figure 2B). For pentanal, the concentration increased to 119 ng/g, 184 ng/g and 185 ng/g when stored during 6 months at 20 °C, 20 °C with exposure to light and 40 °C, respectively (Figure 2D).

In contrast to the large increases observed in the concentrations of short chained aldehydes, concentrations of the two aldehydes with a longer chain, hexanal and benzaldehyde, only increased slightly to 69 ng/g (but significantly after 6 months at 20 °C with exposure to light and 40 °C) and 17 ng/g (Figure 2F and 2H). No significant increases were observed for the two ketones, 2-pentanone and 2-hexanone, for which their concentrations only increased slightly to 13 ng/g and 15 ng/g (Figure 2C and 2E). These low concentrations are not expected to affect product odour as individual compounds although they may contribute to a cocktail effect.

The alcohol, 1-octanol increased significantly during storage at 40 °C to 1803 ng/g after 6 months storage. At this high concentration it is expected to affect product odour. At the other storage conditions, 1-octanol also increased significantly after 6 months storage to 102 ng/g, 279 ng/g and 222 ng/g at 20 °C, 20 °C with exposure to light and 40 °C, respectively (Figure 2G). The exact ODT value for 1-octanol in topical skin formulation has not been determined, but the ODT value for 1-heptanol has been measured in topical skin formulation and was found to be 170±23 ng/g [6]. The volatility of 1-octanol is expected to be lower than 1-heptanol. Therefore, a slightly higher ODT value may be expected for 1-octanol than for 1-heptanol. Nevertheless, it is likely that the observed concentrations after 6 months (279 ng/g, 222 ng/g and 1083 ng/g) may affect product odour.

Comparison of PV results with PV data obtained in our earlier study [1], showed that the oxidative stability was lower for topical skin formulations having a low lipid content than in topical skin formulations with a high lipid content. The PV was up to 21.9 meq/kg and 1.44 meq/kg in topical skin formulations having a low and high lipid content, respectively. However, the concentration of volatile oxidation and degradation products revealed that the oxidative stability of topical skin formulations with a high lipid content was lowest. The concentration of butanal increased to 154.35 ng/g and 408.72 ng/g in topical skin formulations having a low and high lipid content, respectively. The higher PV for topical skin formulations with a low lipid content may be related to a faster conversion from primary to secondary oxidation products. The hypothesis that the low fat topical skin care formulations would have a lower oxidative stability than the high fat products could thereby not be confirmed. Studies in simple emulsions [11, 12, 13] obtained the opposite result namely that decreasing lipid content increased oxidation. The reason for these contradicting results may be related to effects on lipid oxidation from the numerous raw materials used for topical skin formulations e.g. addition of polymers, which may make it difficult to compare the effect of the lipid content in different types of products. Therefore, more studies are needed to investigate this issue.

Lipid oxidation in selected raw materials

A broad screening for volatile compounds in mainly lipid containing raw materials was conducted. The concentration of product relevant to volatile compounds increased notably in five of the raw materials used for the prototype skin formulations during 3 months storage, namely, glycerine and lecithin applied in PCF and PSF, and tricaprylin and tricaprin, coconut oil and polyacrylate crosspolymer-6 for PCF only.

The main focus was on volatile compounds initially present and also appearing during storage both in topical skin formulations and raw materials. An explanation to the appearance of the volatile compounds in the raw material is suggested based on fatty acid composition (degree of unsaturation) and other studies reported in literature.

The raw materials; tricaprylin and tricaprin, glycerine and coconut oil contained several aldehydes (Figure 3). The concentration of volatile compounds increased particularly in tricaprylin and tricaprin, and coconut oil during accelerated storage at 40 °C for 3 months.

The increasing concentration of butanal in both prototype skin formulations may be related to two raw materials, tricaprylin and tricaprin and glycerine. The raw material, tricaprylin and tricaprin, also contained 1-pentanol and 2-pentanone after accelerated storage. Therefore, the increase in the concentration of 1-pentanol in PCF may be related to this raw material.

The three aldehydes, pentanal, hexanal and benzaldehyde, and one ketone, 2-hexanone, originated partly from three raw materials, tricaprylin and tricaprin, glycerine, and coconut oil. Several other volatile compounds increased in these raw materials but not in the products, namely 3-methylbutanal, 3-methyl-1-butanol, 2-heptanone, heptanal, octanal and nonanal. However, 6 out of 9 volatile compounds that increased during storage in PCF also appeared and increased in the raw materials.

The increasing concentration of volatile compounds in tricaprylin and tricaprin can be related to its fatty acid composition. tricaprylin and tricaprin mainly contained fatty acids with a shorter chain length than C14. It contained the saturated fatty acids (>99 %; 57.02 % of 8:0 and 42.30 % of 10:0) and unsaturated fatty acids (< 0.5 %). Even though the degree of unsaturation was low, tricaprylin and tricaprin oxidized to a large extent during accelerated storage. This may be due to the fact that monounsaturated fatty acids can undergo autooxidation at elevated temperature.

The humectant raw material, glycerine was a relatively stable raw material. As described previously [1], glycerine can oxidize to aldehydes such as glyceraldehyde, which may react with other molecules through the mechanism described by Jungermann and Sonntag [26]. However, impurities in the raw material (0.5 %) may also contribute to the volatile compounds developing during accelerated storage.

The other raw material for which the concentration of volatile compounds increased during storage was coconut oil. Again, it can be explained by its fatty acid composition. Coconut oil mainly

contained the saturated fatty acids (> 90 %; 7.65 % of 8:0, 5.81 % of 10:0, 45.68 % of 12:0, 18.29 % of 14:0, 9.89 % of 16:0, 2.87 % of 18:0). However, it also contained unsaturated fatty acids; mono-unsaturated (> 7 %; 7.30 % of 18:1 n-9) and polyunsaturated (< 2 %; 1.83 % of 18:2 n-6). The polyunsaturated fatty acids are highly susceptible to autoxidation, which may explain the large increase observed in the concentration of volatile compounds.

In addition to the lipid containing raw materials, other raw materials, which were present in a concentration above 1 %, were also screened for volatile compounds during accelerated storage. Amongst those, lecithin and polyacrylate crosspolymer-6 had increasing concentrations of at least 6 of the 9 products relevant volatile compounds (Figure 4).

After 3 months of accelerated storage, a broad variety of volatile compounds appeared in both raw materials. In lecithin and polyacrylate crosspolymer-6, the concentration of butanal, 2-pentanone (only lecithin), pentanal, 2-hexanone, hexanal and benzaldehyde increased. Therefore, these raw materials may partly contribute to the increasing concentrations observed in the topical skin formulations. Several other volatile compounds increased in the texture modifying raw materials, but not in the prototype skin formulations: 3-methylbutanal, 2-heptanone, heptanal, octanal and nonanal.

None of the raw materials contained butane, pentane, hexane or octanenitrile. However, a nitrile containing compound was found in polyacrylate crosspolymer-6, namely tetramethylbutanedinitrile. It was therefore speculated that butanenitrile appearing in the PCF during storage may be related to decomposition of the nitrile containing impurities in polyacrylate crosspolymer-6.

A literature search showed that other authors have studied the formation of butanenitrile (and other nitrile containing compounds), but most of the reactions suggested to lead to the formation of butanenitrile require high temperature above 176.85 °C [27]. The high temperature reaction conditions required exclude the reactions from taking place in this study as butanenitrile is appearing at low tempeture at room temperature. However, an alternative reaction route to butanenitrile may have occurred. Tetramethylbutanedinitrile has been suggested by other authors as a by-product from polymer and plastic production. In polymer and plastic production, azobisisobutyronitrile (AIBN) is often used as an intiator of polymerisation. After AIBN has fullfilled its purpose in polymer and plastic production, it decomposes to form 2-cyanoprop-2-yl radicals or/and tetramethylbutanedinitrile as by-products (Figure 5). Several authors have reported

isobutanenitrile as a secondary by-product from AIBN, but no authors have reported that they have detected butanenitrile [28, 29].

Since the formation of isobutanenitrile requires less energy than the formation of butanenitrile [30], it is suprising that this compound could not be detected, whereas we were able to detect butanenitrile. Another possibility is that butane, pentane, hexane and octanenitriles are migrants from plastic packaging. Since, butane, pentane, hexane and octanenitriles appear in prototype skin formulations stored in both plastic and glass packaging, migration does, however, not seem to be a plausable explanation. More studies are needed to fully understand the reaction routes leading to the detected nitriles.

Linking volatile oxidation products in PCF/ PSF and raw materials together

The volatile compounds both present in the prototype skin formulations, PCF and PSF, and in raw materials are summarized in Table 2. In brief, the increasing concentration of butanal in PCF and, especially, PSF during storage may originate from all the selected raw materials except coconut oil. However, the concentrations were conspicuously higher and above ODT value after accelerated storage in tricaprylin and tricaprin, and polyacrylate crosspolymer-6. Therefore, it is most likely that butanal originated from these two raw materials.

It is not surprising that pentanal increased in all raw materials. This was also the case in our previous study [1]. The concentration was above ODT value in all raw materials except glycerine after 3 months accelerated storage. The volatile aldehyde in PSF, hexanal, was present in all raw materials after 3 months accelerated storage. Especially, tricaprylin and tricaprin, and coconut oil had high concentrations of hexanal at 1681 ng/g and 3976 ng/g. Benzaldehyde increased in PSF to 17 ng/g after 6 months storage. It was possible to link benzaldehyde to all raw materials.

The appearance of butanenitrile was surprising; it is usually not observed in studies of lipid oxidation. A likely explanation to its appearance has been suggested, but more studies are needed to identify the exact route of formation from impurities in polyacrylate crosspolymer-6.

The alcohol 1-pentanol increased in one raw material, namely tricaprylin and tricaprin. The other alcohol that increased in PCF, 1-octanol, was not to linked to any of the raw materials. As observed previously [1], the two ketones, 2-pentanone and 2-hexanone, were only present in low concentrations in both PSF and raw materials.

Despite the product odour changing during storage in topical skin formulation, the product odour was still deemed acceptable (within product range by an expert panel).

Therefore, it was possible to link eight out of nine volatile compounds found in the prototype skin formulations to raw materials. GSK Toxicology group has assessed the human safety impact of the volatiles included in this study. At the determined levels these substances do not raise any toxicological concern, neither locally or systemically.

Conclusion

This study explored lipid oxidation and oxidative degradation in two topical skin formulations (PCF and PSF) containing a low lipid content. In an earlier study, we investigated lipid oxidation and oxidative degradation in a topical skin formulation containing a high lipid content. Comparison of these two studies revealed that the oxidative stability measured by PV decreased with decreasing lipid content for topical skin formulations. However, the opposite was observed for the concentration of volatile oxidation and degradation products. Thus, the concentration of the volatile compounds was higher in the topical skin formulations with a high lipid content than in the topical skin formulations with a low lipid content. These results are contrary to those for simple emulsions. The higher stability of the topical skin formulations with a low lipid content may be related to their high complexity due to the large number of raw materials, which can affect lipid oxidation such as Polyacrylate crosspolymer-6. However, more studies are needed to investigate this difference between simple emulsions and topical skin formulations.

Similar to our previous findings for topical skin formulations with a high level of lipids, several secondary volatile oxidation products were present initially and more were formed during 6 months of storage.

Selected raw materials were explored in order to link volatile compounds affecting the quality in the topical skin formulation to raw material(s) and eight out of nine volatile compounds found in topical skin formulations could be linked to their presence in raw materials. Thus, well-known lipid oxidation products and non-enzymatic browning products found in PSF and PCF were suggested to originate from tricaprylin and tricaprin, and in particular coconut oil because of its unsaturated nature.

Butanenitrile appeared during storage in PCF. This compound has not been reported in other lipid oxidation studies either in model emulsions, food emulsions or in topical skin formulations. Since the concentration of butanenitrile was low it most likely did not affect product odour, but it is still

important to explore the mechanism behind its formation. A possible link between butanenitrile and the decomposition of the initiator used for production of polyacrylate crosspolymer-6 was identified. However, more studies are needed to determine the exact reaction route from this ingredient to butanenitrile.

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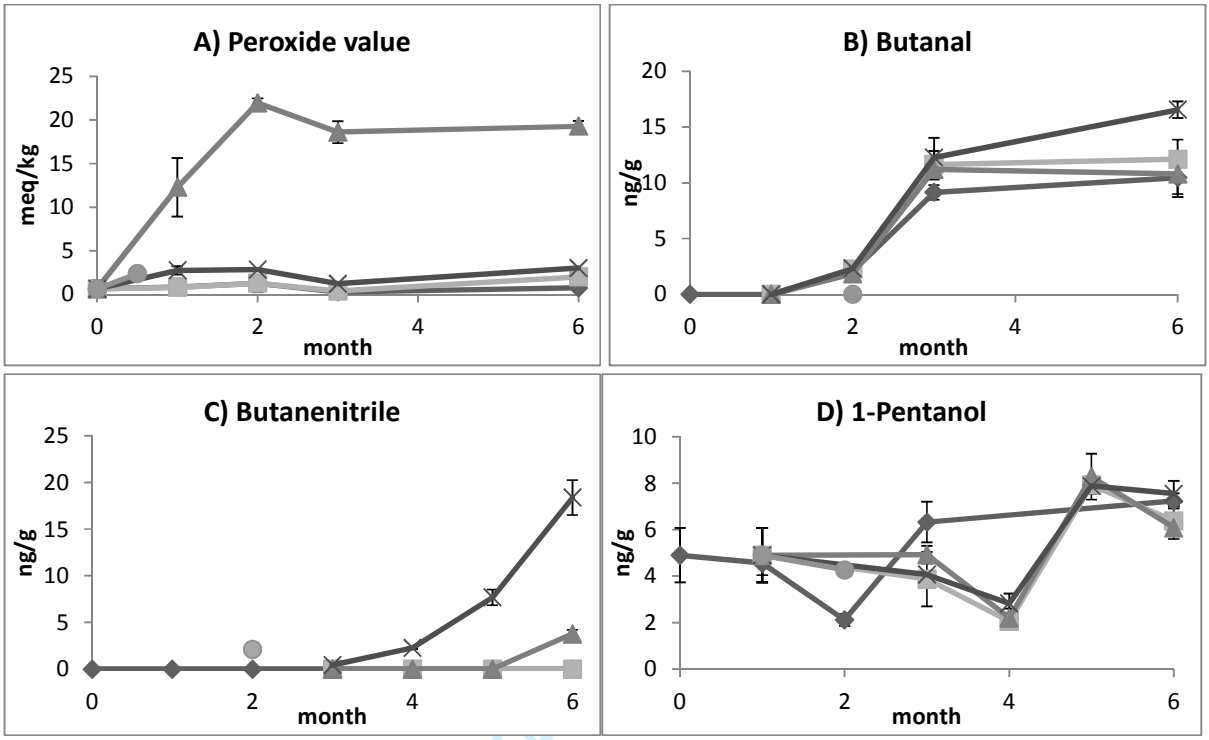

Figure 1. Lipid oxidation and degradation products in PCF during 6 months storage at 5 °C (◆), 20 °C (■), 20 + light (▲), 40 °C (×) and 50 °C (●). The development of A) Peroxide value in meq/kg, B) Butanal, C) Butanenitrile and D) 1-Pentanol in ng/g.

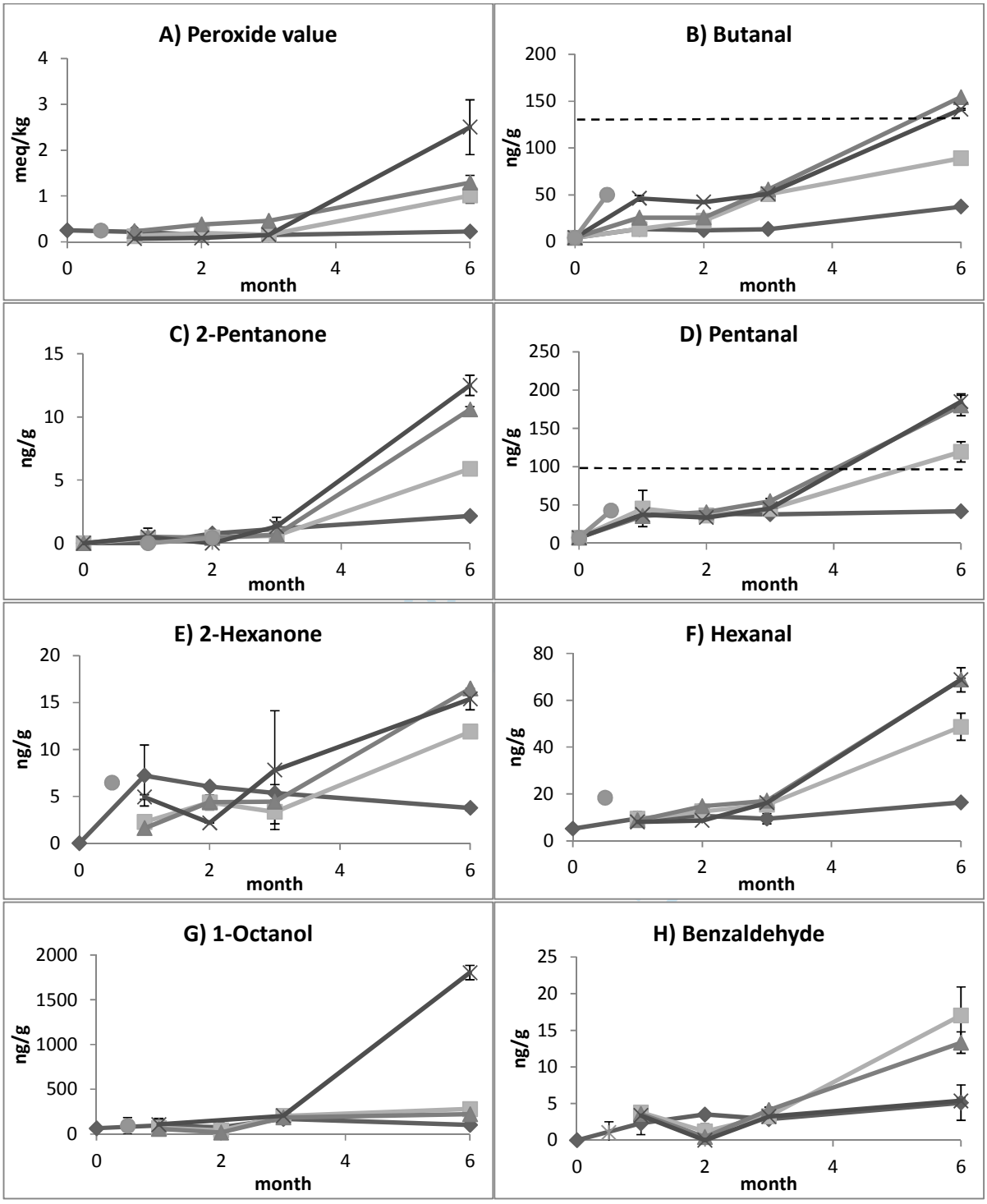

Figure 1. Lipid oxidation and degradation products in PSF during 6 months storage at 5 °C (◆), 20 °C (■), 20 + light (▲), 40 °C (×) and 50 °C (●). The dotted line indicates the exact ODT value (determined in product) (butanal and pentanal). The development of A) Peroxide value in meq/kg, B) Butanal, C) 2-Pentanone, D) Pentanal , E) 2-Hexanone, F) Hexanal, G) 1-Octanol and H) Benzaldehyde in ng/g.

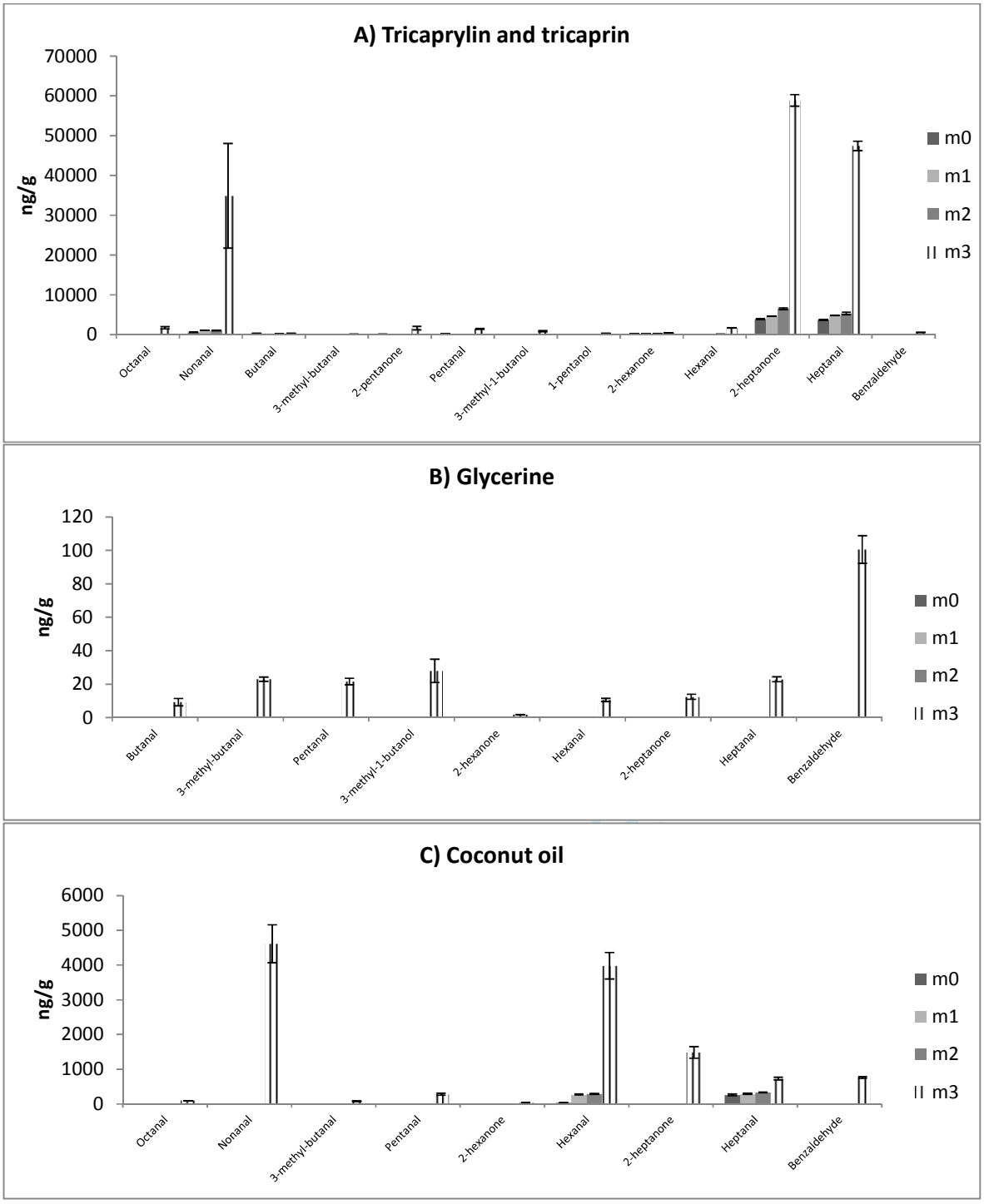

Figure 1. Volatile compounds [ng/g] present in skin conditioning and emollient raw materials during 3 months storage at 40 ºC. A) tricaprylin and tricaprin, B) glycerine, and C) coconut oil.

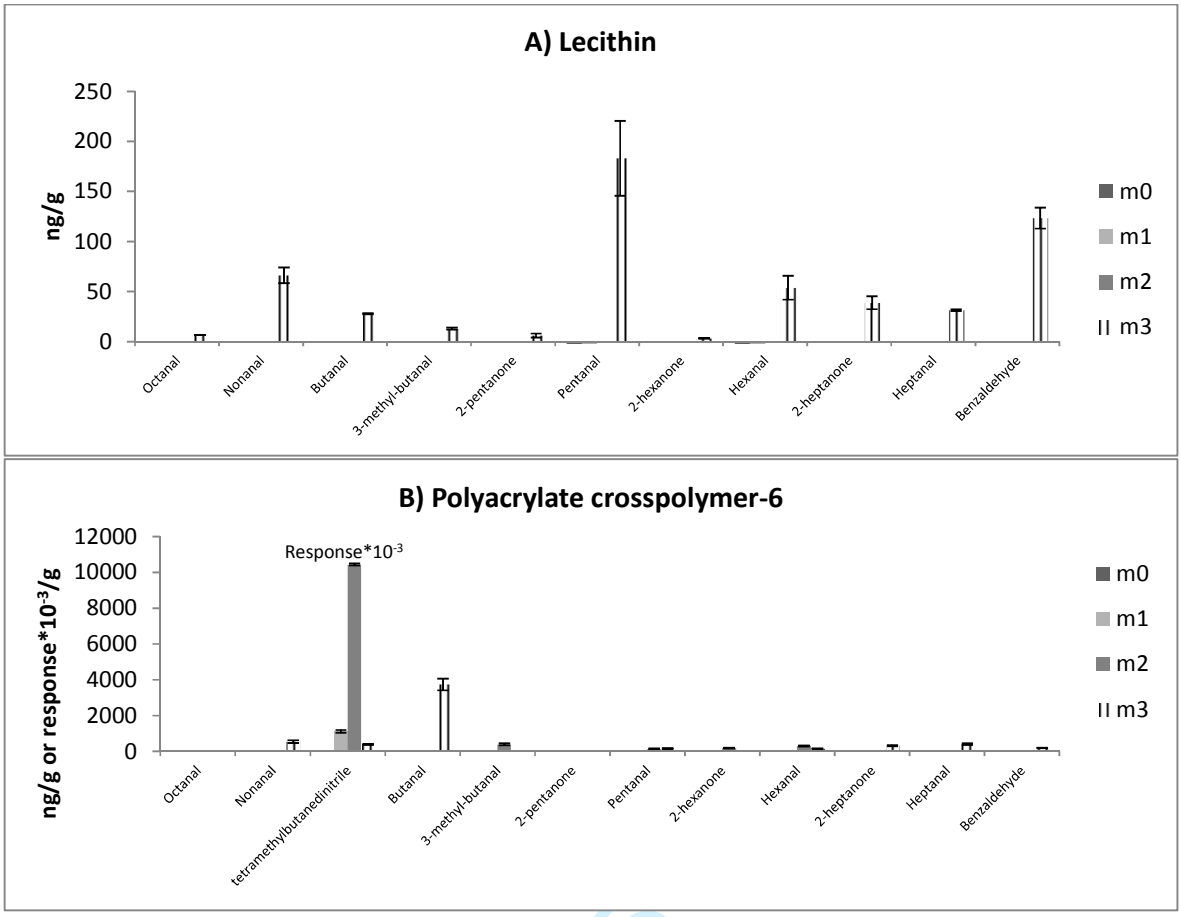

Figure 1. Volatile compounds [ng/g and response*10^{-3}/g(only tetramethylbutanedinitrile)] present in texture modifying raw materials during 3 months storage at 40 ºC. A) Lecithin and B) Polyacrylate crosspolymer-6.

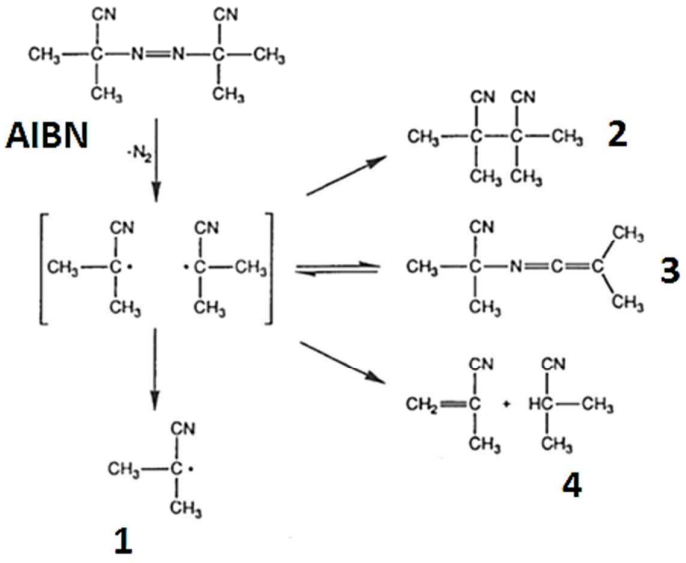

Figure 1. AIBN decomposes to form 1. 2-cyanoprop-2-yl radicals, 2. Tetramethylbutanedinitrile, 3. Dimethyl-N-(2-cyanoprp-2-yl)ketenimine and 4. isobutanenitrile. A modification of Krstina *et al*. [29].

Table 1. The modifications for sample preparation, collection and water evaporation

Samples	Preparation	Collection	Evaporation
PSF	1 g sample. Incubation at 45 °C for 5 min.	50.0 mL/min at 45 for 10 min	50 ml/min at 25 °C for 22 min.
Tricaprylin and tricaprin Glycerine Coconut oil	1 g sample. Incubation at 60 °C for 4 min.	50.0 mL/min at 60 for 20 min	-
Lecithin Polyacrylate crosspolymer-6	1 g of sample and water were mixed(1:1). Incubation at 45 °C for 5 min.	50.0 mL/min at 45 for 10 min	50 ml/min at 25 °C for 22 min.

Table 2. Summary of volatile compounds present in both products and raw materials. + = present, ++ = present above ODT value in raw material (only available for butanal and pentanal), and - = absent.

Volatile compounds/ Raw material	Butanal	Pentanal	Hexanal	Benzaldehyde	Butanenitrile	1-Octanal	1-Pentanol	2-Pentanone	2-Hexanone
Tricaprylin and tricaprin	++	++	+	+	-	-	+	+	+
Glycerine	+	+	+	+	-	-	-	-	+
Coconut oil	-	++	+	+	-	-	-	-	+
Lecithin	+	++	+	+	-	-	-	+	+
Polyacrylate crosspolymer-6	++	++	+	+	-(?)	-	-	+	+

Appendix 5: Article V

International Journal of Cosmetic Science

Predicting long-term oxidative stability in prototype skincare formulations using various lipid oxidation-initiators

Journal:	*International Journal of Cosmetic Science*
Manuscript ID	ICS-2017-2199
Manuscript Type:	Original Article
Keywords:	Oxygraph, volatile oxidation products, peroxide values, Emulsions and Formulation stability

Predicting long-term oxidative stability in prototype skincare formulations using various lipid oxidation-initiators

Abstract

OBJECTIVE: The purpose of this study was to identify an effective lipid oxidation initiator which could predict, within one month, the long-term oxidative stability of a prototype skincare formulation.

METHODS: Four initiators (below) were examined in three steps:

1) Reaction kinetics using Oxygraph;
2) Effect of adding an initiator on the product's physical and oxidative stability in prototype skincare formulations by visual observation, peroxide value (PV) and headspace GC-MS determination of volatile oxidation products; and
3) Ability to differentiate unstable vs. stable prototype creams by initiator addition.

The four initiators explored were:

- $FeCl_2/H_2O_2$,
- $FeCl_3$/ascorbic acid,
- 2,2'-Azobis(2,4-dimethylvaleronitrile) (AMVN) and
- 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH)

RESULTS: In Oxygraph, the initiator systems $FeCl_2/H_2O_2$ and $FeCl_3$/ascorbic acid were good accelerators of oxygen consumption. The addition of $FeCl_2/H_2O_2$ to prototype formulations did not affect the physical stability. However, the addition of $FeCl_3$/ascorbic acid to prototype formulations resulted in phase separation and $FeCl_3$/ascorbic acid was therefore deemed unusable. Moreover, the addition of AAPH or AMVN resulted in an increased and decreased viscosity, respectively.

In the oxidation stability study, PV increased significantly when AMVN was added. However, the PV remained low for the other initiators and the control (no initiator). The secondary volatile oxidation product, butanal, increased most with the $FeCl_2/H_2O_2$ addition. Three out of the four initiators did not have the ability to rank the stable and unstable formulations in accordance with the result obtained for volatile oxidation products after 42 days of storage at 20°C of formulations without initiator. Only, $FeCl_2/H_2O_2$ was able to rank the formulations in accordance with the oxidative stability observed for volatile oxidation products after 42 days of storage.

CONCLUSION: $FeCl_2/H_2O_2$ showed potential as an initiator to predict the oxidative stability of skincare formulations, but more studies are needed to confirm the result in a broader range of products over a longer time.

Keywords

Oxygraph, volatile oxidation products, peroxide values, Emulsions and Formulation stability

Introduction

In the development of new products, product stability is tested in order to determine the shelf life, which typically ranges from six months to three years for cosmetic products. Performing temperature-accelerated stability tests typically takes 3 to 6 months, which constitutes a large part of the development time. Many prototype skincare formulations (PFs) fail during this process - often in the late stage - from instability, e.g. physical separation or lipid oxidation. It would therefore save time to predict which products may be unstable due to lipid oxidation as early as possible.

It is well known that certain reactive species are able to initiate lipid oxidation faster than elevated temperature (1). We hypothesised that the addition of such molecules to a PF may be able to comparatively predict the level of resistance to oxidation in different PFs, and therefore help to identify lead PF candidates at an earlier stage in product development. The studies reporting on the use of oxidation initiators mainly focused on accelerating oxidation to investigate the effect of antioxidants and other factors affecting stability, but did not investigate the ability to predict a product's long-term oxidative stability. A literature review revealed four different approaches to accelerate lipid oxidation with potential applications in PFs. The approaches included two non-radical initiator systems and two radicals, respectively:

- $FeCl_2/H_2O_2$,
- $FeCl_3$/ascorbic acid,
- 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), and
- 2,2'-Azobis(2,4-dimethylvaleronitrile) (AMVN).

The two initiator systems, $FeCl_2/H_2O_2$ and $FeCl_3$/ascorbic acid, both follow the same principle of radical formation as they are based on iron´s ability to produce reactive peroxyl and alkoxyl radicals. In lipid oxidation, metal ions catalyse the decomposition of hydroperoxides by electron transfer. To further accelerate the decomposition, a Fenton oxidant is included in addition to iron in order to produce highly reactive hydroxyl radicals.

The Fenton oxidants are compounds such as ascorbic acid and H_2O_2 (1–3). The Fenton reaction for iron and H_2O_2 is shown in Equation 1 below.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$$

Equation 1. Fenton reactions for H_2O_2. Adopted from Chevion (3)**.**

The effective concentrations of the two initiator systems, $FeCl_2/H_2O_2$ and $FeCl_3$/ascorbic acid, were reported to be 1 mM $FeCl_2$ and 2 mM H_2O_2, and 0.10 mM $FeCl_3$ and 25 mM ascorbic acid, respectively (2).

AAPH is a water-soluble radical, which has been reported to accelerate oxidation when added in concentrations of 5 to 50 mM. AAPH is distributed in the water phase, and thereby can only initiate lipid oxidation in the interfacial area on the oil droplet surface. On the oil droplet surface, oxidation is initiated through production of peroxyl radicals (2,4). In contrast, AMVN is a lipid-soluble radical, which has been reported to increase the initial rate of hydroperoxide formation when added in concentrations of 2 to 50 mM (5,6). AMVN partitions to the lipid phase of an emulsion, where it reacts immediately with unsaturated fatty acids leading to propagation of lipid oxidation. Hence, the radicals generated by AMVN in the oil droplets are more easily produced compared with AAPH, which only initiates lipid oxidation in the interfacial area (7).

The purpose of this study was to identify an effective lipid oxidation initiator, among the four initiators mentioned above, which could predict long-term stability within one month in a PF. The effectiveness of the four initiators were explored in three steps: 1) reaction kinetics using Oxygraph; 2) the effect of adding an initiator on the product's physical and oxidative stability when PFs were stored for one month at 20°C, and 3) the ability to determine differences in oxidative stability in prototype creams via initiator addition. The results were compared with a control stored at the same temperature without any initiator addition.

Materials and methods

Materials

Initiators

For the experiment 1 on Oxygraph (Table I): ten µl of stock solution containing the two iron-based initiator systems or radicals diluted in water were added to 1 mL of diluted PF (75 % w/w) (Table I).

For the storage experiments 2 and 3 (Table I): A stock solution of the initiator systems was prepared by mixing a minimum amount of water with $FeCl_2/H_2O_2$ or $FeCl_3$/ascorbic acid to obtain the target concentrations. The water addition to the final PFs and prototype creams (PCs) because of initiator addition was 1% (initiator systems added as 4 mL/400 mL). The two radicals were added as neat powders in 2 and 3. Sources for initiator systems and radicals are listed below and concentrations are available in Table I:

- $FeCl_2/H_2O_2$ (Sigma-Aldrich, Darmstadt, Germany)
- $FeCl_3$/Ascorbic acid (Sigma-Aldrich, Darmstadt, Germany)
- AAPH (Sigma-Aldrich, Darmstadt, Germany)
- AMVN (Hangzhou dayangchem, Hangzhou, China)

Prototype formulations

Three PFs were produced by Glaxo Smith Kline (Brentford, UK):

- PF (A) containing ~2% lipids, antioxidants and with a high water content
- PF (B) containing ~12% lipids, tocopherols and organic UV filters
- PF (C) containing ~22% lipids and antioxidants

These PFs were used in the Oxygraph screening to evaluate the effect of initiator addition on the oxygen consumption. Furthermore, a storage experiment was conducted for 1 month to evaluate the effect of initiator addition on oxidative stability. Moreover, PFs with initiators added were used for comparison of the oxidation rate with neat PFs (no initiators added) after six months of storage at 20^{o}C.

A second storage experiment was conducted on a prototype cream (PC) with the purpose of predicting long-term oxidative stability and sample ranking with respect to stability within one month. This prototype was produced according to Poyato *et al.* (8). Poyato *et al.* (8) studied the antioxidant efficacy of extracts from brown alga *Fucus vesiculosus* to enhance oxidative stability of cream. In this study, two types of brown alga *Fucus vesiculosus* extracts, a water extract (WE1) and an ethanol extract (EE1) were added to the water phase of PC.

The experimental design is shown in Table II, where all samples (PFs and PCs) and analyses performed are listed.

Methods

Experiment 1: Reaction kinetics using Oxygraph

The oxygen consumption was determined by measuring the extent of an oxygen-dependent reaction using an oxygen electrode as described by Kristinova *et al.* and Mozuraityte *et al.* (9–11) on an Oxygraph system (Hansatech Instrument Ltd., Norfolk, UK). The oxygen electrode was an electrochemical cell consisting of two electrodes immersed in an electrolyte solution (KCl). By applying a polarising voltage of 700 mV, the electrolyte ionises and initiates a series of electrochemical reactions. The reactions depend on oxygen and the magnitude of the current flow is therefore related to the oxygen concentration in the sample. Due to the high viscosity of the PFs, the evaluation of the four initiators was performed in diluted versions of the PFs (75% (w/w) in water). Initially, a blank sample was analysed. Thereafter, 10 µl of initiator solution was injected while stirring. The initiator concentration was gradually increased until a clear effect on oxygen consumption rate was observed or phase separation occurred in the sample. The concentrations range evaluated were 0-1 mM for $FeCl_2/H_2O_2$; 0-10 mM for $FeCl_3$/Ascorbic acid; 0-10 mM for AAPH; and 0-5 mM for AMVN. The oxygen consumption rate (α) was calculated as the slope of the oxygen concentration in the sample over time. The difference, Δ, in oxygen consumption rates (α) between sample without initiator and sample with initiator were calculated in the following way: $\Delta = \alpha_{no\ initiator} - \alpha_{initiator}$ [nmol/mL]. The analyses were conducted in triplicate with results reported as an average ± standard deviation.

Experiment 2: Storage experiment with PFs

Initiators were added to freshly produced PFs (A, B and C). A storage experiment was conducted at 20°C for 1 month with sampling points after 0, 3, 6, 9, 12, 16, 21, and 30 days of storage. After sampling at the different time points, the samples were stored at 5°C until analysis. The initiators were added individually in the following concentrations: 1 mM $FeCl_2$ and 2 mM H_2O_2, 50 mM AAPH, and 50 mM AMVN. In parallel, neat PFs without initiators were stored for 3 months at 20°C.

Experiment 3: Storage experiment with PCs

Initiators were added to freshly produced PCs (with and without antioxidants). A storage experiment was conducted at 20°C for 1 month with sampling points after 0 and 30 days of storage. After sampling at the different time points, the samples were stored at 5°C until analysis. The initiators were added individually in the following concentrations: 1 mM $FeCl_2$

and 2 mM H_2O_2, 50 mM AAPH, and 50 mM AMVN. In parallel, neat PCs without initiators were stored for 42 days at 20°C.

Physical stability

The effect of initiator addition on the physical stability was assessed visually on three parameters: emulsion stability was evaluated by visual inspection for oil droplets on the top of the cream; colour changes were assessed by comparison to reference; and viscosity changes were evaluated by the ability of the formulation to flow when the bottle was manipulated (by hand) compared to a reference.

Peroxide value

Oil was extracted from the PFs prior to PV analysis. Oil extraction was performed as described by Bligh and Dyer (12) but with a reduced amount of solvent as described by Iverson *et al*. (13). The method was used to extract neutral lipids, polar lipids and free fatty acids from the samples. The primary oxidation products were measured according to the method by Shantha and Decker (14). In brief, the hydroperoxides were quantified by colorimetric determination of iron thiocyanate. The PV was measured using a spectrophotometer (UV mini 1240, Shimadzu, Duisburg, Germany) at a wavelength of 500 nm. A mixture of chloroform/methanol (7:3) solvent was used as blank.

GC-MS

The quantification of volatile compounds generated under storage of the PFs with initiators added was conducted by the method described by Thomsen *et al.* (15). Briefly, the volatile compounds were collected automatically by thermal desorption unit/dynamic headspace and analysed on GC 6890N Series - MS 5973 inert mass-selective detector (Agilent Technologies, Santa Clara, USA). A DB1701 column (30m x ID 0.25mm x 0.5µm film thickness, J&W Scientific, Folsom, CA, USA) helium gas flow (1.3 ml/min) was used for separation. GC temperature program: initial 45°C for 5 min, then increasing with 5°C/min to 90°C, and then with 7°C/min to 220 °C and held for 4 min. MS-settings: EI mode, 70 eV, mass to charge ratio (m/z) scan between 30 and 250. The oxidation rate for each volatile compound was calculated as shown in Equation 2.

$$\text{Oxidation rate [\%]} = \frac{c_{end} - c_{start}}{c_{start}} \times 100\%$$

Equation 2. Calculation of oxidation rate [%].

Results and discussion

Experiment 1: Reaction kinetics using Oxygraph

It was possible to add the two initiator systems, $FeCl_2/H_2O_2$ and $FeCl_3$/ascorbic acid, in high concentrations by injecting into the PFs without phase separation of the PFs. The $FeCl_2/H_2O_2$ initiator system addition gave rise to the largest increase of oxygen consumption. However, both systems increased oxygen consumption compared with the PF without initiator (Figure 1).

The radicals, AAPH and AMVN, could only be added in concentrations of 5-10 mM by injection into the cell in the Oxygraph without generating immediate physical instability in the PFs (phase separation). At higher concentrations, phase separation occurred immediately. However, a reduction in Δ, and thereby, an increased oxygen consumption was still observed when AAPH or AMVN were added in low concentrations.

The initiator systems, $FeCl_2/H_2O_2$ and $FeCl_3$/ascorbic acid, were able to initiate lipid oxidation in all three PFs in the Oxygraph experiment. However, the increases in oxygen consumption rates differed between the PFs with the added $FeCl_2/H_2O_2$. Hence, PF C had the highest decrease in Δ followed by PF B and lastly PF A. In a Oxygraph study on liposome dispersions by Mozuraityte *et al.* (9), iron was added at time 0, and after 10 mins a metal chelator was added. In their study, they were able to detect a clear effect of pH and chelator type on the oxygen consumption rates. The different compositions of the PFs in the present study may explain the difference in the oxygen consumption rates upon addition of $FeCl_2/H_2O_2$. Hence, the high lipid content in PF C may make it more sensitive to iron-catalysed oxidation than PFs A and B. Kristinova *et al*. (10) also demonstrated that the oxidative stability in marine emulsions stored at 30°C can be predicted by Oxygraph studies. Therefore, the Oxygraph results may suggest that PF C is less stable at room temperature than PFs A and B upon addition of $FeCl_2/H_2O_2$.

The Fe^{2+} added as $FeCl_2/H_2O_2$ gave rise to higher oxygen consumption in lower concentrations than Fe^{3+} added as $FeCl_3$/ascorbic acid. In both cases, oxygen consumption increased with increased iron concentrations. Two studies in marine emulsions by Kristinova *et al*. (10,11) also showed an increased oxygen consumption with increased iron concentration and they also found that Fe^{2+} increased oxygen consumption rates more than

Fe^{3+}. The authors explained the higher oxygen consumption as a consequence of faster decomposition of LOOH by Fe^{2+} than Fe^{3+}. This may also be the case in our study.

The radicals, AMVN and AAPH, were able to accelerate lipid oxidation to a lesser extent than the initiator systems. This may be related to the fact that lipid oxidation is dependent on a redox reaction in which the transition metals, Fe^{2+} and Fe^{3+}, are an active part of the further decomposition of LOOH. The decomposition results in generation of secondary oxidation products, which include radicals that further accelerate the propagation of lipid oxidation. The LOOH formation by AAPH and AMVN does not include the redox reaction, and therefore, we suggest that this explains the lesser ability to accelerate lipid oxidation.

Experiment 2: Effect of adding initiator on product stability (1 month of storage)

Physical stability

The initiator addition must not affect the PF's physical stability because physical instability can affect the oxidative stability significantly (16). The physical stability was assessed visually (Table III).

As shown in Figure 1, the initiator system $FeCl_2/H_2O_2$ was a very good accelerator of oxygen consumption. In addition, it was possible to add this initiator system to all three PFs and still maintain physical stability of the product. The initiator system $FeCl_2/H_2O_2$ did not change viscosity initially and the PFs remained physically stable for 30 days of storage. However, it was not possible to add $FeCl_3$/ascorbic acid in PF without the occurrence of phase separation. Even though several different approaches were examined, it was not possible to dissolve both iron and ascorbic acid in any of the PFs. Thus, $FeCl_3$/ascorbic acid was not a compatible initiator system, and therefore it was not used in the subsequent storage experiments. It was possible to add both AAPH and AMVN in all three PFs and still maintain the physical stability of the product. However, the product viscosity changed (observed visually). Viscosity decreased when AAPH was added, and increased when AMVN was added. Moreover, PFs with AMVN showed early signs of phase separation as oil appeared on PF B surface after 16 days of storage. PF B, however, was not completely separated after 30 days of storage. Even though some changes were observed in the viscosity of the formulations, these initiators were included for further studies because the formulations did not separate completely.

Oxidative stability

In the PFs with initiators added, lipid oxidation was measured by PV and volatile compounds. PV was initially below 2 meq/kg oil and remained below 2 meq/kg in all PFs with

$FeCl_2/H_2O_2$. Moreover, PV was below 5 meq/kg oil in all PFs with AAPH. However, addition of AMVN significantly increased PV in all three PFs to 21, 4 and 10 meq/kg oil after 30 days of storage, in PFs A, B and C, respectively. The neat PFs A, B and C had an initially low PV below 2 meq/kg oil and it remained below this value for six months of storage. The higher PV in PFs with the added AMVN may be related to hydrophobic properties of the radical. Hence, this hydrophobic radical was expected to be located in the oil droplets together with the unsaturated fatty acids. This increased the probability that formation of lipid hydroperoxides was initiated by AMVN (2,17).

The results from the PVs led to the conclusion that $FeCl_2/H_2O_2$ and AAPH were the best at accelerating lipid oxidation in a pattern comparable to long-term stability of the PFs stored for 6 months based on the PV measurements.

Earlier studies have shown that butanal, pentanal and 3-methyl-1-butanol (from hydrolysis of UV filter: only PF B) are good markers for lipid oxidation and degradation in the PFs used in the present study (18–20). The concentration of butanal increased significantly in all three PFs between 15 to 21 days of storage (Figure 2). This indicates that it may be possible to reduce the storage period from 30 to 21 days in this type of accelerated oxidation experiment. The same pattern was observed for pentanal and 3-methyl-1-butanol (data not shown). The increase in butanal concentrations was clearly more dependent on the initiator system added to the PFs than on the type of formulation. The most efficient initiator system was $FeCl_2/H_2O_2$ in all three PFs.

No correlation was observed between the lipid content of PFs and PV or the butanal concentration. PF C had the highest lipid content at 22% compared with 12% and 2% in PFs B (omit UV filter) and A, respectively. There was no significant difference in concentrations of the oxidation products between PFs with different lipid contents when the same initiator was used. This finding was in contrast to the observations from the Oxygraph study.

The oxidation rates of the volatile compounds butanal, pentanal and 3-methyl-1-butanol (only PF B) were calculated for neat PFs (no initiator) after six months of storage and after one month of storage with initiators. Both PFs without and with initiators were stored at 20°C (Figure 3).

Ideally, the initiators must be able to initiate and progress oxidation within one month of storage to at least the same level as neat PFs after 6 months.

In PF A, the oxidation rate for butanal increased more when stored for one month with $FeCl_2/H_2O_2$, AMVN or AAPH added than in the neat PF A stored for six months. For pentanal, the same pattern was observed for $FeCl_2/H_2O_2$ and AAPH. However, the radical, AMVN, was not able to reach the same oxidation rate as the neat PF A (no initiator).

As observed for PF A, $FeCl_2/H_2O_2$ was the best accelerator of lipid oxidation as evaluated by formation of butanal and pentanal in PF B. In contrast, the radicals, AAPH and AMVN, only gave rise to low oxidation rates, which were lower than those of the neat PF B (no initiator) for butanal and pentanal. $FeCl_2/H_2O_2$ was the only initiator that was able to accelerate the hydrolysis of the UV filter to yield 3-methyl-1-butanol to a larger extent than in the neat PF A (no initiator) (20).

Similar to the findings in PF B, only $FeCl_2/H_2O_2$ was able to reach and exceed the oxidation rates of butanal and pentanal of the neat PF C (no initiator).

In summary, all three initiators were able to accelerate oxidation, but only $FeCl_2/H_2O_2$ was able to provide oxidation rates that exceeded those obtained with the three PFs without initiator when stored at 20°C for 6 months. Moreover, only $FeCl_2/H_2O_2$ was able to accelerate the hydrolysis of the UV filter. However, the ability to accelerate lipid oxidation and degradation is not the same as the ability to reveal long-term stability. Therefore, the ability to reveal unstable PFs faster was examined in the following section.

Experiment 3: Ability to differentiate unstable formulations from stable formulations by initiator addition

Earlier studies have shown the clear antioxidative ability of *Fucus vesiculosus* extract leading to a clear difference in PCs, with and without extracts, oxidation rates after 56 days of storage (8). To further confirm these findings, a new study was conducted by Hermund *et al.* (21). In parallel with this study, initiators were added to the PCs with two different *Fucus vesiculosus* extracts (EE1, WE1) and to PC without extract (REF) to evaluate the initiators' ability to accelerate oxidation and rapidly predict the ranking of the PCs' oxidative stability at 20°C. The study by Hermund *et al.* (21) showed that the oxidation rate was lowest in PC with EE1 and WE1 extract for pentanal and heptanal, respectively. The PC without initiator was stored at 20°C for 42 days because time did not allow a six-month storage experiment. Overall, the PC REF had the highest oxidation rate for the PCs without initiators (Figure 4).

For pentanal, $FeCl_2/H_2O_2$ and AMVN (no data for AAPH due to limited sample amount available) were able to accelerate oxidation in such a way that the REF PC had the highest oxidation rate. Only $FeCl_2/H_2O_2$ resulted in the same ranking of PC with antioxidants as PC without initiator, namely, that WE1 and EE1 were ranked as second and third. These findings led to the conclusion that $FeCl_2/H_2O_2$ was able to both accelerate oxidation and result in the same ranking of the PCs as no initiator.

For heptanal, only $FeCl_2/H_2O_2$ was able to accelerate oxidation and obtain the highest oxidation rates for REF PC.

Summary

The Oxygraph results may suggest that PF C is less stable at room temperature than PFs A and B with the addition of $FeCl_2/H_2O_2$. However, this was not the result of the storage experiment both with and without $FeCl_2/H_2O_2$, where PF B had the lowest stability followed by PFs C and A. These results showed that Oxygraph cannot be used to assess the oxidative stability in complex emulsion systems such as skincare formulations. This is properly related to the PFs were diluted in water. The water addition may have removed the matrix's effect on lipid oxidation. Therefore, a storage experiment is necessary to determine the oxidative stability.

The three initiators' abilities to predict long-term stability were evaluated. The lipid-soluble radical, AMVN, led to a significantly higher PV but not a significantly higher concentration of the volatile compounds compared with the other initiators. The higher PV was most likely related to the hydrophobic properties of AMVN, which led to its partitioning into the oil droplets together with the unsaturated fatty acids and thereby increased probability of lipid oxidation. However, the high PV and the ranking of PFs were not comparable to those obtained for PFs without initiator.

The hydrophilic initiators, $FeCl_2/H_2O_2$ and AAPH, were able to initiate PV in the same pattern as PFs without initiator. These hydrophilic initiators were present in the water phase and most likely initiated lipid oxidation in the interfacial area as generally observed in emulsions.

The results for the volatile compounds showed that AMVN did not result in the highest formation rate of butanal even though it resulted in the highest PV. AAPH resulted in a low PV and a lower formation rate of butanal than $FeCl_2/H_2O_2$. This confirms that the hydrophobic properties of the initiator increased LOOH formation, and therefore PV, but the

lack of contribution to the redox reaction led to a lower decomposition to volatile oxidation products (10,11). However, it also shows that AAPH did not contribute to the redox reaction in terms of decomposition of LOOH to secondary oxidation products, which led to slower formation of volatile oxidation rates for AAPH compared with FeCl2/H2O2, as was also the case for AMVN.

Moreover, for the volatile compound butanal, the sample ranking in PFs with no initiators and PFs with added radicals, AAPH and AMVN, were not the same. The sample ranking of the butanal in PFs with no initiators was B>C>A, whereas it was B>A>C when AAPH or AMVN were added. However, the sample ranking in PFs with no initiators was the same as observed for PFs with added $FeCl_2/H_2O_2$.

A study by Baron *et al.* (17) examined the effect of tocopherol, trolox, carotenoids astaxanthin and canthaxanthin in emulsions using initiators. The emulsions were incubated with antioxidants and oxidation was initiated either with AAPH, $FeCl_3$/ascorbate or $FeCl_2$/EDTA/H_2O_2. The formation of aldehydes was measured by GC-MS after 30 min and 360 min. They observed a significant increase in the concentration of the volatile aldehydes: butanal, t-2-pentenal, t,t-2,2-hexadienal and t,t-2,4-heptadienal in all samples without antioxidants. These volatile aldehydes were formed from linolenic acid methyl ester oxidation when the initiator was added. The effectiveness of the initiators to accelerate oxidation in the study by Baron *et al.*(17) was ranked in the following way: $FeCl_3$/ascorbate> $FeCl_2$/EDTA/H_2O_2> AAPH. The greater ability of $FeCl_2$/ H_2O_2 to accelerate oxidation than AAPH was also observed in the present study. Unfortunately, it was not possible to add $FeCl_3$/ascorbic acid to PFs and PCs because it led immediately to phase separation. Moreover, Baron *et al.*(17) observed a significantly improved oxidative stability when antioxidants were added in their model emulsion. This was also the outcome in this study for complex emulsions, PCs, where the addition of *Fucus* vesiculosus led to an increased oxidative stability. However, Baron *et al.*(17) did not perform a long-term storage experiment for comparison and therefore they, in contrast to our study, could not make any conclusions on the ability of the initiators to predict long-term stability.

Conclusion

The ranking of PCs clearly showed that the most promising initiator was $FeCl_2/H_2O_2$ because it was able to predict oxidative stability and imitate formulation ranking for pentanal.

The initial Oxygraph screening showed that $FeCl_2/H_2O_2$ and $FeCl_3$/ascorbic acid were efficient accelerators of lipid oxidation as observed from the increased oxygen consumption in the diluted PFs.

In neat PFs, the physical stability was not affected by $FeCl_2/H_2O_2$ addition. However, $FeCl_3$/ascorbic acid resulted in phase separation in all PFs investigated in this study. Therefore, $FeCl_3$/ascorbic acid was deemed unusable. The physical stability of emulsions was maintained, but viscosity was affected by addition of AAPH (viscosity decrease) and AMVN (viscosity increase).

The oxidative stability measured by PV showed that the formulation with $FeCl_2/H_2O_2$ and AAPH both had a low and stable PV for 30 days. However, the AMVN addition to all PFs resulted in an increased PV. After six months of storage, the neat formulations without initiators also had a low and stable PV. Therefore, $FeCl_2/H_2O_2$ and AAPH were the initiators that resulted in the most similar pattern.

Secondary volatile oxidation product analysis also confirmed the same initiator efficacy ranking.

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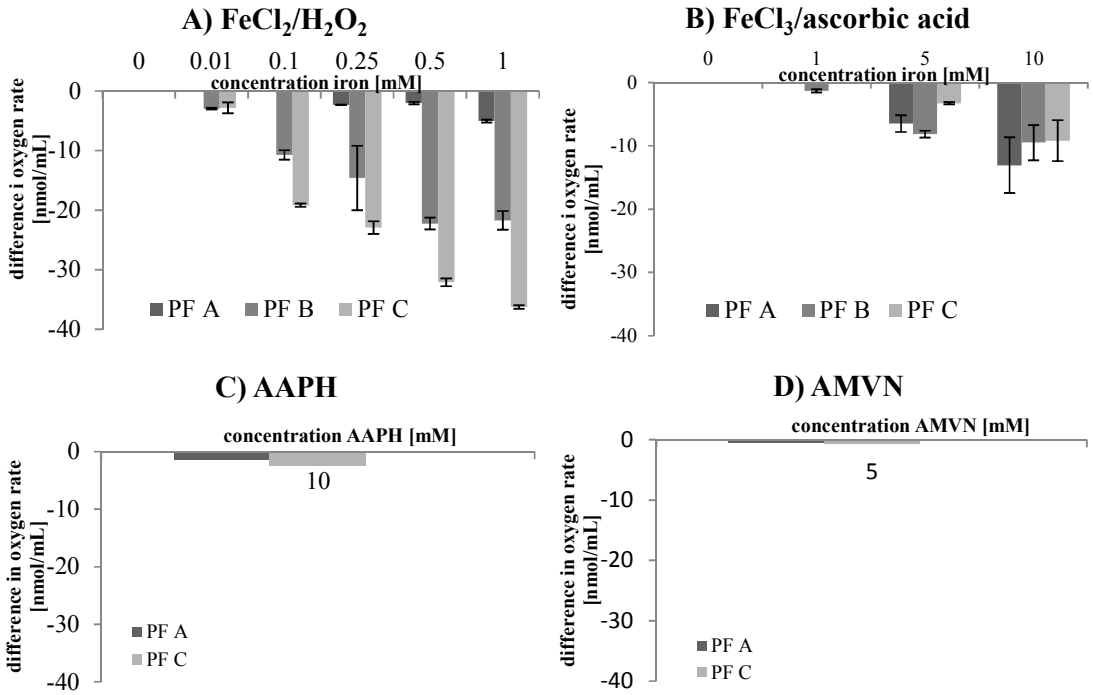


Figure 1. The difference in oxygen consumption rate compared with the no initiator [nmol/mL] in the PF A, B and C due the initiator addition estimated using Oxygraph. A) $FeCl_2/H_2O_2$ and B) $FeCl_3$/Ascorbic acid initiator systems are used, and C) AAPH and D) AMVN radicals are used as initiators.

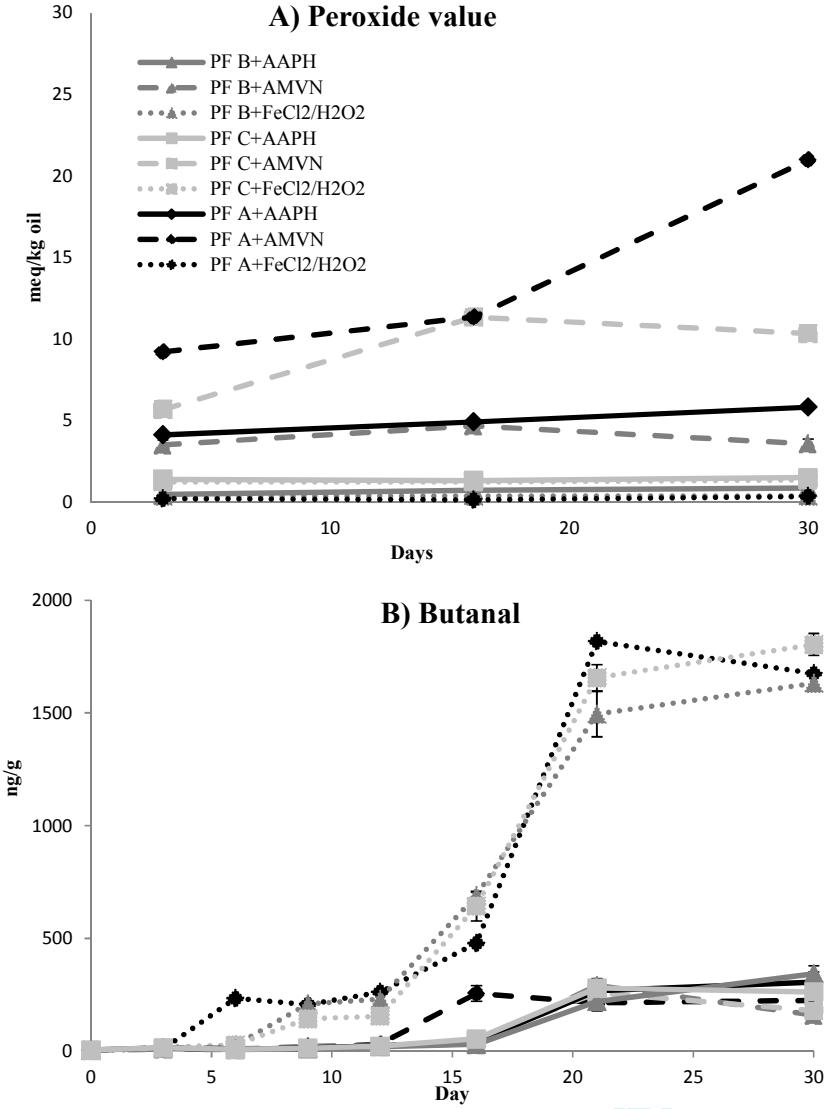


Figure 2. The development of A) PV [meq/kg oil] and B) butanal [ng/g] in PFs A, B and C with added initiators for 30 days of storage.

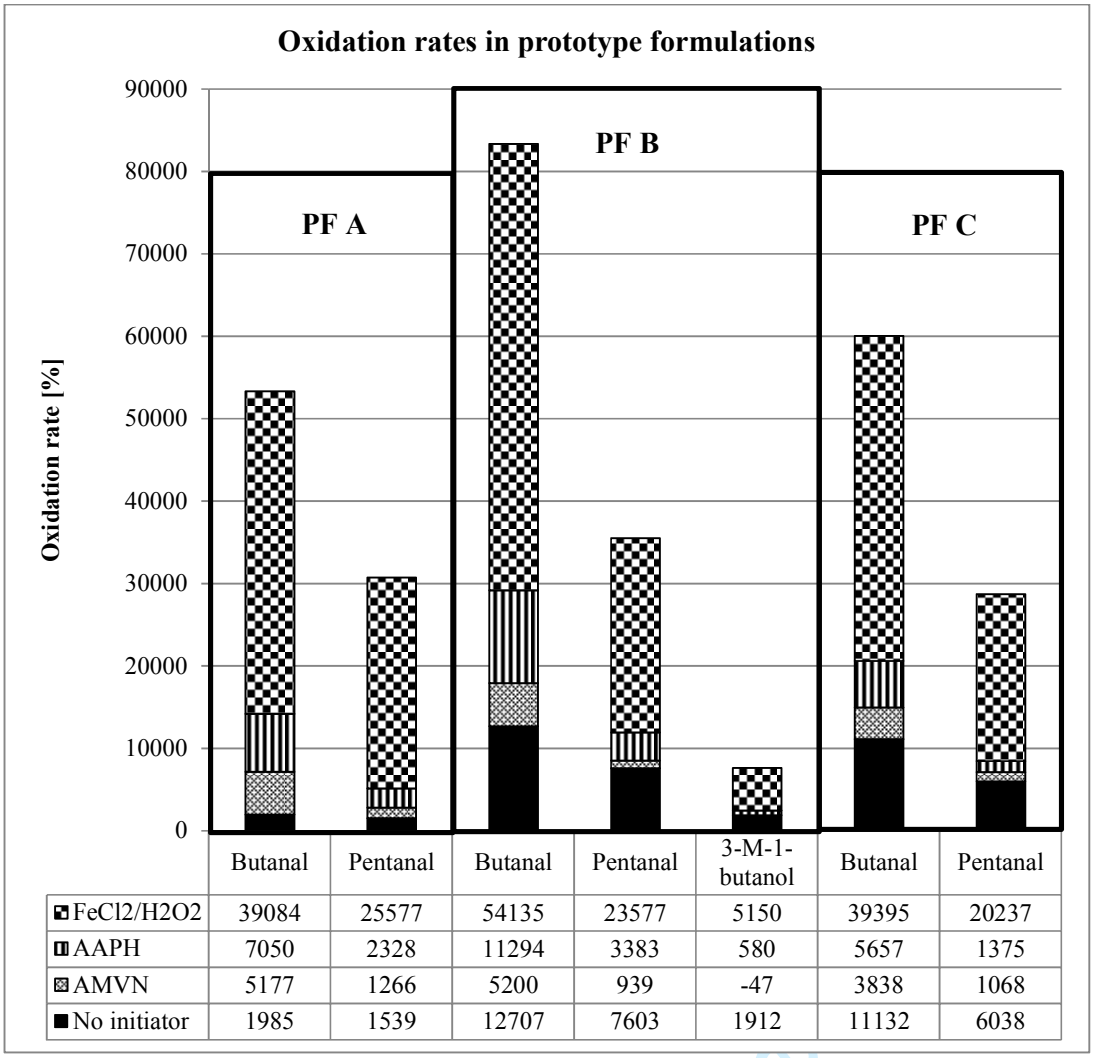
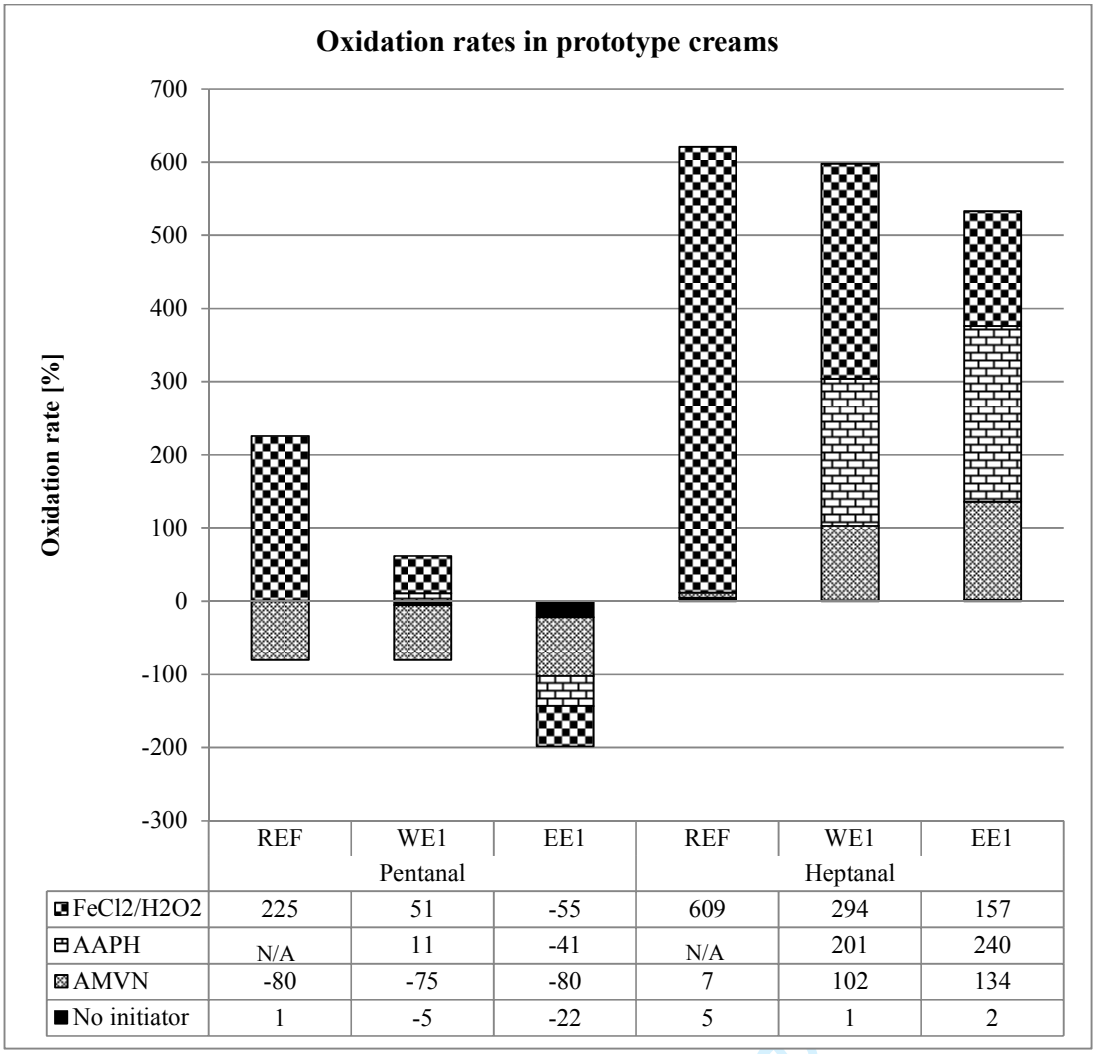


Figure 3. Oxidation rate [%] of butanal, pentanal and 3-methyl-1-butanol (3-m-1-butanol) in the neat PFs with no initiator stored for six months and PFs with initiators stored for one month.



	REF	WE1	EE1	REF	WE1	EE1
	Pentanal			Heptanal		
FeCl2/H2O2	225	51	-55	609	294	157
AAPH	N/A	11	-41	N/A	201	240
AMVN	-80	-75	-80	7	102	134
No initiator	1	-5	-22	5	1	2

Figure 4. The oxidation rates [%] for pentanal and heptanal in PCs; reference (REF), *Fucus vesiculosus* water extract (WE1) and *Fucus vesiculosus* ethanol extract (EE1). Lipid oxidation was accelerated by AAPH, AMVN and $FeCl_2/H_2O_2$. No initiator refers to PC stored at 20°C for 42 days without addition of initiator.

Table I. An overview of the initiator concentrations, iron or radical, included in experiments (EX) on (1) reaction kinetics using Oxygraph, (2) effect of adding initiator on the product's stability within one month in PFs and (3) ability to differentiate unstable vs. stable PCs by initiator addition.

Experiment/ Formulation	EX 1: Oxygraph	EX 2: Product stability	EX 3: Ability to differentiate
$FeCl_2/H_2O_2$	0, 0.25, 0.5 and 1 mM (0, 25, 50 and 100 mM)*	1/2 mM (0.1/0.2 M)*	1/2 mM (0.1/0.2 M)
$FeCl_3$/Ascorbic acid	0, 1, 5 and10 mM (0, 100, 500 and 1000 mM)*	0.1/25 mM (0.01 M/2.5 M)*	-
AAPH	0 and 10 mM (0 and 1000 mM)*	50 mM	50 mM
AMVN	0 and 5 mM (0 and 500 mM)*	50 mM	50 mM

*stock solutions

Table II. An overview of samples included in experiments (EX) and the analysis conducted on (1) reaction kinetics using Oxygraph, (2) effect of adding an initiator on the product's stability for one month in PFs and (3) ability to differentiate unstable vs. stable PCs by initiator addition.

Experiment/ Formulation	EX 1: Oxygraph	EX 2: Product stability			EX 3: Ability to differentiate
		Physical stability	Peroxide value	GC-MS	GC-MS
PF A	x	x	x	x	
PF B	x	x	x	x	
PF C	x	x	x	x	
PC REF					x
PC WE1					x
PC EE1					x

Table III. Visual changes observed in physical stability due to addition of initiators in PFs.

Initiator / Formulation	$FeCl_2/H_2O_2$	$FeCl_3$/ascorbic acid	AAPH	AMVN
PF A	No	Brown and phase separation occurred	No	No
PF B	No	Brown and phase separation occurred	Lower viscosity	Higher viscosity Oil appeared on surface
PF C	No	Brown and phase separation occurred	Lower viscosity	Higher viscosity

Appendix 6: Article VI

Research Article

Antioxidant effect of water and acetone extracts of *Fucus vesiculosus* on oxidative stability of skin care emulsions

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A water and an acetone extract of the Icelandic brown algae *Fucus vesiculosus* were evaluated as potential natural sources of antioxidant compounds in skin care emulsions. To assess their efficacy in inhibiting lipid oxidation caused by photo- or thermoxidation, they were stored in darkness and room temperature as control conditions, and compared to samples stored under accelerated conditions (light and room temperature, or darkness and 40°C). The presence of extracts in the skin care emulsions induced remarkable colour changes when the emulsions were exposed to light, and more extensively under high temperature. High temperature also caused greater increments in the droplet size of the emulsions. The analysis of the tocopherol content, peroxide value and volatile compounds during the storage revealed that, whereas both water and acetone extracts showed (at 2 mg/g of emulsion) protective effect against thermooxidation, only the water extract showed antioxidant activity against photooxidation.

Practical applications: This research is the basis of developing natural antioxidants derived from seaweed to limit lipid oxidation in skin care products.

Keywords: brown algae / cosmetic emulsion / lipid oxidation / skin care emulsion

Received: February 6, 2016 / Revised: June 6, 2016 / Accepted: June 13, 2016

DOI: 10.1002/ejlt.201600072

1 Introduction

Naturally derived ingredients combined with carrier agents, preservatives, surfactants, humectants and emulsifiers are commonly used in skin care products. A natural ingredient is based on botanically sourced ingredients currently existing in nature (such as herbs, roots, essential oils and flowers), in order to reduce synthetic compounds in the final product. Nowadays, there is an increasing interest in natural ingredients [1] because of the negative perception of the synthetic ones. Thus, the evolution of the cosmetic industry to adapt products to the trends of the 21st century consumer has given rise to new challenges.

Emulsions are the most common type of delivery system used in cosmetics, with creams and lotions being the best known. Skin care emulsions enable a wide variety of active ingredients to be quickly delivered to skin. In this sense, there are many factors that can potentially influence the physical and oxidative stability of these emulsions, such as fatty acids and ionic composition, type and concentration of antioxidants and prooxidants, emulsion droplet size and interfacial properties [2–5].

Lipid oxidation can occur in skin care emulsions [6, 7] and can be triggered or enhanced by light and/or high temperatures. Moreover, the high content of vegetable oils, e.g. almond oil, in skin care emulsion formulations might contribute to induction of lipid oxidation, causing unpleasant odours, colour changes and in consequence, low quality products [6, 7].

Therefore, it is important to limit lipid oxidation and to extend the shelf life of skin care products using natural

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Abbreviations: AE, acetone extract; **PV,** peroxide value; **RF,** reference; **TTC,** total tocopherol content; **VC,** volatile compounds; **WE,** water extract

antioxidants. In addition, some natural antioxidants can give the skin product added functional value. It has recently been suggested that the use of natural antioxidants, such as vitamins A and E, in skin care formulations could provide a preventive therapy for skin photoaging [8, 9]. Moreover, beauty-improving formulations of skin care emulsion with seaweed extracts or micro algae added have also been reported [10].

Vitamin E is one of the most used natural antioxidants in skin care products, usually added due to its radical scavenging activity [9]. However, in highly complex matrices containing trace metals, such as cosmetics products [11], other antioxidant properties such as metal chelating ability might be of relevance. Therefore, to stabilise lipid-rich skin care products, extra addition of antioxidants might be necessary.

Naturally derived antioxidants from various plants and marine algae have shown great potential in improving oxidative stability in these kinds of products. A high variety of bioactive compounds, such as pigments, sulphated polysaccharides, proteins and polyphenols, have been described for different types of Danish brown and red algae by Farvin and Jacobsen [12]. Especially, the high content of phlorotannins, the major polyphenolic compounds in brown algae, has been related to high antioxidant activity, as these compounds can work both as radical scavengers and metal chelators [13, 14]. Furthermore, phlorotannins have been shown to possess biological activity of potential medicinal value making them valuable in development of nutraceutical, pharmaceutical and cosmetic products [15, 16]. Balboa et al. [1] successfully used a *Sargassum muticum* extract to improve the oxidative stability of oil-in-water model emulsions with cosmetic purposes. Farvin and Jacobsen [12] found that compared to other Danish brown alga species, *Fucus vesiculosus* had higher phenolic content and exhibited the highest antioxidant activity in vitro. Wang et al. [14, 17] found that the high in vitro antioxidant activity of extracts derived from the Icelandic *F. vesiculosus* were related to a high phenolic content and identified the phlorotannin tetramer, fucodiphloroethol E, to be the main contributor to this activity. Moreover, Hermund et al. [18] and Karadağ et al. [19] studied the application of Icelandic *F. vesiculosus* extract as potential antioxidant against lipid oxidation in fish-oil-enriched food products, i.e. food emulsions such as mayonnaise, milk and granola bars added preemulsified fish oil, and found promising results.

Whereas the in vitro antioxidant properties of *F. vesiculosus* have been widely studied [14, 20], applied studies on the antioxidant activity of *F. vesiculosus* extracts to hinder lipid oxidation are sparse [18, 19, 21, 22].

The aim of this study was to evaluate the antioxidant properties of two extracts obtained from Icelandic brown algae *F. vesiculosus* (water and acetone extract) in terms of assessing their efficacy to inhibit lipid oxidation during the storage of skin care emulsions, at room temperature in darkness and under two different accelerated conditions (photo- and thermooxidation).

2 Material and methods

2.1 Materials

The ingredients for the formulation of the skin care emulsion were purchased from Urtegaarden (Allingåbro, Denmark). Lanette wax has a composition of C16: 45–55% and C18:45–55% from coconut oil. VE (vegetable emulsifier) is fat from palme oil and it has a composition of 60–70% monoglycerider, free glycerol 1.5% and free fatty acids 1.5%. All solvents used were of high-performance liquid chromatography (HPLC) grade and purchased from Lab-Scan (Dublin, Ireland). External standards were purchased from Sigma–Aldrich (Steinheim, Germany).

2.2 Extraction

The two extracts used in this study were provided by Matís in Iceland and have been used in previous studies of foods (water extract previously used by Hermund et al. [18], the acetone extract by Honold et al. [22] and both extract by Karadağ et al. [19]).

The extractions were carried out according to Wang et al. [14, 17]. The seaweed was collected in the Hvassahraun coastal area near Hafnarfjordur, southwestern Iceland, in 2011. At the collecting spot, the seaweed was washed with clean seawater to remove salt, epiphytes and sand attached to the surfaces of the samples and transported to the laboratory. The samples were rinsed with tap water and wiped with paper towel. The samples were freeze-dried, pulverised into powder and stored at −80°C prior to extraction.

The extracts were produced as follows: 5 g of the algal powder was mixed with 100 mL of distilled water or 70% aqueous acetone v/v. Hereafter, these were incubated on a platform shaker (InnovaTM 2300, New Brunswick Scientific, Edison, NJ) for 24 h at 200 rpm and at room temperature. The mixture was centrifuged at 2168*g* for 10 min at 4°C and filtered with Whatman no. 4 filtre paper. Each extraction was conducted in duplicate. The extracts were stored at −80°C until use.

The water extract (WE) composition was as follows: phenolic content, 14.73 g gallic acid equivalent/100 g extract; chlorophylls, 0.46 μg/mg extract; xanthophylls, 2.17 μg/mg extract; carotenes, 1.72 μg/mg extract; iron, 4.39 μg/mg extract and copper, 0.91 μg/mg extract. The acetone extract (AE) composition was as follows: phenolic content, 18.55 g gallic acid equivalent/100 g extract; chlorophylls, 0.85 μg/mg extract; xanthophylls, 0.75 μg/mg extract; iron, 9.53 μg/mg extract and copper, 1.21 μg/mg extract. The composition of the seaweed extracts was determined and reported in Hermund et al. [18] and Honold et al. [22]. The composition

of potential antioxidant or prooxidant compounds was as follows: total phenolic content (g GAE/100 g dry extract): WE, 18.4 ± 0.1, AE, 23.2 ± 1.1, protein (w/w% dry extract): WE, not detected, AE, 2.3 ± 0.0, tocopherol (μg/g dry extract): WE, α: 19.0 ± 1.9, β: 2.9 ± 0.0, γ: 6.2 ± 0.2, δ: 24.5 ± 1.2, AE, α: 4.0 ± 0.3, β: 1.9 ± 0.7, γ: 2.5 ± 0.8, δ: 12.9 ± 0.6, iron (μg/g dry extract): WE, 4.4 ± 1.0, AE, 9.5 ± 1.1. Pigments (μg/mg dry extract): chlorophylls: WE, 0.5 ± 0.0, AE: 0.8 ± 0.1, carotenoids: WE, 3.9 ± 0.9, AE: 0.8 ± 0.1.

Antioxidant properties (at a concentration of 1.5 mg dry extract/mL water) of the two seaweed extracts were as follows: DPPH radical scavenging activity (%): WE, 93.6 ± 0.5, AE, 101.5 ± 0.9, metal chelating ability (%): WE, 75.6 ± 10.8, AE, 28.9 ± 6.7, reducing power (OD700): WE, 0.8 ± 1.1, AE, 1.6 ± 0.1.

2.3 Skin care emulsion production and storage conditions

The two *F. vesiculosus* extracts, WE and AE were applied to the skin care emulsion in two concentrations, 1 and 2 mg/g [equivalent of phenolics in WE (0.15 in 1 mg and 0.29 in 2 mg) and AE (0.19 in 1 mg and 0.37 in 2 mg)) of skin care emulsion (concentrations 1 and 2, respectively). The concentrations have previous shown to increase the stability of fish-oil-enriched foods [18]. Thus, five different types of skin care emulsions were finally obtained: RF (reference, without extract), WE1, WE2, AE1 and AE2. Table 1 shows all the ingredients for the water phase and the oil phase (including the extract).

The ingredients were weighted in individual pots and heated to 70–75°C. The oily phase was slowly poured into the water phase under powerful steering (9.500 rpm, Ultra-Turrax® T25basic). After the homogenisation process, the emulsions were cooled to room temperature. The skin care emulsions were packed in transparent 50 mL containers. Then the samples were stored under three different conditions: room temperature (21.2 ± 0.7°C) and darkness (A0), room temperature (24.4 ± 0.3°C) and light (A+) and high temperature (42.3 ± 1.5°C) and darkness (H0). The samples were analysed at different storage times (0, 7, 21, 35 and 56 days).

Table 1. Formulation of the different samples

Water phase	
Demineralised water	52.5 g/100 g
Aloe vera water	10.0 g/100 g
Glycerin	6.3 g/100 g
MF fat	3.6 g/100 g
Natriumbenzoat	0.6 g/100 g
F. vesiculosus extract (water or acetonic)	1 or 2 mg/g
Oil phase	
Almond oil	21.8 g/100 g
Lanette wax	2.0 g/100 g
VE fat	1.8 g/100 g
Vitamin E	0.9 g/100 g

2.4 Lipid extraction

Lipids were extracted from the skin care emulsions according to the method described by Iverson et al. [23] based on the method of Bligh and Dyer [24]. For each sample, two oil extractions were performed and analysed independently. Resulting lipid extracts were used as starting material for the analysis of peroxides, fatty acid composition and tocopherol content.

2.5 Fatty acid composition (fatty acid methyl esters, FAME)

The fatty acid composition of the oil phases was determined after fatty acid methylation and analysis by GC-FID. The Bligh and Dyer [24] lipid extract from skin care emulsion, corresponding to 30–60 mg lipid, were weighted in vials. A total of 100 μL toluene, 200 μL heptane with 0.01% v/v BHT and 100 μL internal standard (C23:0) (2% w/v) were added. One millilitre of BF_3 in methanol was added to the lipid extract mixture and the lipids were methylated in a one-step procedure using a microwave oven (Multiwave3000 SOLV, Anton Paar, Graz, Austria) with a 64MG5 rotor. The settings for the microwave were 5 min at 500 W followed by 10 min cooling. The fatty acid methyl esters (FAMEs) were washed with 1 mL saturated NaCl and 0.7 mL heptane with 0.01% v/v BHT. The heptane phase was transferred to a GC vial and FAMEs were analysed by GC (HP 5890A, Agilent Technologies, Palo Alto, CA, USA) according to AOCS [25]. For separation DB127-7012 column (10 mL × ID 0.1 mm × 0.1 μm film thickness, Agilent Technologies) was used. Injection volume was 0.2 μL in split mode (1:50). The initial temperature of the GC-oven was 160°C. The temperature was set to increase gradually being as follows: 160–200°C (10.6°C/min), 200°C kept for 0.3 min, 200–220°C (10.6°C/ min), 220°C kept for 1 min, 220–240°C (10.6°C/min) and kept at 240°C for 3.8 min. The measurements were performed at storage days 0 and 56, in duplicates, and the results were given in % of total area.

2.6 Tocopherol content

The lipid extracts from the skin care emulsions were evaporated under nitrogen and dissolved in heptane. The samples were analysed by HPLC (Agilent 1100 Series, Agilent Technology) according to AOCS [25] to quantify the contents of α-, β-, γ- and δ-tocopherols. These tocopherol homologues were separated using a silica column (Waters, Dublin, Ireland, 150 mm, 4.6 mm, 3 μm silica film). A stock solution added 10 mg tocopherols (mixture of α-, β-, γ- and δ-tocopherols) per litre was prepared to determine the retention time of the tocopherols and the peak areas of the

given standards. The peak areas of the standard solution were used to calculate the tocopherol content of the samples. The analyses were done in duplicates and results were reported as μg tocopherol/g skin care emulsion.

2.7 Peroxide value (PV)

PVs of the lipid extract of the skin care emulsions were determined at all sampling points. This was done according to the method by Shantha and Decker [26], based on the formation of an iron–thiocyanate complex. The coloured complex was measured spectrophotometrically at 500 nm (Shimadzu UV1800, Shimadzu Scientific Instruments, Columbia, MD, USA). The analyses were done in duplicate and the results were expressed in milliequivalents peroxides per kg oil (meq O_2/kg oil). In addition, for every sample, oxidation rates were calculated as follows:

$$\text{Oxidation rate } (\%) = \frac{\left(\text{PV}_{\text{day 35 or 36}} - \text{PV}_{\text{day 1}}\right)}{\text{PV}_{\text{day 1}}} \times 100$$

2.8 Volatile compounds (VC)

Tenax GR^{TM} packed tubes were used to collect volatile compounds by dynamic headspace. The collection of the volatile compounds was carried out using 4 g of emulsion [including 30 mg internal standard (30 μg/g of 4-methyl-1-pentanol in ethanol)] and 20 mL of distilled water. The volatile secondary oxidation products were collected at 45°C under purging with nitrogen (flow of 150 mL/min) for 30 min, followed by flushing the Tenax GR^{TM} packed tube with nitrogen (flow of 50 mL/min for 5 min) to remove water. The trapped volatiles were desorbed using an automatic thermal desorber (ATD-400, Perkin- Elmer, Norwalk, CT) connected to an Agilent 5890 IIA model gas chromatograph equipped with a HP 5972 mass selective detector. The settings for the MS were: electron ionisation mode, 70 eV, mass to charge ratio (m/z) scan between 30 and 250 mAU. Chromatographic separation of volatile compounds was performed on a DB1701 column (30m × ID 0.25mm × 0.5 μm film thickness, J&W Scientific, Folsom, CA, USA) using helium gas flow (1.3 mL/min).

The temperature programme was as follows: 3 min at 35°C, 3°C/min from 35 to 120°C, 7°C/min to 120 – 160°C, 15°C/min 160–200°C and hold for 4 min at 200°C.

The auto sampler collector setting details were: 9.2 psi, outlet split: 5.0 mL/min, desorption flow: 60 mL/min. The analysis was performed in triplicate in all sampling points and the results were given in ng/g of emulsion.

The quantification of the different volatiles was done by the use of a calibration curve prepared from the following external standards dissolved in ethanol: pentanal (calibration range, c.r.: 0.007–3.77 mg/g), hexanal (c.r.: 0.005–2.69 mg/g), heptanal (c.r.: 0.008–4.15 mg/g), *trans*-2-heptenal (c.r.: 0.005–2.95 mg/g), octanal (c.r.: 0.006–3.11 mg/g), *trans*-2-octenal (c.r.: 0.005–2.91), 1-octen-3-ol (c.r.: 0.006–3.01 mg/g) and 2-ethyl-1-hexanol (c.r.: 0.006–3.19 mg/g). About 1 μL of every solution prepared at different concentrations, was added to a Tenax GR^{TM} tube and flushed with nitrogen (flow of 50 mL/min for 5 min) to remove the solvent. Then, the volatiles were analysed in the same way as for the samples. Results for each compound were expressed as ng/g of extract, and oxidation rates were calculated as follows:

$$\text{Oxidation rate } (\%) = \frac{\left(\text{VC}_{\text{day 35 or 56}} - \text{VC}_{\text{day 1}}\right)}{\text{VC}_{\text{day 1}}} \times 100$$

2.9 Droplet size distribution

The size of fat globules in the o/w emulsion systems was determined by laser diffraction using a Mastersizer 2000 (Malvern, Inc.,Worcestershire, UK).

The skin care emulsion was diluted 1:9 in SDS buffer (10 mM NaH_2PO_4, 5 mM SDS, pH 7) prior to analysis. Droplets of the diluted skin care emulsion was added to recirculation water (3000 rpm) reaching an obscuration of 12–14%. The set-up used was the Fraunhofer method, which assumed that all sizes of particles scatter light with the same efficiency and that the particles are opaque and transmits no light. The refractive index (RI) of sunflower oil at 1.469 and water at 1.330 were used as particle and dispersant, respectively. Measurements were performed on days 0 and 56, in triplicates. Results were given as surface area mean diameter D(0.9), which indicates that 90% of the volume of the oil droplets is smaller than this value.

2.10 Colour determination

Colour of skin care emulsions was measured using a digital colourimeter (Chromameter-2 CR-200, Minolta, Osaka, Japan) to obtain the colour coordinates L^*, a^* and b^*. These values were used to calculate the euclidean distance value $\left(\Delta E = \sqrt{\left(L_2^* - L_1^*\right)^2 + \left(a_2^* - a_1^*\right)^2 + \left(b_2^* - b_1^*\right)^2}\right)$ that allowed two types of comparisons: comparison of samples with and without extracts, and also comparison of the initial colour of every sample to that detected along the storage. The measurements were performed in triplicates.

2.11 Statistical processing

Mean and standard deviation of results obtained were calculated. For each parameter, one way ANOVA with Tukey-b post hoc multiple comparisons was used in order to evaluate the significant differences among samples and treatments. Within each type of sample, the differences between 0 and 56 days were evaluated by Student t-test.

The statistical analysis of data was done using the SPSS 15.0 program (SPSS, Inc., Chicago, IL, USA). Significance level of $p \leq 0.05$ was used for all evaluations.

3 Results and discussion

3.1 Physical changes

3.1.1 Droplet size determination

The distribution of oil droplets in the skin care emulsions was determined at the beginning and the end of storage (Fig. 1).

D(0.9) value was selected to highlight the differences among samples and treatments. This value indicates that 90% of the volume of droplets is smaller than this value. When an increment in this parameter was observed, a destabilisation of the emulsion system had occurred. Regarding the accelerated storage conditions, D(0.9) tended to increase in the presence of light (A+), but a significant increase in D(0.9) was only observed at high temperature (H0). Hence, in all emulsions stored at high temperature, this increased D(0.9) caused a large destabilisation of the emulsions, leading to an evident syneresis at the end of the storage (visual evaluation). Due to this observed syneresis effect in H0 stored samples at day 56, only samples stored up to 35 days were considered for further analysis in this case. Moreover, WE2 and AE2 showed the highest D(0.9) increments, highlighting that the presence of high doses of these extracts in the skin care emulsions under high temperature, could influence the physical stability of the emulsions. In the case of A+ stored samples, only the addition of 2 mg/g of AE seemed to decrease the stability of the skin care emulsions, although no syneresis was observed.

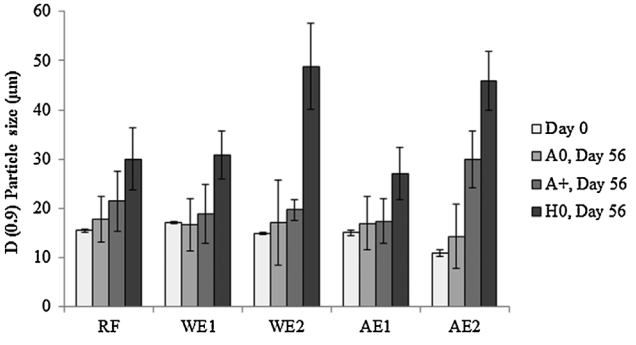

Figure 1. Droplet size distribution on cosmetic emulsions with or without *F. vesiculosus* extract (water or acetonic) after 56 days of storage under accelerated conditions or at room temperature and dark. RF, reference; WE1, water extract (1 mg/g); WE2, water extract (2 mg/g); AE1, acetone extract (1 mg/g); AE2, acetone extract (2 mg/g); A0, darkness and room temperature; A+, light and room temperature; H0, darkness and high temperature.

3.1.2 Colour

In order to evaluate the influence of the presence of some pigments (carotenoids, xantophylls and chlorophylls) on the colour of the emulsions, euclidean distance value (ΔE) was calculated before the storage (day 0). Thus, when comparing colour between the emulsions containing extract and the RF emulsion at day 0, the calculated ΔE were 3.90, 6.80, 4.93 and 7.59 for WE1, WE2, AE1 and AE2, respectively. All these values were higher than 2, leading to conclude that clear colour differences were noticed between the samples containing extract and the RF [27], with a strong influence of the concentration and type of *F. vesiculosus* extract applied. The instrumental colour data confirmed that whereas no differences in lightness (L^*) and redness (a^*) were found, yellowness (b^*) was significantly higher ($p < 0.05$) in the samples containing extract compared to the RF. These colour differences were dose dependent and higher in the AE containing emulsions as compared to WE ones. Hence, WE had a higher content of pigments (chlorophylls, 0.46 μg/mg extract; xanthophylls, 2.17 μg/mg extract; carotenes, 1.72 μg/mg extract) compared to AE (chlorophylls, 0.85 μg/mg extract; xanthophylls, 0.75 μg/mg extract), this may explain why the colour change was higher for AE.

These colour differences pointed out that the use of seaweed extracts did not perfectly mimic the characteristics of conventional skin care emulsions, due to the presence of pigments.

Additionally, the evolution of the colour was also measured during the storage conditions, and ΔE were also determined (Fig. 2), comparing, in this case, each sample with their own colour at day 0. Results pointed out that the five emulsions did not maintain the colour during storage, as observed by the ΔE increments in all cases. The increment was higher in the samples with higher amount of extract (WE2 and AE2). Particularly light, and mainly temperature (40°C), induced remarkable colour changes in the samples containing extracts, whereas the lowest colour changes in the RF sample at high temperature was observed. These changes were a consequence of an increment in a^* value and a reduction in the L^* value (data not shown), as the samples became more brown over time. The storage conditions could induce oxidative reactions that might affect pigments such as fucuxanthin and chlorophylls and produce colour changes in the samples. However, this deserves more research.

3.2 Oxidative changes

Almond oil (*Prunus amygdalus*) is one of the most valuable skin care oils due to its penetrating, moisturising and restructuring properties, and high content of unsaturated fatty acids. It can be used for numerous skin problems because of their anti-inflammatory, emollient, sclerosant and cicatrising effects [28]. Therefore, in the present study, almond oil was used in the formulation of the oil-in-water

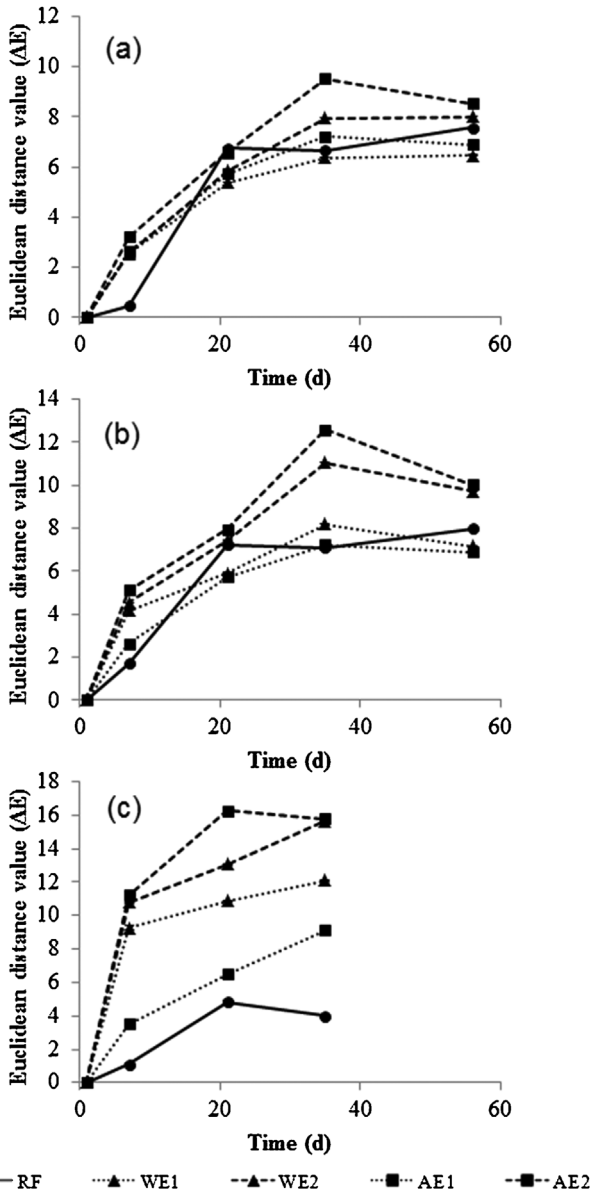

Figure 2. Euclidean distance value of the cosmetic emulsions calculated along the storage. It compares colour at each time of storage to its color at day 0. (a) A0, room temperature and darkness; (b) A+, room temperature and light; (c) H0, high temperature and darkness. RF, reference; WE1, water extract (1 mg/g); WE2, water extract (2 mg/g); AE1, acetone extract (1 mg/g); AE2, acetone extract (2 mg/g). Error bars indicate SD of the measurements.

skin care emulsions. However, the susceptibility of the unsaturated lipids present in almond oil to oxidation might be a major cause of quality deterioration and rancidity in the skin care emulsion. The lipid profile of the samples was determined at the beginning and at the end of the storage in every sample, and it was observed that it remained unchanged during the storage period (data not shown), with oleic acid as the major fatty acid, followed by linoleic, and the saturated ones, palmitic and stearic acid. On average, the fatty acid composition was as follows: 14:0, 0.27%; 16:0, 12.0%; 16:1 (ω-7), 0.11%; 18:0, 12.1%; 18:1 (ω-9), 53.8%; 18:1 (ω-7), 0.66%; 18:2 (ω-6), 18.4%; 18:3 (ω-3), 0.08%; 20:0, 0.28%; 20:1 (ω-11), 0.47%; 20:4 (ω-6), 0.31%; 22:1 (ω-9), 0.17%.

3.2.1 Tocopherol content during storage

Four tocopherol homologues were detected in the skin care emulsions, α-, β-, γ-, δ-tocopherol. The most abundant one at the beginning of the storage was γ-tocopherol (4895 ± 151 μg tocopherol/g skin care emulsion) followed by δ- (1657 ± 94 μg tocopherol/g skin care emulsion), α- (1329 ± 72 μg tocopherol/g skin care emulsion) and β-tocopherol (116 ± 10 μg tocopherol/g skin care emulsion), respectively. The addition of seaweed extract did not affect the content of tocopherols. The high level of tocopherols is mainly due to exogenous vitamin E added to the formula, but the raw material almond oil also contributed.

Similar changes were observed in the four homologues, so the sum of all of them was calculated, and represented as the total tocopherol content (TTC) along the storage (Fig. 3). The TTC decreased in all samples during storage, and the highest rate of decrease was observed at the high temperature conditions (H0).

It is worthy to highlight, that the AE showed the highest tocopherol protective effect at all storage conditions, with AE2 being the best concentration.

However, WE showed protective effects only when exposed to light (up to 50 days for WE1) and high temperature storage conditions (up to 20 and 35 days for WE1 and WE2, respectively).

This protective effect of WE and AE on tocopherols could be due to a synergistic effect between tocopherol and phenolic compounds or pigments, contributing to the regeneration of tocopherol in skin care emulsions containing extracts.

3.2.2 Peroxide value (PV)

The autoxidation of unsaturated fatty acids is an autocatalysed chain reaction through free radical intermediates, and can be accelerated during storage by exposure to light, temperature and in presence of redox metals. On that basis, the primary oxidation compounds, expressed as the peroxide content of the skin care emulsions stored in the different conditions, were determined (Table 2).

At the beginning of the storage, WE2 and AE2 samples showed slightly higher PV values than RF samples ($p < 0.05$). This could be a consequence of the presence of trace metals in the extracts (iron and copper) [18, 22] which promoted, together with the temperature of processing (70–75°C), oxidative reactions at an initial stage. The iron and copper

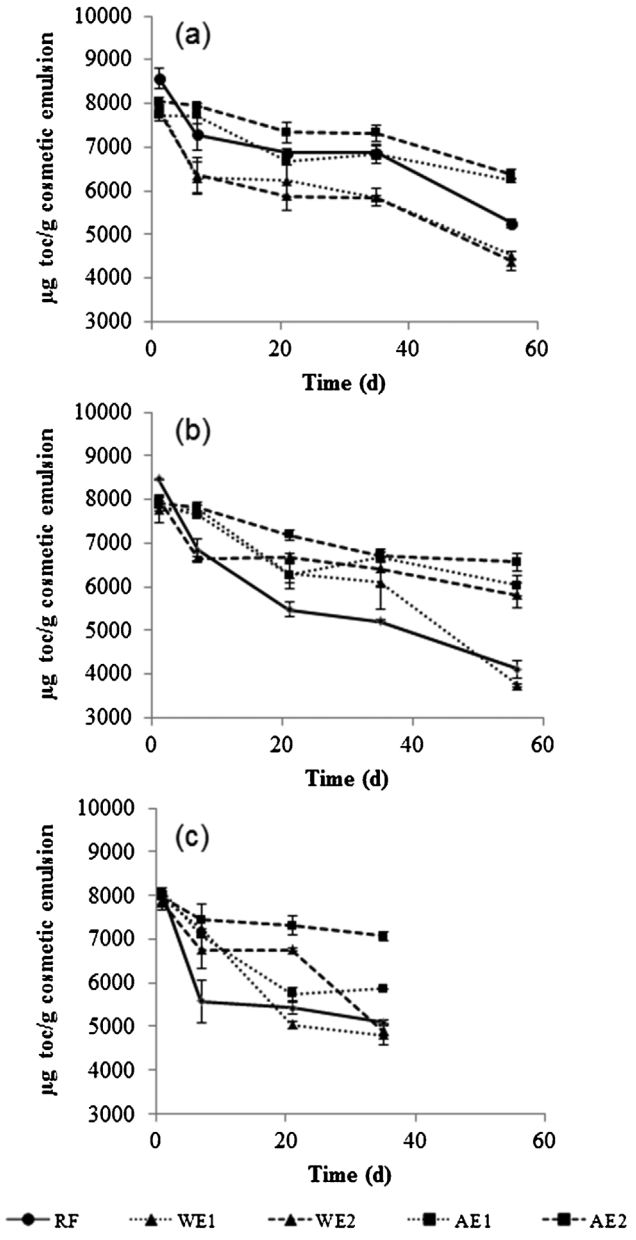

Figure 3. Total tocopherol content (µg tocopherol/g cosmetic emulsion) in emulsions with WE or AE including a control without any extract during storage. (a) A0, room temperature and darkness; (b) A+, room temperature and light; (c) H0, high temperature and darkness. RF, reference; WE1, water extract (1 mg/g); WE2, water extract (2 mg/g); AE1, acetone extract (1 mg/g); AE2, acetone extract (2 mg/g). Error bars indicate SD of the measurements.

content in the extracts was measured initially; AE (iron, 9.53 µg/mg extract and copper, 1.21 µg/mg extract) and WE (iron, 4.39 µg/mg extract and copper, 0.91 µg/mg extract). This initial difference may explain why WE and AE had slightly higher PV than RF. During storage, significant increments in PV were found in all samples ($p < 0.05$).

At A0 storage conditions an increase in the oxidation rate, between day 1 and 56, was found in WE2 (151%) and AE2 (154%) compared to RF (108%). It is well known that interactions between lipid hydroperoxides and transition metals can induce the formation of secondary oxidation compounds. Consequently, metal chelating capacity is claimed as one of the important mechanisms of antioxidant activity [2, 29]. Regarding this, several studies showed that *F. vesiculosus* extracts, containing phlorotannins, had good ferrous ion-chelating capacity [12, 17]. In this sense, the presence of phlorotannins may form complexes with metals and inactivate their catalytic effects in promoting lipid hydroperoxide decomposition. Due to this antioxidant effect, an accumulation of peroxide compounds in the extract containing samples might take place and consequently lead to a lower formation of secondary oxidation compounds, as will be discussed below.

Regarding A+, after 56 days of storage, while samples containing AE showed the highest (AE2) or not significant differences (AE1) on PV, both WE samples had lower PV than RF 1 ($p < 0.05$). This could be due to the higher content of carotenoids in WE, as carotenoids are well known inhibitors of free radical chain reactions caused by photooxidation process [30].

Moreover, in the case of high temperature conditions it should be pointed out that a higher oxidation rate, between day 1 and 35, was found in RF (185%), compared to WE2 (102%) and AE2 (110%). The high content of phlorotannins in the extracts, with radical scavenger activity could inhibit lipid oxidation initiation by radicals.

The antioxidant properties, both metal chelating and radical scavenging capacity, can be related to the structure of the phlorotannins. Previously, we have found that using less polar solvents such as acetone these extract more amphiphilic phenolic compounds are favoured compared to water. In a recently submitted manuscript [31], the relationship between the phlorotannin structure and the antioxidant capacity have been investigated. This study showed that more polymerized (six phloroglucinol units), and thereby more polar phlorotannins, are less efficient antioxidants, compared to smaller (less polar) oligomer phlorotannins (three phloroglucinol units). Although, no standards are available for the phlorotannins, with exception of the monomer (phloroglucinol), therefore, it is not possible to quantify the different phlorotannin isomers. Conversely, it is hypothesised that the water extracts consists of the highly polymerised and more polar phlorotannins, whereas acetone extracts to a larger extent the less polar oligomers.

3.2.3 Volatile compounds

Odour deterioration of lipid containing products is caused mainly by the presence of volatile secondary oxidation compounds, which have an impact on odour at extremely low concentrations. Compounds formed from decomposition of

Table 2. Effect of adding water or acetonic *F. vesiculosus* extract on PV (meq O_2/kg oil) of cosmetic emulsions under accelerated stored conditions or room temperature and dark

	Peroxide value (meq O_2/kg oil)					
	Day 0	S.C.	Day 7	Day 21	Day 35	Day 56
RF	5.52 ± 0.51^{A}	A0	6.85 ± 0.29^{Ba}	7.31 ± 1.00^{Aa}	9.48 ± 0.45^{Aa}	11.62 ± 0.31^{A}
		A+	8.22 ± 0.52^{Ab}	11.71 ± 1.07^{Bb}	13.11 ± 1.19^{ABb}	$18.93 \pm 0.63^{B,***}$
		H0	11.49 ± 0.84^{ABc}	11.65 ± 0.29^{Ab}	15.01 ± 0.78^{ABb}	17.52 ± 0.49^{b}
WE1	5.19 ± 0.29^{A}	A0	6.09 ± 0.04^{ABa}	6.97 ± 0.45^{Aa}	8.53 ± 0.36^{Aa}	10.77 ± 1.25^{A}
		A+	8.11 ± 0.05^{Ab}	10.58 ± 0.59^{ABb}	12.37 ± 0.70^{ABb}	$15.67 \pm 1.29^{A,**}$
		H0	10.11 ± 1.11^{Ac}	13.08 ± 1.33^{Ac}	14.31 ± 0.07^{Ac}	21.74 ± 1.93^{c}
WE2	6.58 ± 0.69^{B}	A0	12.89 ± 0.67^{Da}	11.95 ± 0.22^{Ba}	13.96 ± 0.32^{Ca}	17.67 ± 1.38^{C}
		A+	14.08 ± 0.21^{Da}	12.40 ± 0.69^{Ba}	14.38 ± 1.28^{Ba}	$16.42 \pm 1.42^{A,\ ns}$
		H0	13.05 ± 1.60^{BCa}	11.38 ± 0.19^{Aa}	13.97 ± 0.19^{Aa}	15.8 ± 1.07^{a}
AE1	6.28 ± 0.65^{B}	A0	14.26 ± 0.29^{Ea}	13.36 ± 1.03^{Bb}	15.34 ± 0.35^{Db}	18.78 ± 0.71^{C}
		A+	13.16 ± 0.32^{Ca}	10.90 ± 1.15^{ABa}	10.34 ± 0.52^{Aa}	$23.97 \pm 0.49^{C,***}$
		H0	13.73 ± 1.71^{Ca}	16.58 ± 1.61^{Bc}	16.47 ± 1.73^{Bb}	19.43 ± 2.27^{a}
AE2	6.63 ± 0.75^{B}	A0	11.31 ± 0.28^{Cb}	17.03 ± 0.47^{Cb}	12.36 ± 0.87^{Ba}	15.14 ± 0.95^{B}
		A+	9.58 ± 0.23^{Ba}	9.43 ± 0.35^{Aa}	17.36 ± 1.99^{Cb}	$18.89 \pm 0.55^{B,**}$
		H0	10.94 ± 0.21^{ABb}	10.43 ± 0.78^{Aa}	14.05 ± 1.05^{ABab}	15.69 ± 0.88^{a}

RF, reference; WE1, water extract (1 mg/g); WE2, water extract (2 mg/g); AE1, acetone extract (1 mg/g); AE2, acetone extract (2 mg/g); S.C., storage condition; A0, darkness and room temperature; A+, light and room temperature; H0, darkness and 40°C.
Different capital letters in the same column denote significant differences between samples for each storage condition ($p < 0.05$).
Different small letters in the same column denote significant differences among storage conditions for each sample ($p < 0.05$).
Level of significance for the Student t test comparing storage conditions at day 56: ns = not significant ($p > 0.05$); $^{**}p < 0.01$; $^{***}p < 0.001$.

lipid hydroperoxides during storage can either react with unsaturated lipids to form stable and innocuous alcohols, or undergo fragmentations into aldehydes and ketones causing rancidity in unsaturated matrices [32]. Major volatile compounds identified from the headspace of the fifteen samples throughout the storage were: four alkanals (pentanal, hexanal, heptanal and octanal), two alkenals (*trans*-2-heptenal and 2-octenal) and two alcohols (1-octen-3-ol and 2-ethyl-1-hexanol). These compounds represent groups of secondary oxidation compounds resulting mainly from the autooxidation of oleic, linoleic and α-linolenic acid [33–35]. Hexanal and 2-octenal showed the highest initial concentrations (248 ± 99 and 222 ± 52 ng/g emulsion, respectively). However, others such as pentanal and heptanal showed greater differences among samples and also more evident variations during storage compared to their initial concentrations. This was the reason why they were selected to follow their evolution during the whole storage (Fig. 4).

During accelerated storage conditions (A+ and H0), the peroxides decomposition generated higher volatile amounts than in the A0 stored samples, so there was a higher transformation rate from hydroperoxides to secondary oxidation products due to thermo- and photooxidation processes. Furthermore, results showed that temperature had significantly higher effect than light on the formation of volatile compounds, with higher absolute amounts of both aldehydes at the end of the storage.

Regarding the presence of extracts, the concentration of pentanal and heptanal varied between skin care emulsions at day 0 and during the storage. On one hand the highest amounts of extracts contributed to increase the pentanal and heptanal concentration at the beginning of all storages conditions. This could be due to the presence of these compounds in the extract itself. Hermund et al. [18] observed higher amounts of some volatile compounds (1-penten-3-ol and 1-penten-3-one) in milk emulsions containing *F. vesiculosus* extracts.

On the other hand, in the samples with the highest extract content (WE2 and AE2), pentanal showed significantly lower concentrations in all samples compared to RF at the end of the storage (reduction up to 72% in AE2 samples at A0 storage conditions), whereas heptanal amount was lower than RF only at the end of storage at high temperature (19% reduction). On the other hand, the presence of antioxidant modified the timing of volatile compound formation. Thus, even though the presence of extract at the beginning of the storage resulted in higher amounts of pentanal and heptanal in all samples, lower oxidation rates were observed during storage in these samples.

In particular, in the light stored samples, lower oxidation rates for pentanal and heptanal were found in WE2 (6.7 and 69%, respectively) and AE2 (−35.2 and 40%, respectively) compared to RF (144 and 211%, respectively). Moreover, at high temperature, AE2 showed the best results against the

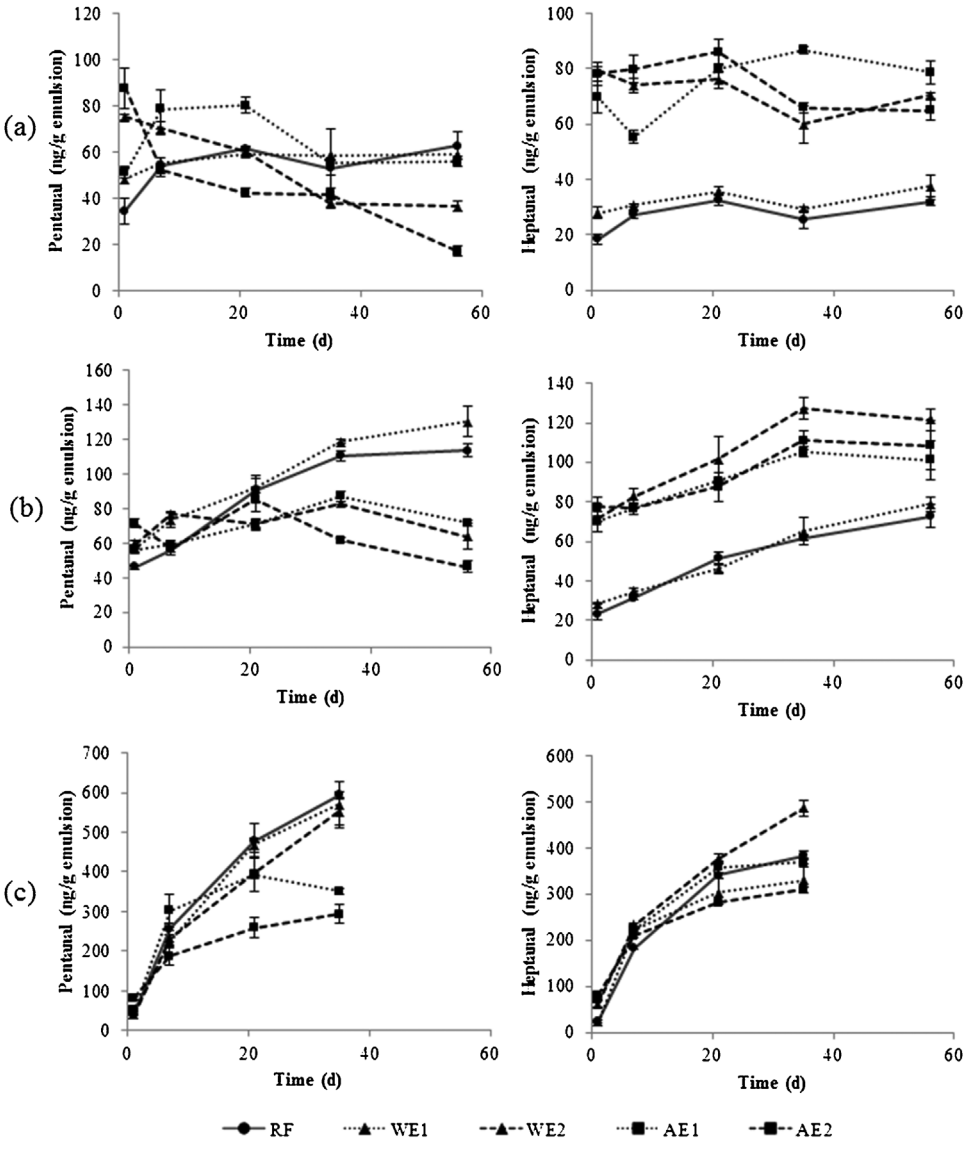

Figure 4. Development of volatile compounds, pentanal and heptanal (ng/g emulsion), during the storage. (a) A0, room temperature and darkness; (b) A+, room temperature and light; (c) H0, high temperature and darkness. RF, reference; WE1, water extract (1 mg/g); WE2, water extract (2 mg/g); AE1, acetone extract (1 mg/g); AE2, acetone extract (2 mg/g). Error bars indicate SD of the measurements.

formation of pentanal and heptanal, with oxidation rates of 261 and 281%, respectively, compared to the rates calculated for RF (1251 and 1419%). Finally, it is worth noticing that at A0 stored conditions, while RF showed an increment of pentanal (81%) and heptanal (71%) between days 1 and 56, the highest extract concentration samples lead to a reduction compared to their initial amounts. These results were in agreement with the accumulative effect observed in PV in these samples. The presence of the extract decreased the hydroperoxide decomposition rate to volatile secondary oxidation compounds at all storage conditions, with AE2 being the most efficient extract. This information helps to elucidate the antioxidant mechanism of those extracts, which may influence the protection of the peroxides decomposition to secondary oxidation products. However, more studies are needed to confirm these findings.

4 Conclusions

The type of antioxidant extract was a key factor in controlling oxidation processes of skin care products influenced by light or temperature. Whereas both water and acetone extracts of Icelandic *F. vesiculosus* showed (at 2 mg/g of emulsion) protective effect against thermooxidation, only the water extract showed antioxidant activity against photooxidation.

Therefore, the presence of highly antioxdative phlorotannin (radical scavenging activity and iron-chelating capacity) in the extracts presumably contributed to decreasing the lipid oxidation. Moreover, the higher carotenoids content in the water extract could inhibit free radical chain reactions caused by the photooxidation process. The Icelandic *F. vesiculosus* extracts were effective in protecting highly-unsaturated skin care emulsions but gave rise to colour changes particularly when stored at high temperature.

We thank PIUNA (Plan de Investigación de la Universidad de Navarra) and Ministerio de Economía y Competitividad (AGL2014-52636-P and Red de Excelencia Consolider- PROCARSE AGL2014-51742-REDC) for their contribution to the financial support of this work. C. Poyato is grateful to "Asociación de Amigos de la Universidad de Navarra" for the grants received.

The authors have declared no conflict of interest.

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Appendix 7: Article VII

Application of active ingredients derived from brown algae *Fucus vesiculosus* for skin care products.

Hermund, D.B., Heung, S.Y., **Thomsen, B.R.**, Akoh, C.C. and Jacobsen, C.

In preparation.

Appendix 8: Article VIII

J Am Oil Chem Soc (2016) 93:929–942
DOI 10.1007/s11746-016-2837-2

ORIGINAL PAPER

Comparison of Three Methods for Extraction of Volatile Lipid Oxidation Products from Food Matrices for GC–MS Analysis

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Received: 4 June 2015 / Revised: 13 April 2016 / Accepted: 17 April 2016 / Published online: 28 April 2016

Abstract The aim of this study was to compare three different collection methods; purge and trap, solid phase micro extraction and automated dynamic headspace/thermal desorption, all followed by GC–MS analysis used for the measurements of concentrations of volatile oxidation products in three different food matrices, namely oil, emulsion and milk. The linearity ranges of calibration curves obtained by the three different methods were compared for oil samples. Overall, the results showed that the three collection methods were comparable, although there were large differences in the linearity range of the calibration curves depending on the collection method. However, some challenges were observed for solid phase micro extraction and automated dynamic headspace/thermal desorption, namely, competition problems and overestimation of concentration by calibration curves, respectively. Based on the results, we suggest mainly to apply solid phase micro extraction on simple matrices and to be cautious with more complex matrices such as enriched milk and highly oxidized oils. Thereby, the study confirmed some challenges observed by other authors regarding competition problems on the fiber when using solid phase micro extraction. Furthermore, we observed that purge and trap, and automated dynamic headspace/thermal desorption were excellent for extraction of volatile compounds in all three matrices. However, automated dynamic headspace/thermal desorption calibration curves did provide an overestimation for oil samples so results must be interpreted with caution.

Keywords Lipid oxidation · Volatile oxidation products · a-DHS · P&T · SPME

Introduction

Lipid oxidation is one of the most important quality deteriorating processes of lipid bearing foods. It results in—among other reaction products—an important increase in saturated and unsaturated aldehydes and ketones (secondary volatile oxidation products). These secondary volatile oxidation products are of special interest because they can seriously affect the flavor and odor of the food product even in low concentrations [1]. Since these quality changes are important, the concentration of secondary volatile oxidation products should be measured during storage of the products [2]. Oxidative flavor deterioration can be investigated by two main approaches, namely by chemical or by sensory analysis [3–5]. The chemical approach includes the use of unspecific methods such as analysis of thiobarbituric acid reactive substances and the anisidine value [6]. However, due to their unspecificity and low sensitivity, these methods are in many cases not suitable for complex food matrices [7]. Secondary volatile oxidation products can also be determined by specific methods such as GC–MS. Because of the complicated matrices, direct injection techniques on GC–MS are normally considered unsuitable for this type of analysis and collection of volatile compounds before injection into the GC–MS is therefore required. Several different

Electronic supplementary material The online version of this article (doi:10.1007/s11746-016-2837-2) contains supplementary material, which is available to authorized users.

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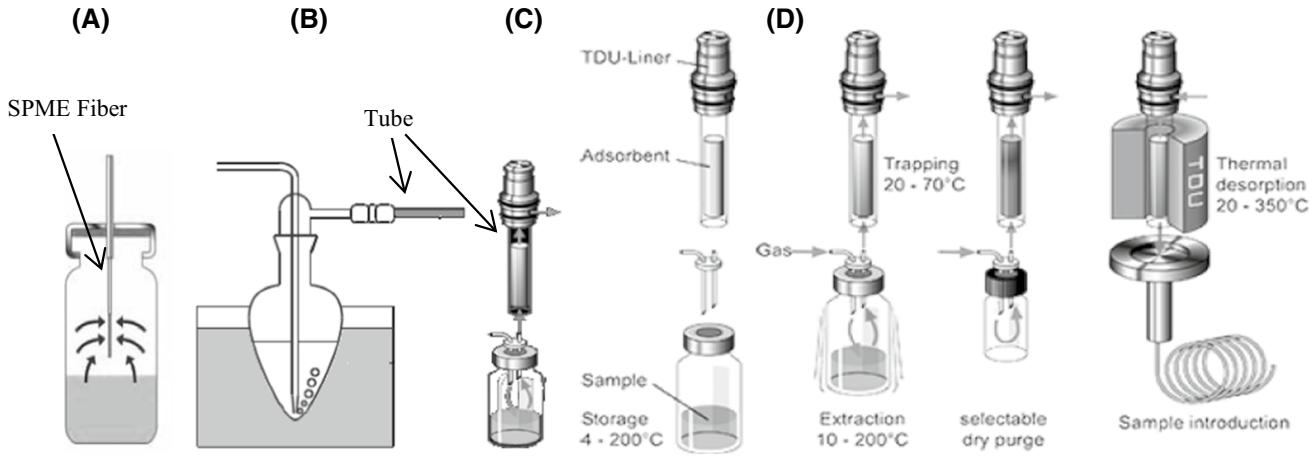

Fig. 1 **a** Schematic description of the three collection methods; SPME (**a**), P&T (**b**) and a-DHS (**c**). The principles of a-DHS collection method (**d**). Adapted from Gerstel [15]

methods are available for this purpose. The focus of this study is on different collection methods and the comparability of the results obtained. The methods compared were manual purge and trap (P&T), solid phase micro extraction (SPME) and automated dynamic headspace (a-DHS). The principles of the collection methods are shown in Fig. 1a–c.

The principle of P&T collection: volatile compounds are released from food matrices by purging through them with an inert gas such as nitrogen at an elevated temperature. The state of equilibrium between the volatile compounds in the sample and headspace is continuously disturbed by purging nitrogen through the sample. Thereby, the release of the volatile compounds from the sample to the headspace is increased. Hereafter, the released volatile compounds are adsorbed on a thermal desorption (TD) tube containing adsorbing material such as Tenax®. It is expected that the amount of adsorbent is large enough to avoid breakthrough of any adsorbed component, and thus all released components from the matrix are quantitatively trapped. If necessary, water residues are removed by purging the TD tube with nitrogen. The sample preparation and collection is performed manually. Then, the TD tube is inserted into an automatic thermal desorption unit (ATD) and the TD tube is heated, which transfers the volatile compounds from the TD tube to a focusing cold trap. Lastly, the volatile compounds are transferred from the cold trap to GC–MS [8]. This allows detection of volatile compounds, which are only present in the sample in trace amounts [9]. P&T has been shown to have a tendency towards detecting more volatile compounds compared to SPME [10–12].

The principle of SPME: volatile compounds adsorbed by a fiber, and TD of trapped compounds into an injector. The sample is first incubated in a closed vial to obtain equilibrium of volatile compounds between sample and headspace. The fiber is now introduced into the headspace of the vial and waits until equilibrium has been reached between headspace and fiber or till a well-defined time period where a certain percentage of up-take of volatile compounds has been reached [13]. However, Górecki *et al.* [14] found that some volatile compounds may interfere with the absorption and cause a reduction of other volatile compounds extracted from the sample. They found that the interfering volatile compounds had a higher affinity for the fiber coating, and thereby the interfering volatile compounds were present in higher concentration on the fiber than in the sample. Adsorption is a competitive process where the composition of matrix and the extraction conditions may highly affect the amount extracted of the individual volatile compounds. The conclusion of a study by Górecki *et al.* [14] was that SPME performed well in matrices of constant composition.

Compared to P&T, a-DHS is a new automated headspace purging technique where the volatile compounds are extracted and concentrated on the DHS station located on the auto sampler unit. Unlike P&T technique, only the headspace of sample in a-DHS is purged with the controlled and inert nitrogen, and not the sample matrix. Thereby, the volatile compounds are extracted from the headspace and collected on the adsorbent. During collection, the sample and the adsorbent are thermostated and the vial agitated in the DHS station. After collection, water residues are removed by purging with nitrogen through a drying vial (step 3 in Fig. 1d). Hereafter, the adsorbent is transferred and injected into the GC–MS through a thermal desorption unit. The entire method does not require manual work other than weighing the sample into the vial [15]. Another difference

compared to the P&T method is that the distances (number of transfers and storage time) that volatile compounds have to pass from the sample to injection are very short. Moreover, volatile compounds are transferred directly from the Tenax tube via the small thermal desorption unit to the GC liner. In contrast, volatile compounds released from the cold trap in the automatic thermal desorption unit used in P&T are transferred to the GC via a long transfer line. Hence, the risk of losing volatile compounds during transfer in the a-DHS unit is lower.

Only few studies have investigated the effect of the collection method on the overall quantitation of the volatiles, the changes measured during storage and the concentration of the individual volatile compounds in different food matrices. Moreover, to the best of our knowledge no studies have compared the new a-DHS method with SPME and P&T. The aim of this study was therefore to compare the three methods for collection of volatile compounds from neat fish oil, fish oil-in-water emulsion or fish oil enriched milk. Several matrices were selected to explore the usability of each collection method in both simple and complex matrices. All collection methods and GC temperature programs were optimized for each food, individually either prior to or as part of the present study. The volatile compounds were analyzed by GC–MS and results calculated in ng/g. The samples used in the present study were part of other experiments reported elsewhere where formation of volatile oxidation products during storage was investigated using P&T GC–MS. Therefore, collection and analysis of the samples were done at different time points for the different products.

Materials and Methods

The Three Food Matrices

Fish Oil

Six different commercial fish oils were purchased locally. The names of the products are known to the authors but will remain anonymous in the article and referred to as product 1–6. The oil samples were from six different brands of fish oil capsules and cod liver oils. Initial peroxide values (PV) in the oils were 2.95 ± 0.11, 4.7 ± 0.14, 1.75 ± 0.04, 0.37 ± 0.01, 1.71 ± 0.03 and 2.29 ± 0.05 meq/kg in oil 1–6, respectively. The oils were incubated in darkness at 40 °C for 4 days ($n = 1$).

Three blends of antioxidants and fish oil, AO 1, AO 2 and AO 3, were used for a standard addition experiment, which was performed to study observed differences in results between the P&T and a-DHS methods ($n = 1$).

Fish Oil Enriched Emulsion

Materials

Fish oil was supplied by Maritex Norway (subsidiary of TINE BA, Norway). Fish oil had a PV of 0.16 meq peroxides/kg oil and contained 234.46 μg α-tocopherol/g oil, 3.65 μg β-tocopherol/g oil, 109.27 μg γ-tocopherol/g oil and 45.40 μg δ-tocopherol/g oil. The fatty acid composition was as follows: 14:0, 3.5 %; 16:0, 10.0 %; 16:1n-7, 9.2 %; 16:3n-4, 0.5 %; 18:0, 2.0 %; 18:1n-9, 16.9 %; 18:1n-7, 4.8 %; 18:2n-6, 1.9 %, 18:3n-4, 0.9 %; 18:4n-3, 2.7 %; 20:1n-11 + n-9, 12.43 %; 20:4n-3, 0.7 %; 20:5n-3 (EPA), 9.2 %; 22:1n-11, 0.8 %; 22:1n-9, 5.3 %, 22:5n-3, 1.0 % and 22:6n-3 (DHA) 10.3 %. Caffeic acid was from Sigma–Aldrich (Steinheim, Germany). Chemicals were from Merck (Darmstadt, Germany). All solvents were of HPLC grade and purchased from Lab-Scan (Dublin, Ireland). Butyl caffeate was synthesized in an acid catalyzed reaction with caffeic acid and 1-butanol as described elsewhere [16].

Production and Storage Conditions

The emulsions used in this study originated from a study by Sørensen *et al.* [17]. Details about antioxidants effect are described in their publication. In short, an oil-in-water emulsion of 94 % 10 mM acetate imidazole buffer (pH 7), 5 % fish oil, 1 % CITREM (DuPont) as emulsifier, and 100 μM antioxidant was prepared. Three codes were prepared: Control sample: emulsion with methanol (CON), Emulsion with caffeic acid dissolved in methanol (CAC0) and Emulsion with butyl caffeate dissolved in methanol (CAC4). Traces of methanol were evaporated with nitrogen. These emulsions were prepared in two steps: pre-emulsification and homogenization. Pre-emulsification with an Ultra-Turrax (Janke & Kunkel IKA-Labortechnik, Staufen, Germany) and thereafter homogenized in a table homogenizer from GEA Niro Soavi Spa (Parma, Italy) at a pressure of 80 and 800 bar in second and first stage of the homogenization, respectively. The emulsion was recirculated four times in the homogenizer. The emulsions (100 g) were stored in 100-mL blue cap bottles at room temperature in darkness ($n = 1$). Samples were taken on days 0, 1, 2, 4, 6, 9, 12 and 15, and flushed with N_2 and stored at −40 °C until analysis.

Fish Oil Enriched Milk with Seaweed Extract Added

Materials

Conventional milks with a fat content of 0.5 and 1.5 % were purchased locally. Fish oil was supplied by Maritex

Table 1 Overview of sample codes for all three foods; fish oil, fish oil enriched emulsion and fish oil enriched milk with seaweed extract added

Food matrix	Fish oil	Fish oil enriched emulsion	Fish oil enriched milk with seaweed extract added
Samples	Oil 1	REF	CON
	Oil 2	WEI1	CAC0
	Oil 3	WEI2	CAC4
	Oil 4	WEI3	
	Oil 5		
	Oil 6		

A/S a subsidiary of TINE, BA (Sortland, Norway). Initial PV, tocopherol and fatty acid composition of the fish oil are as described above for the fish oil used in fish oil enriched emulsion. Apart from fish oil, the milk was also supplemented with seaweed extract. Two different seaweed extracts from *Fucus vesiculosus* supplied by Matis ohf. (Reykjavik, Iceland) were used.

Production and Storage Conditions

The two milks with a fat content at 0.5 and 1.5 % were mixed (1 kg:1 kg) to obtain a total milk fat content of 1 %. Subsequently, the milk was heated to 72 °C for 15 s, hereafter fish oil (5 g/kg) and the seaweed extracts (0, 1.0 and 2.0 g/kg) were added. Immediately after addition of fish oil and seaweed extract, the mixture was homogenized at 250 bar using a two valve table homogenizer from GEA Niro Soavi Spa (Parma, Italy). The mixture was recirculated four times. The milk emulsion was stored in the dark at 5 °C in 250 mL sterilized bottles ($n = 1$). Samples were taken at day 0, 6 and 12, flushed with N_2 and stored at −40 °C until analysis. Seven codes were prepared: Control sample, milk emulsion only (REF), milk emulsion with increasing concentrations of water extract seaweed (WEI1, WEI2 and WEI3) and milk emulsion with increasing concentrations of ethyl acetate extract seaweed (EAE1, EAE2 and EAE3). Detailed information on the samples and antioxidant effect can be found in an article by Hermund *et al.* [18].

An overview of the samples included in the experiment is summarized in Table 1.

Three Different Volatile Collection Methods

Optimization of Collection Conditions and GC Temperature Programme

For oil collected by P&T and SPME, and milk collected by P&T, the collection conditions have been optimized in connection with earlier studies in our laboratory (no additional optimization has been conducted for this study). For SPME on the milk and emulsion and for a-DHS on all three products, optimization was carried out prior to the present study. The optimal conditions is a compromise between obtaining the highest possible GC–MS response of as many compounds as possible and at the same time avoiding that lipid oxidation will take place during the collection. Therefore, we aimed at using as low temperature as possible. The collection conditions for the individual methods and products are described below.

GC temperature programs were optimized to obtain the best possible separation of the volatile compounds from the different products.

P&T (Purge and Trap)

The P&T collection was performed as described by Rørbæk and Jensen [8], but optimized to the following conditions for each food matrix. Volatile compounds s from 4 g of sample mixed with 30 mg of internal standard solution (30 µg/g of 4-methyl-1-pentanol in rapeseed oil) were collected on Tenax GR® (Perkin-Elmer, Norwalk) for 30 min at 60 °C for oil and for 30 min at 45 °C for milk, respectively ($n = 3$). In all cases, a nitrogen flow of 150 mL/min for 30 min was used at room temperature. Water was removed by a 15 min nitrogen flow at 50 mL/min after collection from milk samples. The collected volatile compounds were desorbed from the TD tubes by using an automatic thermal desorber (ATD-400, PerkinElmer, Norwalk, CT, USA) connected to an Agilent 5890 IIA model (Palo Alto, CA, USA) GC equipped with a MS HP 5972 mass selective detector. The settings for the MS were: electron ionization mode, 70 eV, mass to charge ratio (*m/z*) scan between 30 and 250. Chromatographic separation of volatile compounds was performed on a DB1701 column (30 m × ID 0.25 mm × 0.5 µm film thickness, J&W Scientific, Folsom, CA, USA) using helium gas flow (1.3 mL/min).

The GC oven program applied on milk samples: the initial temperature 45 °C for 5 min, then the temperature increased by 1.5 °C/min to 55 °C, then increased by 2.5 °C/min to 90 °C. Finally, increased by 12 °C/min to 220 °C and kept there for 4 min.

The GC oven program applied on oil samples was: the initial temperature 35 °C for 3 min, then the temperature

increased by 3 °C/min to 120 °C, then increased by 7 °C/min to 160 °C. Finally, increased by 15 °C/min to 200 °C and kept there for 4 min.

The P&T method was not applied on emulsion samples.

SPME

One gram of sample with 30 mg of internal standard solution (30 µg/g of 4-methyl-1-pentanol in rapeseed oil) was mixed for 30 s in a 10-mL vial ($n = 3$). The sample was placed in the SPME tray and equilibrated for 3 min at 60 °C. Extraction of volatile compounds was performed in the headspace for 45 min for emulsion and milk, and for 55 min for oil at 60 °C while agitating at 500 rpm. The extraction of volatile compounds was made by a SPME fiber 50/30 µm CAR/PDMS 57,295-U (Supelco, Bellefonte, USA) installed on a GERSTEL MPS 2, with SPME option (Gerstel GmbH & Co. KG., Mülheim an der Ruhr, Germany).

The extracted volatile compounds were transferred from the SPME fiber to the GC HP 6890 (Agilent Technologies, USA) in the split/splitless injector set at 220 °C. The injection was done in splitless mode. The glass liner used had an internal diameter of 0.75 mm from Agilent (for fast transfer of the components). Subsequently, the separated volatile compounds were detected by MS HP 5973 inert mass-selective detector (Agilent Technologies, USA). The same GC-column and MS-setting as described for P&T were applied. The GC oven temperature program had an initial temperature of 35 °C for 3 min, with increment of 3.0 °C/min to 140 °C, then increment of 5.0 °C/min to 170 °C and increment of 10.0 °C/min to 240 °C and then this temperature was kept there for 8 min (same temperature program for all food matrices).

A-DHS

In short, the volatile compounds were automatically collected and injected by an a-DHS system from Gerstel (Gerstel GmbH & Co. KG., Mülheim an der Ruhr, Germany) and detected on the same GC–MS system as mentioned above under "SPME". Approximately 1 g of sample together with 30 mg of internal standard solution (30 µg/g of 4-methyl-1-pentanol in rapeseed oil) was mixed on a whirly mixer for 30 s in a 10 mL vial ($n = 3$).

The automation sequence was: the sample was incubated for 4 min at a temperature of 60 °C for oil and emulsion, and 45 °C for enriched milk. Agitation was applied at 300 rpm (agitator on time: 10 s, agitator off time: 1 s). Thereafter, the volatile compounds were collected by purging nitrogen at 50 ml/min through the headspace of the vial for 20 min. The volatile compounds were trapped in TD tubes containing Tenax GR 300 (Gerstel GmbH & Co. KG., Mülheim an der Ruhr, Germany). In order to remove the water from the tubes for the emulsion and milk samples, a 30 mL/min purge flow was applied for 3 min for emulsion and a 50 mL/min purge flow for 44 min for milk. The volatile compounds were desorbed from TD tubes in the thermal desorption unit (initial temp 40 °C, then 720 °C/min to 280 °C kept there for 5 min) via the focusing trap CIS 4 at 10 °C during the focusing period and subsequently heated with 720 °C/min to 280 °C and transferred to the GC 6890 N Series (Agilent Technologies, Santa Clara, USA). The individual volatile compounds were analyzed using a MS 5973 inert mass-selective detector (Agilent Technologies, Santa Clara, USA).

The same GC-column, MS-setting and GC oven temperature program as described for "SPME" were applied.

The volatile compounds were identified individually by the MS-library Wiley 138K (John Wiley and Sons, Hewlett-Packard), comparison of the mass spectra obtained for the samples with the mass spectra of the external standards, and quantified by comparison with an external standards calibration curve.

Calibration Curves

Calibration Curves for Fish Oil Collected by P&T, SPME and a-DHS

External standards used for calibration curves in the oil study (collection by P&T, SPME and a-DHS): butanal, 2-ethylfuran, 2-(*E*)-butenal, 1-penten-3-one, pentanal, 1-penten-3-ol, 2-(*E*)-pentenal, hexanal, 2-(*E*)-hexenal, heptanal, 4-(*Z*)-heptenal, 2,4-(*E*,*E*)-hexadienal, 2-heptanal, 2,4-(*E*,*E*)-heptadienal, 2-octenal, nonanal, 2,6-(*E*,*E*)-nonadienal, decanal, 2-(*E*)-decenal and 2,4-(*E*,*E*)-decadienal dissolved in rapeseed oil. The standard solution was diluted in rapeseed oil to obtain different concentrations of the volatile compounds ($n = 3$). Concentrations ranged from approximately 10 ng/g up to 4500 ng/g for evaluation of the short chain volatile standards up to (6–7 carbon), and 10 ng/g up to 200,000 ng/g for the long chain volatile standards (>6 carbon).

Calibration Curves for Emulsion Collected by P&T, SPME and a-DHS

External standards used for calibration curves in the emulsion study (collection by P&T, SPME and a-DHS): 1-penten-3-one, hexanal, 4-(*Z*)-heptenal, 2-pentylfuran, 2,4-(*E*,*E*)-heptadienal and nonanal supplied by Sigma Aldrich. The standards were dissolved in ethanol and added to an emulsion in concentrations from 10–1200 ng/g ($n = 3$).

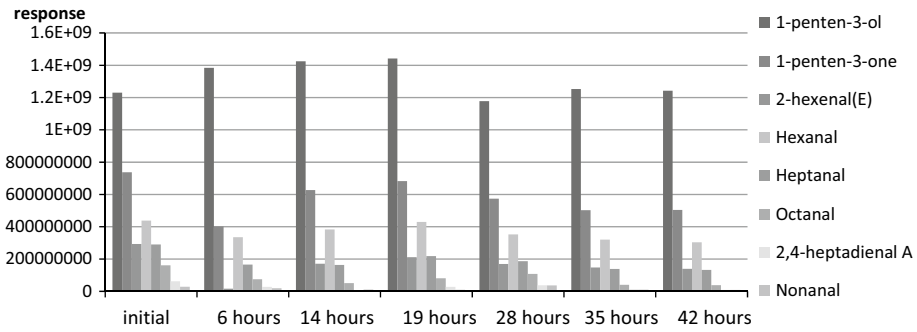

Fig. 2 The result of a standard solution analyzed with a-DHS collection of volatile compounds every 7 h for 42 h ($n = 1$)

Calibration Curves for Milk Collected by P&T, SPME and a-DHS

External standards used for calibration curves in the milk study (collection by P&T, SPME and a-DHS): 2-(*E*)-butenal, 1-penten-3-one, 1-penten-3-ol, 2-(*E*)-hexenal, hexanal, heptanal, octanal, 2,4-(*E*,*E*)-heptadienal and nonanal dissolved in milk (1.5 % lipid for P&T and 1 % for a-DHS and SPME) at a concentration of 10–500 ng/g (a-DHS up to 30,000 ng/g) ($n = 3$). For a-DHS and SPME, the calibration curves had to be parallel shifted to pass (0,0) to obtain positive concentration values.

Standard Addition Study Conducted for P&T and a-DHS Collection

For AO 1, AO 2 and AO 3 samples collected by P&T and a-DHS quantification of 2-ethylfuran was also performed by standard addition. These compounds were added to oils in three concentrations: 25, 125 and 250 ng/g. In all cases the two concentrations nearest to the amount in the sample were used for quantification. For example if a sample was calculated to have 50 ng/g by all three concentrations; only sample +25 ng/g and sample +125 ng/g were used for final quantification.

Effect of Storage Time in the Autosampler Prior to a-DHS Analysis

The standard solution containing1-penten-3-ol, 1-penten-3-one, 2-(*E*)-hexenal, hexanal, heptanal, octanal, 2,4-(*E*,*E*)-heptadienal and nonanal was added to milk (1 % fat) at 2000 ng/g and was used to investigate the effect of sample storage prior to analysis on a-DHS.

Statistical Analyses

Statistical analyses were performed with Graphpad prism version 6 (GraphPad Software, Inc., California, USA). Data presented in Figs. 4, 6 and 7 were analyzsed by a two-way ANOVA with a Tukey *post hoc* test. Data presented in Fig. 8 was analyzsed by a two-way ANOVA with a Bonferroni *post hoc* test. In this analysis, the row factor was collection method and column factor was sampling point.

Results and Discussion

Investigating Effect of Sample Storage Prior to Analysis on a-DHS

One unknown parameter in relation to a-DHS is the effect of storing the samples in the vials in the cooling tray at 10 °C of the autosampler for up to 24 h prior to analysis. In order to evaluate if it had an effect on the volatile response, a standard solution at 2000 ng/g dissolved in milk was analyzed approximately every 7 h for 42 h. The results are shown in Fig. 2.

In general, the changes in the volatile response during storage prior to analysis were only marginal, but there was to be a tendency towards a slight decrease in the response after 28 h of storage. Thereby, it is assumed safe to store the samples in the cooling tray at 10 °C for 24 h prior to analysis.

Calibration Curves: A Comparison of Linearity Range and Detection Limit

The calibration curves obtained by using the three different volatile collection systems varied widely both in range of the linearity of the curve and in the intersection with y-axis (Table 2). The results presented in Table 2 are marked as follows (1) Numbers in italics means that it may be possible to detect the volatile compound in lower concentrations than indicated (i.e. the linearity limit is lower) as we did not test lower concentration than the one indicated in the table, (2) Numbers in bold-face print means that it may be possible to detect the volatile compound in higher concentrations than indicated (i.e. the linearity limit is higher) as we did not test concentrations higher than that indicated in the table, and (3) Number without any changes in style mean the detection/linear limit. The calibration curves and the

Table 2 The linearity range of the calibration for oil collected with P&T, SPME and a-DHS, respectively

	P&T		SPME		a-DHS	
	ng/g		ng/g		ng/g	
	Min	Max	Min	Max	Min	Max
Butanal	*12*	650	*35*	<200	*35*	300
2-Ethylfuran	*14*	750	*40*	<200	*40*	2500
2-(*E*)-Butenal	*16*	875	*50*	1400	*50*	3000
1-Penten-3-one	*15*	750	*45*	1000	*45*	3000
Pentanal	*12*	750	*40*	<200	*40*	2500
1-Penten-3-ol	*12*	675	*40*	<200	*40*	**3500**
2-(*E*)-Pentenal	*15*	875	*45*	1400	*45*	**4500**
Hexanal	*13*	750	*40*	1000	*40*	**3800**
2-(*E*)-Hexenal	75	**1250**	*45*	2500	*45*	**4000**
Heptanal	80	**1250**	*50*	2500	*50*	**4500**
4-(*Z*)-Heptenal	70	**1250**	*40*	2000	*40*	**4000**
2,4-Hexadienal	90	**1600**	*55*	5000	*55*	**5500**
2-Heptenal	75	**1250**	*45*	2500	*45*	**4300**
2,4-Heptadienal a	170	50,000	*100*	40,000	*100*	**210,000**
2,4-Heptadienal b	170	12,500	*100*	20,000	*100*	**210,000**
2-Octenal	350	27,500	*50*	20,000	*50*	**210,000**
Nonanal	350	27,500	*50*	20000	*50*	**210,000**
2,6-Nonadienal a					1000	**200,000**
2,6-Nonadienal b	350	**50,000**	265	40,000	500	**200,000**
Decanal	350	37,500	250	40,000	1200	**200,000**
2-(*E*)-Decenal	350	**50,000**	600	45,000	1800	**230,000**
2,4-Decadienal a					5000	**190,000**
2,4-Decadienal b	950	**50,000**	1500	**95,000**	2750	**190,000**

(1) Numbers in italics means that it may be possible to detect the compound in lower concentrations than indicated (i.e. the linearity limit is lower), (2) Numbers in bold face means that it may be possible to detect the compound in higher concentrations than indicated (i.e. the linearity limit is higher) and (3) Number without any changes in style means detection/linear limit

detection limits, defined as a *s/n* ratio of 3:1, also depended on the sample matrix: oil, emulsion or water (milk). In the following, the main focus is on calibration curves collected from oil as a broader range of concentrations and volatile compounds were applied. Furthermore, most challenges were observed for oil. All calibration curves for oil are available in supplementary material, S1.

For oil, the linearity range and the detection limit for volatile standards collected with P&T, SPME and a-DHS varied depending on the chain length of the volatile standard (Table 2). When collected with P&T, volatile standards (C_4–C_9) with a short chain length (up to C_6) were possible to detect when diluted to approximately 15 ng/g, but we did not attempt to analyze lower concentrations. Therefore, the detection limit of these compounds was possibly below 15 ng/g. With a long chain length (C_7–C_9), detection was not possible with the low concentrations. Thus, the detection limit was higher for these volatile standards. In contrast to P&T, SPME and a-DHS could detect volatile standards with a chain length up to $C_{8–9}$ in concentrations from 50 ng/g. However, the detection limit increased dramatically for volatile standards with a chain length above C_8 and more than one double bond for all three methods. The linearity range of the volatile standards diluted in oil was the smallest for a short chain length and the largest for a long chain length using all three collection systems. However, a-DHS seemed to have the largest linearity area compared to P&T and SPME irrespective of the chain length of the volatile standard.

Butanal was a special case. Figure 3a–c shows the calibration curves for butanal dissolved in oil and collected with P&T, SPME, and a-DHS, respectively. P&T had a clear linearity between response and concentration over a large range (Fig. 3a), whereas both SPME and a-DHS had a challenge with butanal dissolved in oil (Fig. 3b, c). For SPME the response did not increase with an increase in concentration when the concentration was above 180 ng/g. This may be related to saturation of the fiber. A decrease

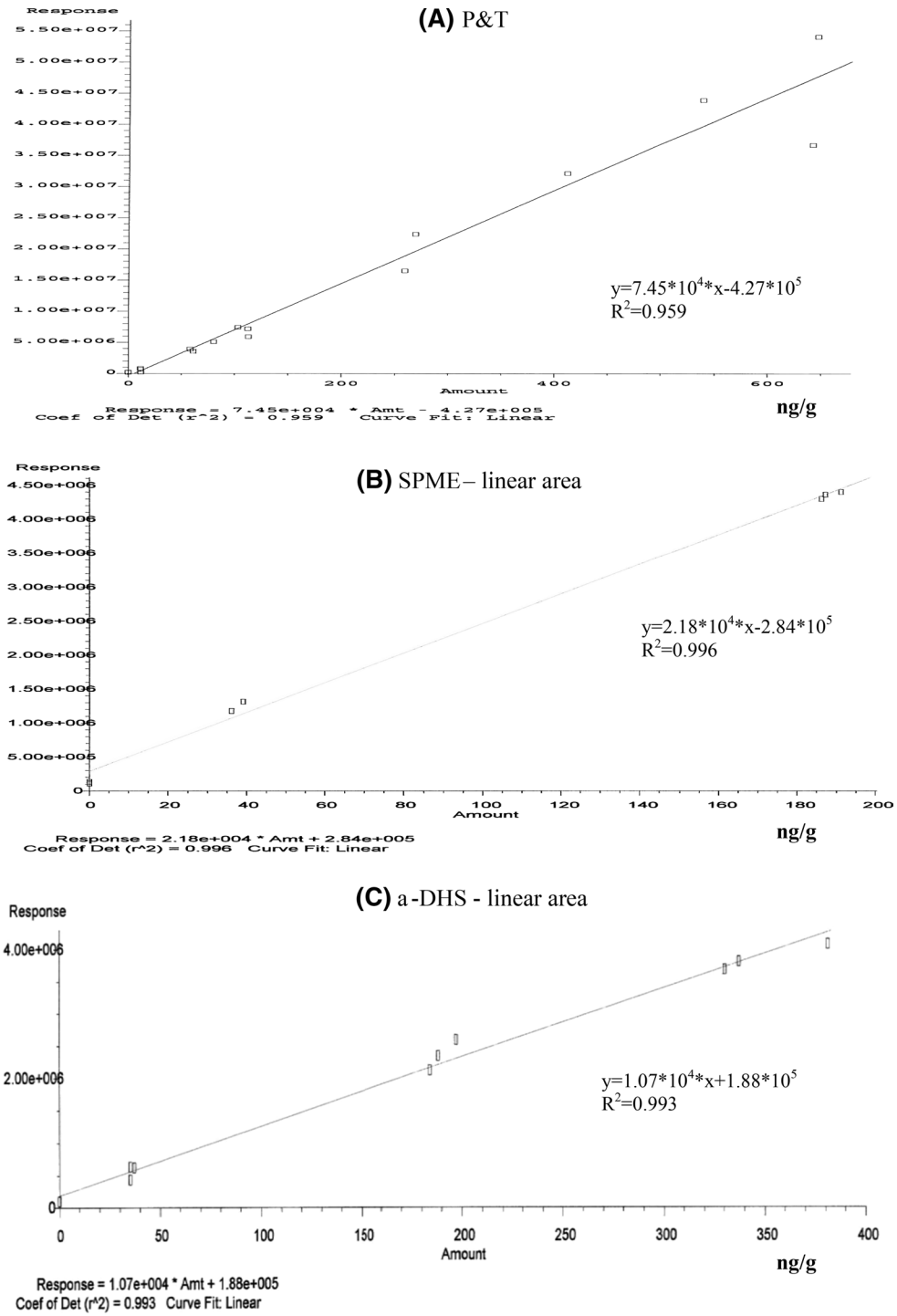

Fig. 3 The calibration curves obtained from butanal dissolved in oil analyzed by **a** P&T, **b** SPME corrected to linear fit by narrowing the concentration range and **c** a-DHS corrected to linear fit by narrowing the concentration range. The amount is ng/g

in response was observed for a-DHS. Linearity of the calibration curve was not observed for concentrations above 350 ng/g. This observation was surprising and it has not been possible to find a reasonable explanation. For both methods, linearity was only observed for the three lowest concentrations.

However, for a-DHS this problem was not observed for most of the other volatile compounds whereas this was the case for SPME. Hence, a-DHS only had problems with non-linearity within the concentration range tested for 5 out 20 volatile compounds; butanal, 2-ethyl furan, 1-penten-3-one and pentanal. In comparison to a-DHS, SPME had problems with non-linearity within the concentration range tested for 19 out 20 volatile compounds; butanal, 2-ethylfuran, 2-(*E*)-butenal, 1-penten-3-one, pentanal, 1-penten-3-ol, 2-(*E*)-pentenal, hexanal, 2-(*E*)-hexenal,

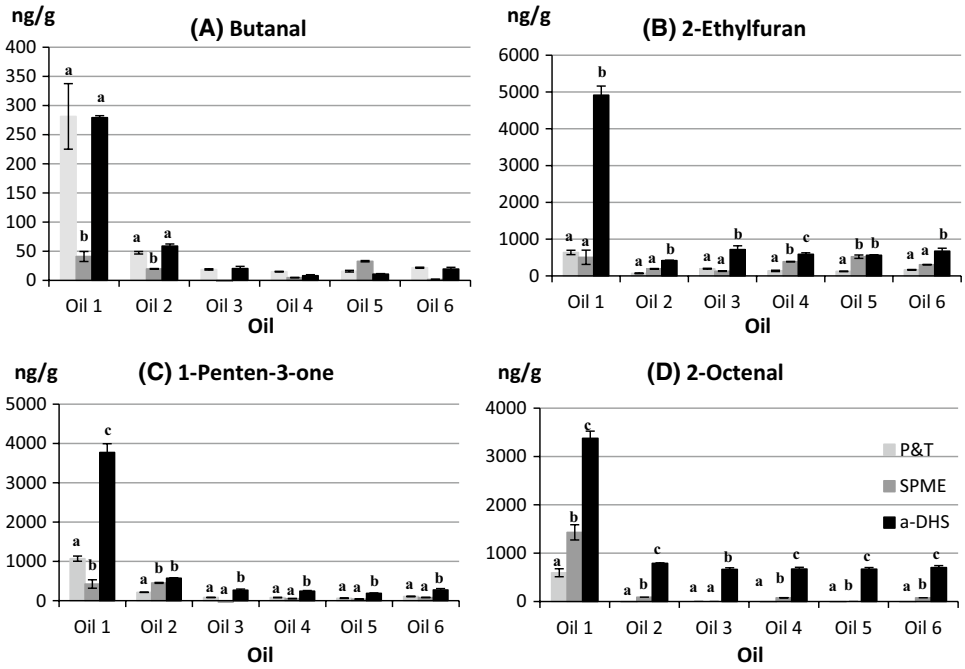

Fig. 4 Comparing volatiles from six different fish oils collected with P&T, SPME and a-DHS after 4 days of storage. The amount is an average of three measurements in ng/g and standard deviations are indicated by *error bars*. **a** Butanal, **b** 2-ethyl furan, **c** 1-penten-3-one and **d** 2-octenal

heptanal, 4-(*Z*)-heptenal, 2,4-(*E*,*E*)-hexadienal, 2-heptanal, 2,4-(*E*,*E*)-heptadienal, 2-octenal, nonanal, 2,6-(*E*,*E*)-nonadienal, decanal and 2-(*E*)-decenal.

For emulsion and milk, calibration curves were only evaluated in a narrow range (see supplementary material, S2). The linearity range for the volatile standards collected with SPME followed the pattern observed for volatile standards dissolved in oil (data not shown): volatile standards with short chain length had narrower linearity range than long chain length volatile standards. Moreover for emulsion, the linearity range for volatile standards dissolved in emulsion was not as wide as observed for oil.

The absolute sensitivity of the Hewlett Packard 5972MSD (used for P&T) and 5973MSD (used for SPME and DHS experiments) differs by a factor 2–3, with the 5973MSD as the most sensitive. This could have influenced the differences in LOD between P&T *versus* a-DHS and SPME. Comparison of LOD for the two most similar collection methods a-DHS and P&T does, however, not reveal a clear pattern. For C6–C9 aldehydes (exclusive of nonadienals), P&T had higher LOD than a-DHS whereas for nonadienal and longer chain aldehydes, the opposite was the case. Therefore, differences in sensitivity have most likely not played a significant role in the observed differences between the different collection methods.

General observations from all three food matrices were that SPME provided a smaller linearity range than both P&T and a-DHS. The calibration curves obtained with SPME were in some cases not suitable at all for a linear fit. Other authors have also reported that SPME has a small linearity range [19]. Lee *et al.* [20] observed detection limits ranging from 1×10^{-5} to 9.3×10^{-4} mg/kg for all volatile compounds. Volatile compounds with low molecular weight yielded relatively poor results using SPME GC–MS. Marquez *et al.* [21] used a-DHS GC–MS for the determination of aromatic esters in sweet wines and quantified them by using internal calibration curves prepared by diluting wines. These were prepared for each ester with linear regression equations having R^2 ranging from 0.9894 to 0.9981. In agreement with our study Lee *et al*- also had some challenges with low molecular weight volatile compounds [20, 22]. Moreover, Papastergiadis [23] had problems with recovery of volatile standards when using SPME for quantification of aldehydes from lipid oxidation in foods. A poor recovery may affect the calibration curves and cause linearity problems.

Another observation was that the calibration curves for the long chain volatile standards did not pass through (0,0) meaning that they either intercepted (x, 0) or (0, y). If the calibration curve intercepted the y-axis, samples with a small response were likely to result in negative concentrations. In contrast, if the calibration curve intercepted the x-axis, it was possible to obtain a positive concentration when the response from the sample was 0. In this study, both tendencies were observed for all three collection methods. However, the most often observed tendency for P&T was to pass (x, 0) and thereby provide a concentration even though the response was 0. In contrast to P&T, SPME and a-DHS had tendency to pass (0, y) and thereby provide a negative concentration for low responses. Marquez *et al.* [21] reported in contrast to most authors, the interception point with the y-axis for their calibration curves. The

authors reported the calibration curve's interception point with y-axis was ranging from 9.76×10^5 to 4.93×10^6. Their calibration curves did not pass through (0, 0) even though they had excellent R^2 which can result in the challenge described above.

Quantification and Comparison of Volatile Compounds Collected from Different Methods and Food Matrices

The differences observed in the calibration curves may affect the quantification of the samples. In order to investigate the effect of the collection method on the quantification of samples, concentrations of selected volatile compounds from the three food matrices were quantified during storage.

Comparison of Volatile Collection Methods on Oil Samples

The concentration of the volatile compounds was calculated based on a calibration curve using low concentrations of approximately 10–750 ng/g for P&T and 35–4500 ng/g for SPME and a-DHS of volatile standards dissolved in rapeseed oil for the short chain volatile compounds. The long chain volatile compounds were quantified by calibration curves with higher concentrations of volatile standards dissolved in rapeseed oil approximately 70–50000, 50–95,000 and 50–200,000 ng/g for P&T, SPME and a-DHS, respectively. Selected representative results for oil after 4 days of storage are shown in Fig. 4.

Especially for fish oil 1, SPME provided a lower concentration than both P&T and a-DHS for the volatile compound butanal (Fig. 4). Elmore *et al*. [11] compared SPME with P&T for collection of aroma compounds from cola and diet cola. They discovered that P&T extracted more volatile compounds than SPME. Povolo and Contarini [9] studied P&T and SPME for collection of volatile compounds from milk. They found that P&T provided higher concentration of volatile compounds than SPME. However, SPME collection resulted in a better extraction of the individual long-chain volatile compounds (extracted more).

In general, a-DHS collection provided a higher concentration of especially 2-ethyl furan, 1-penten-3-one and 2-octenal than the two other methods. Hence, a higher concentration was also observed for all volatile compounds except butanal and 2,4-(*E*,*E*)-hexadienal (data not shown). Moreover, it was possible to detect more volatile compounds with a-DHS compared to the two other collection methods (data not shown). However, the higher amount obtained of the volatile compounds by a-DHS collection needed further exploration to establish the reason.

The ranking of the samples with respect to concentration was the same for most volatile compounds. However, SPME had a different ranking of oil 1 and 2 for 1-penten-3-one compared with P&T and a-DHS. In contrast to P&T and a-DHS where oil 1 had the highest concentration, oil 2 had the highest concentration for SPME. The same was observed for 1-penten-3-ol and pentanal (data not shown). Different ranking of the samples was also observed for other volatile compounds; 2-(*E*)-butenal,1-penten-3-ol, 1-penten-3-one, pentanal, heptanal and 2-heptanal (data not shown).

To investigate further the reason behind the big differences in 2-ethyl furan concentrations obtained by P&T and a-DHS, a new experiment with fish oil was carried out. For this evaluation, the samples were collected by both P&T and a-DHS from a matrix that was added an antioxidant. The samples collected with a-DHS were quantified both by external calibration curves made in rapeseed oil as above and standard addition to the sample it-self, whereas only standard addition was used for P&T. The samples were analyzed in triplicates and for the standard addition 2-ethyl furan was added in three concentrations. Figure 5 shows the result of the experiment. It was clear that quantification by the external calibration curve provided a dramatically higher concentration than standard addition for a-DHS. The concentration obtained by standard addition was almost similar for P&T and a-DHS collection. These findings suggest that the higher concentration obtained for a-DHS for 2-ethyl furan was due to the use of external calibration curves instead of standard addition. It is speculated if the observed difference was due to slower absorption of volatile compounds from the rapeseed used for the calibration curves when collecting by a-DHS. However, the use of standard addition is more labor-demanding as the concentration added to sample must be near to the concentration of the quantified compound, otherwise the calculation of the concentration becomes unreliable. Thus, the slope obtained is highly dependent on the concentration range used. This study only investigated 2-ethyl furan. Studies on more volatile compounds are needed to confirm this hypothesis. Quantification using standard addition is recommended for a more reliable quantification.

Comparison of Volatile Collection Methods on Emulsion Samples

The concentrations obtained by SPME and a-DHS when volatile compounds were collected from the emulsion on day 15 are shown in Fig. 6.

Generally, SPME provided a higher concentration for five out of six volatile compounds than a-DHS, but there was not observed any competition problems. Even though SPME provided higher values, the ranking of the samples was the same independent of the collection method

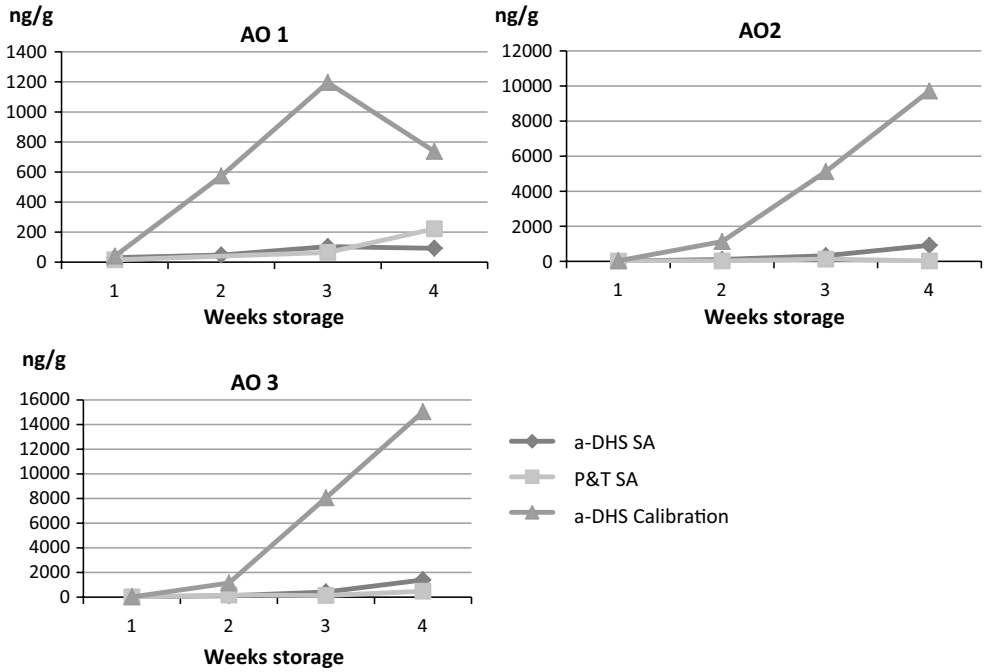

Fig. 5 The calculated amount of 2-ethylfuran in three fish oil samples with different antioxidant mixtures (AO 1, AO 2 and AO 3) collected by either P&T or a-DHS, and calculated using either SA (standard addition) or external calibration curve ($n = 1$)

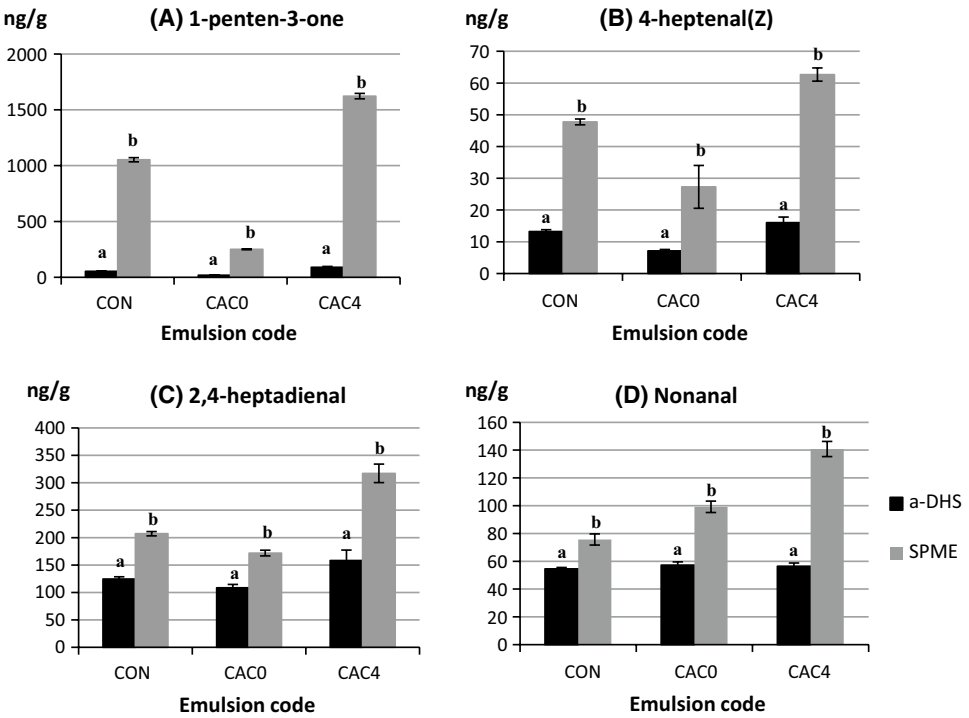

Fig. 6 Comparing concentrations of volatiles (ng/g) from emulsion collected with SPME and a-DHS after 15 days of storage. Emulsion codes: *Con* Control with no antioxidant added, *CAC0* Caffeic acid and *CAC4* Butyl caffeate. The amount is an average of three measurements

applied. The differences in concentrations obtained by the two collection methods deserve further study to understand the reason behind. It was not possible to compare results obtained by P&T in the study by Sørensen *et al*. [17], as volatile compounds in their study were not quantified by external calibration curves.

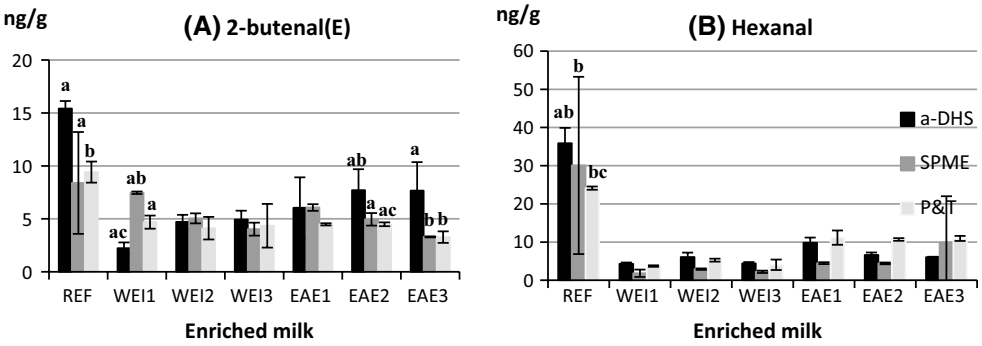

Fig. 7 Comparing volatiles (ng/g) from milk collected with, P&T, SPME and a-DHS after 12 days of storage. The milk emulsions contained two types of seaweed extract: ethyl acetate extract (EAE) and water extract (WEI). These were added in three concentrations low, medium and high marked as 1, 2 and 3 respectively. The amount is an average of three measurements

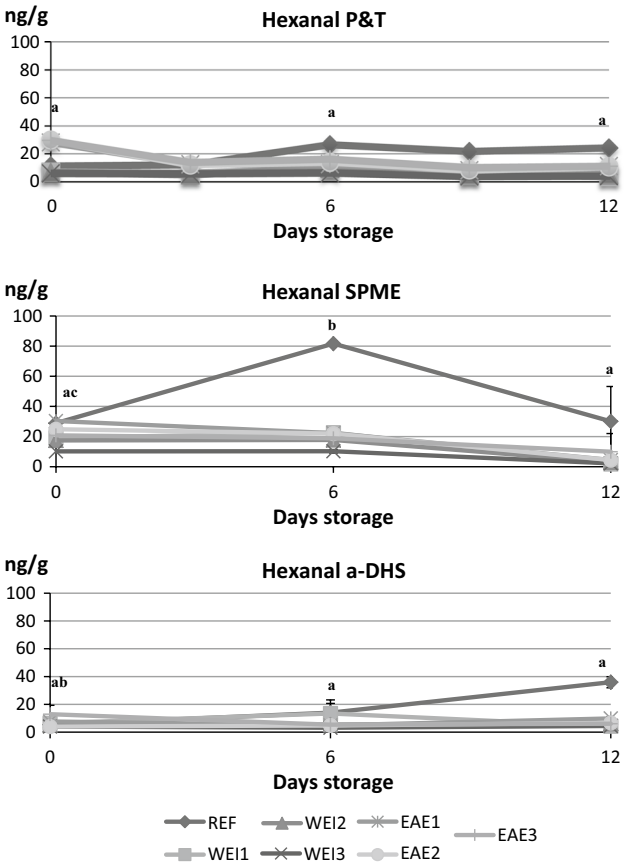

Fig. 8 The concentration of hexanal (ng/g) in fish oil enriched milk during storage. The amount is an average of three measurements. Data for P&T is adapted from Hermund *et al.* [18]

Comparison of Volatile Collection Methods on Milk Samples

The concentration of selected volatile compounds obtained by the three collection systems from milk stored for 12 days at 5 °C are shown in Fig. 7.

When comparing the concentrations of 2-*E*-butenal and hexanal obtained by P&T, SPME and a-DHS collection for each of the samples it was found that concentrations were generally in the same range. While the concentration on day 12 was almost the same for the different collection methods (Fig. 7), the development in hexanal concentrations during storage of the different products varied depending on the collection method (Fig. 8). On a reference sample, SPME collection showed a significant increase at day 6 and then a dramatic decrease at the end of the storage (day 12). In contrast, for both P&T and a-DHS collection, hexanal increased from days 6–12. This specific SPME decrease in hexanal concentration at day 12 is suggested to be due to competition problems on the SPME fiber, meaning that hexanal could not be adsorbed at the fiber at day 12 because other volatile compounds with higher affinity for the fiber had formed in high concentration in the reference sample. This finding was similar to those of Górecki *et al.* and Lu *et al.* [14, 19]. Overall, SPME collection resulted in lower concentration of 1-penten-3-one, 2-hexenal, heptanal and octanal than P &T and a-DHS (data not shown).

Conclusions

Our results showed that when using P&T, SPME and a-DHS for collection of volatile compounds from the three different food systems, the concentration ranges were, with a few exceptions, similar in each food system. Moreover, the three methods resulted in the same ranking of samples except for milk for which a fiber competition problem was apparently observed when using SPME. Therefore, SPME must be applied with caution, particularly in food matrices with a complex and variable composition. The use of a-DHS for collection of volatile compounds s resulted in detection of more volatile compounds s in the oil matrix than both the use of SPME and P&T. Since the collection temperature was similar for all three methods, we suggest this to be due to the shorter transportation routes in a-DHS compared to P&T; together with a more uniform sample preparation time before analysis of the individual samples. The advantages of a-DHS are thus that it both detected more volatile compounds s, and it did not seem to have a competition problem as observed for SPME.

Although P&T is a more validated method, which has provided reliable result for decades, the method requires a lot of manual work which is time consuming and expensive. Furthermore, the long time-frame from preparation to actual analysis (sample (stored until analysis) → TD (stored until analysis) → Automatic sampler with Thermal Desorption → GS–MS) may lead to the loss of some volatile compounds s or lower concentrations compared to the a-DHS (sample (stored until analysis) → TD → GC–MS). However, this study has also highlighted that even though SPME and a-DHS are time saving methods in terms of manual work, there are some issues which need to be addressed:

(1) The challenge regarding competition for adsorption on the SPME fiber observed for milk.
(2) The comparison of P&T, SPME and a-DHS methods provided useful information both regarding the limitation of the collection methods but also the use of calibration curves: a-DHS seemed to provide higher concentrations of volatile compounds s in oil compared to the two other methods applied. However, standard addition showed that this was most probably due to an overestimation provided by the use for external standard calibration curves for quantification. Although the easiest calibration is done with external calibration, our results indicated that standard addition may be a more correct way of calibrating such complicated matrices as food. This again points towards more automated methods as especially manual P&T is heavily demanding in the laboratory. On matrices analyzed as in this article the typical throughput will be around 10 samples/day for manually preparation, whereas the typical throughput with SPME/a-DHS is around 25/day. The mechanisms behind the finding that external calibration curves overestimated the response when a-DHS was used to collect volatile compounds s from oils needs to be further explored. This could be done by isotope dilution, but this would require tedious synthesis of a range of isotope volatile compounds oxidation products as no standards are commercially available.

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Appendix 9: Article IX

Research Article

Improving oxidative stability of liquid fish oil supplements for pets

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Omega-3 polyunsaturated fatty acids have produced beneficial health effects in animals and are recommended by veterinaries to pet patients suffering from osteoarthritis. However, these oils are highly susceptible to lipid oxidation. The objectives of this study were to improve oxidative stability of fish oil by adding vegetable oils, mixed tocopherols and rosemary extract, and to formulate a commercial product according to the results obtained. The formulated product was evaluated against commercial fish oil products. An initial screening for antioxidative effect was performed by using Oxipres equipment. The effect of antioxidant and vegetable oil blends was examined in oils stored at 30 and 40°C by measuring peroxide value, volatile compounds with GC-MS and tocopherol content. Addition of vegetable oil and rosemary extract at high level (4000–6000 ppm) plus 600 ppm of mixed tocopherols increased oxidative stability to the same extent as 2000 ppm mixed tocopherols in Oxipres. Overall, oxidative stability of fish oil or fish oil + vegetable oil blends was improved the most by addition of 5000 ppm rosemary extract and 500 ppm mixed tocopherols. A commercial oil blend with composition optimized based on the results of this study performed better than other commercial marine oils tested.

Practical applications: In some commercial oil blends for pets, a high level of vegetable oils is included in order to increase oxidative stability. In this study, vegetable oils are included at 30% level. At this level of vegetable oil inclusion, the omega-3 EPA and DHA content of the blends is at least 21% of total fatty acids for both fish and tuna oil based blends. In this study we wanted to examine, whether we could reduce the level of vegetable oil inclusion without compromising oxidative stability. This study demonstrates how the oxidative stability of omega-3 PUFA formulations for pets can be improved by combining fish oil with vegetable oils and by adding an antioxidant blend consisting of high concentrations of rosemary extract and tocopherol. The results are also of relevance to the manufacturers of dietary supplements.

Keywords: Omega-3 fatty acids / Peroxide value / Pets / Rosemary extract / Tocopherols / Vegetable oil / Volatile oxidation products

Received: November 30, 2016 / Revised: March 15, 2017 / Accepted: April 13, 2017

DOI: 10.1002/ejlt.201600492

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Abbreviations: AO1, 2000 ppm tocopherol; **AO2,** 2000 ppm rosemary extract plus 600 ppm tocopherol; **AO3,** 5000 ppm rosemary extract plus 500 ppm tocopherol; **DHA,** docosahexaenoic acid; **EPA,** eicosapentaenoic acid; **FO,** fish oil; **LO,** linseed oil; **PUFA,** polyunsaturated fatty acids; **PV,** peroxide value; **RO,** rapeseed oil; **RSI,** relative stability index; **SO,** sunflower oil; **TO,** tuna oil

1 Introduction

Marine omega-3 oils are among the most frequently purchased nutritional supplements for pets [1]. This is partly due to the fact that omega-3 supplements are very popular among people. Omega-3 supplements are used as dietary supplements to support health in people themselves, and therefore likely to be perceived health promoting also in pet animals.

A number of studies document beneficial health effects of fish oil (FO) omega-3 fatty acids in dogs and cats [2, 3].

Today, veterinarians recommend and prescribe foods and supplements comprising high levels of FO omega-3 fatty acids to their pet patients suffering from osteoarthritis. These recommendations are supported by clinical studies demonstrating improved mobility in arthritic dogs after inclusion of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) at 0.8–1.1% of diet dry matter for a period of 12–13 weeks [4–6]. This level of FO inclusion provides 2400–3200 mg/day EPA + DHA for a 20 kg dog.

Farmed and wild salmon as well as ocean fish and fish liver are the most common sources of omega-3 fatty acids in nutritional products for pets. Oil extracted from these marine sources is incorporated in complete diets for pets, both dry and wet. Marine oils are also sold in liquid form packaged in bottles. FO in extruded pet food is exposed to air and therefore at risk of being oxidized [7]. Liquid omega-3 supplements packaged in bottles may be better protected against the oxidizing effect of air and light than the oil incorporated in dry pet food, but there is no published data on the oxidative stability of omega-3 supplements sold to pets.

There is some concern that oxidized lipids and their by-products could be harmful to health [8–10]. Food safety authorities have commissioned risk assessment studies to evaluate the level of risk in humans associated with the consumption of oxidized FO [11, 12]. Due to the paucity of published studies, no firm conclusion on the risk associated with consumption of oxidized FOs in humans could be drawn. Studies in animals have typically been conducted with highly oxidized plant oils or animal fats. Only one study has been conducted in dogs. This study found that oxidized poultry fat depressed growth and impaired immune function as well as bone formation in dog puppies fed oxidized fat from 2 to 6 months of age [13].

Most people have an innate aversion for consuming rancid foods, which effectively self-limits their ingestion of food items that are oxidized. Rancid smell does not appear to produce a strong avoidance reaction in dogs. Given the prevalence of FO use in dogs and the lack of strong avoidance reaction to rancid oils, dogs may become exposed to high levels of oxidized lipids during their lives.

Previously it has been observed that adding vegetable oil to FO improves the oxidative stability of the blend compared to neat FO [14]. Similar findings have been observed when a mixture of rapeseed oil (RO) and FO was added to milk compared to when FO was added alone [15]. Combinations of antioxidants have been shown to provide synergistic effects in maintenance of oxidative stability of oils and emulsions [16–18]. Mixed tocopherols and rosemary extract are often combined for use in the oils and fats industry. The major constituents responsible for the antioxidant effects in rosemary extracts are carnosol and carnosic acid, but other diterpenes such as rosmarinic acid may also contribute to the antioxidant effect [19]. There are a few studies in the literature reporting on the antioxidant effect of a combination of rosemary extract and tocopherols in oils [19, 20]. However, to our knowledge there are no published studies that systematically have assessed the antioxidant effect of different doses of a combination of rosemary extract and mixed tocopherols in FO or in a mixture of FO and vegetable oil.

The first objective of this study was to improve the oxidative quality of FO-based nutritional supplement for dogs. This was done by examining the effect of adding vegetable oil, mixed tocopherols, and rosemary extract on oxidative stability of FO by using different analytical methods. The second objective of this study was to compare the oxidative stability of a commercial FO blend formulated according to the results obtained in the first part of this study against commercial FO products representing the different types of marine oils available on the market.

2 Materials

Rosemary extract (Fortium R20, Kemin Food Technologies, Herentals, Belgium), mixed tocopherols containing 14% of α and at least 56% of β, γ, and δ tocopherols (Tocovet M70®, Fenchem Biotek Ltd., Nanjing, China), FO HO307-9 (Lysi, Reykjavik, Iceland), tuna oil (TO) HT303-5 (Lysi, Reykjavik, Iceland), RO, sunflower (SO), and linseed (LO) oils were purchased locally. The peroxide value (PV), anisidine value, and tocopherol content of the oils are shown in Table 1 and fatty acid composition in Fig. 1.

Commercial marine oil products used were purchased locally in Finland. The oils were popular brands and

Table 1. Initial peroxide value (PV), anisidine value (AV), and tocopherol content of oils used (major isomer in bold).

	Quality			Tocopherols (mg/kg)			
Oil	PV (meq/kg)	AV (KOH/kg)	Total toco.	α	β	γ	δ
Fish oil	1.8 ± 0.0	3.3 ± 0.0	99.9 ± 0.6	**99.9 ± 0.6**	ND	ND	ND
Tuna oil	2.4 ± 0.1	3.7 ± 0.1	386.2 ± 13.9	**205.5 ± 10.3**	3.4 ± 1.3	137.1 ± 1.9	40.2 ± 0.3
Linseed oil	2.1 ± 0.1	0.5 ± 0.0	577.3 ± 7.7	13.0 ± 1.7	144.9 ± 2.3	**409.2 ± 3.2**	10.2 ± 0.4
Rapeseed oil	8.4 ± 3.6	0.3 ± 0.0	593.9 ± 8.6	192.0 ± 2.2	53.7 ± 0.8	**338.6 ± 5.5**	9.6 ± 0.1
Sunflower oil	3.6 ± 0.2	1.0 ± 0.0	610.3 ± 17.8	**579.5 ± 14.1**	17.8 ± 0.8	13.0 ± 3.0	ND

$N = 2$. ND, not detected.

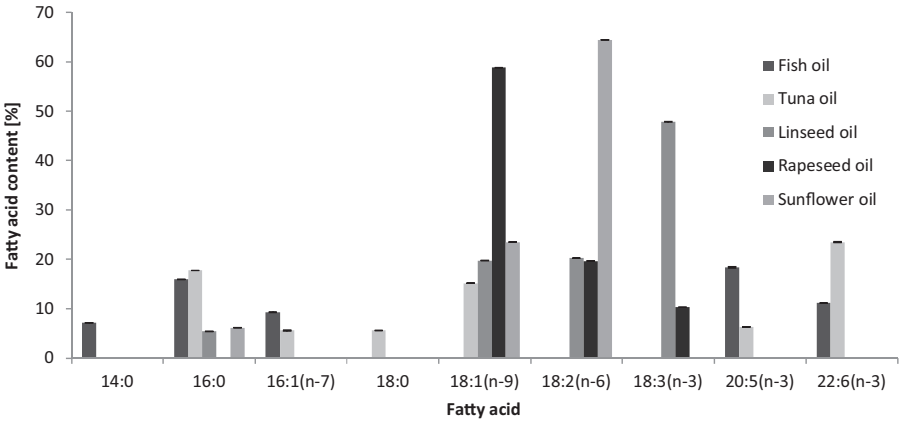

Figure 1. Fatty acid composition of fish, tuna, linseed, rapeseed and sunflower oils for fatty acids present above 5 % (%fatty acid of total fatty acids with C12–24).

represented the most common types of marine oils available on the market. In this set of marine oils, there were three salmon oils: one sourced from farmed salmon (farmed salmon oil), one sourced from wild Alaskan salmon (wild salmon oil), and one sourced from farmed salmon, but having an omega-3 content higher than typically found in farmed salmon oil (premium salmon oil). There were also three FO products, one based on standard 18/12 FO with low level of α-tocopherol (115 μg/g). One based on concentrated FO (concentrated FO) and one comprising a blend of FO + vegetable oil with the most effective antioxidant combination tested in this study (optimized blend). Commercial marine oil products were purchased in duplicate in June 2015. All the oils tested, except for one, had 1 year or more until the best before date. Key compositional data of the oils are provided in Table 2.

We used 18/12-type fish oil (FO, Lysi HO307-9) and tuna oil (TO, Lysi HT303-5) in the optimization study (Table 1). In addition, we used a commercial product consisting of 18/12-type fish oil (Table 2) and low level of tocopoherol (115 ppm α-tocopherol) in the storage stability experiment performed at 40°C.

3 Methods

The study consists of three separate experiments. In the first experiment, we used Oxipres equipment to study oxidative stability of FO as influenced by additions of vegetable oil and antioxidants (Oxipres experiment). In some commercial fish oil blends, vegetable oils are included at 30% level. At this level of vegetable oil inclusion, the omega-3 EPA and DHA content of the blends is at least 21% of total fatty acids for both FO- and TO-based blends used in this study. We aimed to examine, whether we could reduce the level of vegetable oil inclusion without compromising oxidative stability. In the second experiment, we studied oxidative storage stability of FO and FO blends as influenced by additions of antioxidants. This experiment was performed on samples incubated at 40°C for 14 days (Storage stability at 40°C). In the third experiment we studied oxidative storage stability of commercial marine oil samples incubated at 30°C for 14 days (Storage stability at 30°C). The oxidative stability was compared by relative stability index (RSI) which is calculated as (induction time for sample − induction time for neat fish oil)/induction time for neat fish oil × 100%.

Table 2. Commercial marine oil products purchased in Finland in June 2015

				% of total FA	
Product type	Best before	Total tocopherols (mg/kg)	Major tocopherol, % of total (α or γ)	EPA	DHA
Farmed salmon oil	1/2016	137	60 (γ)	2.05	2.65
Premium salmon oil	7/2016	648	70 (α)	6.78	8.11
Wild salmon oil	10/2016	470	43 (γ)	10.75	12.16
Fish oil	5/2017	115	100 (α)	18.86	10.66
Concentrated fish oil	1/2017	3717	78 (α)	37.76	20.85
Optimized blend	10/2016	1099	49 (γ)	13.20	8.29

3.1 Oil and antioxidant blends

The effect of adding vegetable oil in various concentrations to FO on oxidative stability of the blend was explored. The following proportions of oils were tested: 70–100% FO/TO and 0–30% RO/SO. Maximum level of vegetable oil was limited to 30% based on nutritional considerations. Sunflower oil (SO), linseed oil (LO), and rapeseed oil (RO) provide linoleic acid and α-linoleic acid in 2–1 ratio. This was considered as a potentially useful nutritional target. After testing oil without antioxidants, we chose to examine the effect of antioxidant blends consisting of 1000, 2000, 4000, and 6000 ppm of rosemary extract combined with 600 ppm mixed tocopherols on induction time of FO and FO + vegetable oil blends. In addition, a treatment consisting of 2000 ppm of mixed tocopherols alone was included in the test as a positive control. The purpose was to identify the most efficient level of rosemary extract in the antioxidant blend by determining the relative increase in the Oxipres induction time. The five antioxidant blends were mixed with either FO or blends consisting of 70% FO and 30% vegetable oil. In addition to RO, we included a 2:1 mixture of SO and LO in the test. SO and LO were included in the test because the blend was interesting for product formulation reasons.

3.2 Oxipres

Oxipres^{TM} (Mikrolab Aarhus, Denmark) method was used as an initial step in screening a large number of different oil and antioxidant formulations. Our aim was to identify a more manageable number of oil and antioxidant blends for further storage stability testing using a set of analytical methods in assessment of the level of oxidation in oils.

Approximately, 5 g of oil was added to a 200 mL reactor, which was connected to a pressurized oxygen container. The reactor was flushed and filled with oxygen three times in order to remove atmospheric air. The initial pressure was set to approximately 5 bars and the temperature was constant at 50°C for neat oil and 80°C for oil blends. The induction time was determined as the crossing point of the tangents to the curve. The oxygen consumption during incubation, i.e., the pressure was measured once every minute. The analysis was made in triplicate.

Results from this initial screening using Oxipres were used in part to formulate the oil blends and deciding on the levels of antioxidant inclusion for a 14 days storage experiment.

3.3 Storage experiment at 40°C

Antioxidant and oil blends were incubated at 40°C under magnetic stirring and semi-open conditions (covered with tin foil) during 14 days with samples taken after 0, 4, 9, and 14 days of storage. Samples were stored at −40°C until they were analyzed for PV, tocopherols, and volatile compounds.

Motivation to perform these experiments was to improve oxidative stability of commercial FO-based nutritional oils for pets. These commercial oils are blends consisting of FO and vegetable oil (RO or SO + LO) plus 2000 ppm mixed tocopherols. A set of FO and vegetable oil blends was produced in the laboratory based on the results from the first experiment with Oxipres and the specific levels of rosemary extract + tocopherol were decided based on prior use in commercial products (2000 ppm mixed tocopherols), manufacturer's recommendation of maximum inclusion level (2000 ppm rosemary extract), and cost of formulation considerations (5000 ppm rosemary extract + 500 mixed tocopherols equal in cost with 2000 ppm mixed tocopherols). Composition of the oil and antioxidant blends are presented in Table 3.

3.4 Storage experiment at 30°C

Results from the storage experiment carried out at 40°C suggested that this temperature may be too high to be representative for oxidation reactions occurring at lower temperature. Therefore, the second storage experiment was conducted at 30°C. Six commercial marine oils were included in the experiment in duplicates. The oils were incubated at 30°C under magnetic stirring and semi-open conditions

Table 3. Composition of oil and antioxidant blends tested in storage experiment at 40°C

Name	Oil blend	Rosemary (mg/kg)	Tocopherol (mg/kg)
FO + AO1	100% fish oil	0	2000
FO + AO2	100% fish oil	2000	600
FO + AO3	100% fish oil	5000	500
FO + RO + AO1	70% fish oil, 30% rapeseed oil	0	2000
FO + RO + AO2	70% fish oil, 30% rapeseed oil	2000	600
FO + RO + AO3	70% fish oil, 30% rapeseed oil	5000	500
FO + SO + LO + AO1	70% fish oil, 20% sunflower oil, 10% linseed oil	0	2000
FO + SO + LO + AO2	70% fish oil, 20% sunflower oil, 10% linseed oil	2000	600
FO + SO + LO + AO3	70% fish oil, 20% sunflower oil, 10% linseed oil	5000	500

(covered with tin foil) during 14 days. Samples were taken after 0, 4, 9, and 14 days of storage and stored at −40°C until they were analyzed for PV, tocopherols, and volatile compounds.

3.5 Peroxide value

PV was determined at all sampling points in duplicates. This was done as described by Shantha and Decker [21]. The method is based on the formation of a colored iron–thiocyanate complex. The colored change was measured spectrophotometrically on Shimadzu UV1800 (Shimadzu Scientific Instruments, Columbia, USA) at 500 nm.

3.6 Tocopherol content

The content of α-, β-, γ-, and δ-tocopherols was determined according to AOCS method [22] in duplicates. In short, the oil blends were dissolved in heptane and analyzed by HPLC Agilent 1100 Series (Agilent Technology, CA, USA) with a fluorescence detector. These tocopherol homologues were separated using a silica column (Waters, Dublin, Ireland, 150 mm, 4.6 mm, 3 mm silica film). Retention time and the peak area of each tocopherol homolog were determined in a stock solution added 10 mg tocopherols (mixture of α-, β-, γ- and δ-tocopherols) per liter. The peak areas of the stock solution were used to quantify the tocopherol content in the samples.

3.7 Quantification of volatile compounds

Analysis was performed as described by Thomsen et al. [23] for oils. In short, volatile compounds were automatically collected and injected by an automatic-TDU/DHS system from Gerstel (Gerstel GmbH & Co. KG., Mülheim an der Ruhr, Germany) and detected on GC HP 6890-MS HP 5973 (Agilent Technologies).

3.8 Statistics

The data were analyzed statistically using Graph pad prism version 7 (graph pad, La Jolla, USA). A Bonferroni multiple comparison test was employed to evaluate significant changes between samples at each sampling point. The significance level was 0.05, the significant differences are denoted in the Tables and Figures.

4 Results and discussion

4.1 Oxipres experiment

The initial screening using Oxipres was performed in order to reduce the number of oil and antioxidant blends for further testing in storage experiments at 40°C. The screening was executed in two steps; 1) test of vegetable oil and FO blends and 2) optimization of the antioxidant blend based on rosemary extract and mixed tocopherols.

Table 4. Induction time for neat oil blends of fish oil (FO) + rapeseed oil (RO) and tuna oil (TO) + sunflower oil (SO) at 50°C. The level of significant difference is 0.05

	Induction time (minutes)			
	FO + RO		TO + SO	
Fish oil:vegetable oil	ave ± std	RSI, %	ave ± std	RSI, %
100:00:00	2425 ± 35^{a}	–	3900 ± 141^{a}	–
95:05:00	2900 ± 71^{b}	20	4100 ± 0^{ab}	5
90:10:00	2950 ± 71^{b}	22	4425 ± 35^{bc}	13
80:20:00	4050 ± 71^{c}	67	4500 ± 212^{c}	15
70:30:00	5050 ± 71^{d}	108	5263 ± 53^{d}	35

The level of significant difference is 0.05.
Relative stability increase (RSI) obtained by anti-oxidant mixture was calculated.

Increasing the proportion of vegetable oil in the blend increased induction time (Table 4). Induction time was doubled as indicated by the 108% increase in relative stability index (RSI) by increasing the proportion of RO in FO to 30%. Adding 30% SO to TO also produced the largest increase in induction time. However, the relative increase in induction time was lower for additions of SO to TO. It is noteworthy that in this experiment the induction time was higher for neat TO compared to neat FO in spite of the fact that the level of unsaturation was higher in TO compared to FO due to its higher DHA content (Fig. 1) and in spite of the fact that the TO had a slightly higher PV than the FO (Table 1). Higher tocopherol content in TO (386 mg/kg) compared to FO (100 mg/kg) might explain the apparent increase in oxidative stability. Higher relative stability increase was obtained by RO addition to FO compared to that obtained by addition of SO to TO. This finding may be related to the lower degree of unsaturated fatty acids and the higher level of γ-tocopherol in RO than SO. The fact that RO had a higher PV than SO did not override the positive effects of the fatty acid composition and γ-tocopherol. Other studies have also found a protecting effect of mixing vegetable oils with FO. Let et al. [15] compared the oxidative stability of milk enriched with FO or with a mixture of FO and RO (1:1). They found that RO had a protective effect against lipid oxidation that exceeded that of a simple dilution of the FO. In agreement with the hypothesis proposed above, they suggested that the protective effect of RO was due to its high content of tocopherols with a favorable composition. They also proposed that minor constituents of the RO such as sinapic acid could have had a protective effect.

Based on these Oxipres results, we decided to use the highest level of vegetable oil inclusion (30%) in further testing.

Table 5. Induction time for fish oil (FO), fish oil + rapeseed oil (FO+RO) and fish oil + sunflower oil + linseed oil (FO + SO + LO) with increasing concentrations of rosemary extract (RE) and fixed (600 ppm) concentration of mixed tocopherols (MT) or 2000 ppm MT alone at 80°C

Oils							
Induction time (min)	FO: 100%		FO: 70% RO: 30%		FO: 70% SO: 20% LO: 10%		
Antioxidants	ave ± std	RSI	ave ± std	RSI	ave ± std	RSI	Rank
Control	175 ± 7^{a}	–	410 ± 14^{a}	–	320 ± 7^{a}	–	–
RE: 1000 ppm	788 ± 4^{b}	350	878 ± 4^{b}	114	783 ± 25^{b}	145	4–5
RE: 2000 ppm	702 ± 40^{b}	301	965 ± 14^{bc}	135	805 ± 14^{b}	152	4–5
RE: 4000 ppm	888 ± 11^{c}	407	1005 ± 14^{c}	145	1050 ± 14^{d}	228	2–3
RE: 6000 ppm	1030 ± 14^{d}	489	1175 ± 0^{e}	187	1118 ± 11^{d}	249	1
MT: 2000 ppm	913 ± 18^{c}	421	905 ± 21^{bc}	121	940 ± 14^{c}	194	2–3

No antioxidants were added to the control oil. Relative stability increase (RSI) obtained by anti-oxidant mixture was calculated and the effect was ranked.

Similar to the first Oxipres experiment at 50°C, addition of vegetable oil increased induction time at 80°C (Table 5). The first experiment was run at 50°C and the second at 80°C in order to further reduce the analysis time. Reduction in test temperature did not seem to influence the magnitude of vegetable oil's ability to improve FO stability. The effect of adding RO to FO was somewhat greater than adding SO + LO to FO.

In general, the oxidative stability increased with increasing concentrations of rosemary extract. For each oil type, the largest increase in RSI was observed when 6000 ppm rosemary extract plus 600 ppm mixed tocopherols were added followed by 2000 ppm mixed tocopherols added alone and 4000 ppm rosemary extract plus 600 ppm tocopherol. The ability of rosemary extracts to increase oxidative stability in neat oils is in accordance with several studies reported in the literature. For example, Frankel et al. [24] reported an antioxidative effect in neat oil of rosemary extract when added in a concentration of 500 ppm, whereas the effect of 250 ppm rosemary extract was more unclear. Many of the studies reported in the literature have used lower concentrations of rosemary extracts than the ones used in the present experiment. However, Kendrick and Macfarlane [25] evaluated the effect of 2000 ppm rosemary extract in combination with 200 ppm ascorbyl palmitate and 1000 ppm mixed tocopherol in FO by Rancimat at a temperature of 100°C and found that the antioxidant addition increased the induction period from 1.7 to 6.2 h. In their study, the antioxidants were added during the deodorization of the oil.

In the present study, antioxidants had the smallest effect when added to the FO:RO mixture compared to addition to FO:SO:LO and the largest effect when added to the pure FO. Chen et al. [26] evaluated the antioxidative effect of 200 ppm rosemary extracts in canola oil and found that its efficacy was low. Although the study by Chen et al. [26] only evaluated a low concentration of rosemary extract, these results could indicate that addition of an oil with high γ-tocopherol content such as RO could negatively influence the ability of rosemary extract to inhibit oxidation. However, it cannot be ruled out that an even higher increase in RSI would be obtained if higher concentration of rosemary extract was used.

Previous studies have shown that high level of α-tocopherol added to FO (2000 ppm) had a pro-oxidant effect compared to FO without any antioxidants added at 20°C [27]. Kulås and Ackman [28] found that the efficacy of α-tocopherol decreased when added to purified FO in amounts greater than 100 ppm, inversion of activity for γ-tocopherol occurred at 500 ppm level whereas activity of δ-tocopherol increased to additions up to 1500–2000 ppm. Jung and Min [29] observed that the optimal concentrations of α-, γ-, and δ-tocopherols in soybean oil were 100, 250, and 500 ppm, respectively. In this study, the tocopherol mixture used contained 17% α-tocopherol, 1% β-tocopherol, 53% γ-tocopherol, and 29% δ-tocopherol. We did not observe any prooxidative activity of 2000 ppm mixed tocopherol addition, despite the fact that the concentration of α-tocopherol was 340 ppm and that of γ-tocopherol 1060 ppm, which were well above the optimal levels suggested in previous studies. However, it cannot be ruled out that the efficacy of mixed tocopherol would have been higher if a lower concentration had been applied. Nevertheless, the concentration of 2000 ppm of mixed tocopherols was selected on the basis of prior use in commercial liquid oil products since the of improvement of their oxidative stability was the target in this study.

4.2 Storage stability at 40°C

The motivation to perform these experiments was to improve oxidative stability of commercial FO-based nutritional oils for pets (currently marketed under Nutrolin® brand). These

commercial oils were blends consisting of FO and vegetable oil (RO or SO + LO) plus 2000 ppm mixed tocopherols. Composition of the antioxidant blends used in this experiment was based partly on efficacy (Oxipres experiment) and partly on cost of formulation considerations. Mixed tocopherols added to FO or FO blend at 2000 ppm was used as a commercial reference (positive control) and the effect of adding 2000 or 5000 ppm rosemary extract together with a low amount tocopherol (500 or 600 ppm) was studied.

Data on changes in PV during storage at 40°C is summarized in Fig. 2. PVs increased over time with no consistent differences between added antioxidants at the end of the storage period (Fig. 2a, b, and c). Hence, there were no differences in PV when antioxidants were added to neat FO or FO + SO + LO blend (Fig. 2a and c). Addition of only mixed tocopherols to FO + RO resulted in higher PV compared to addition of 2000 or 5000 ppm rosemary extract together with 500 or 600 ppm tocopherol (Fig. 2b). Comparison of the PV responses between different FO blends suggests that the profile of PV response depended on the composition of the oil blend. PV followed a clear zig-zag pattern for the neat FO while the FO blended with SO and LO (FO + SO + LO) resulted in a rather smooth PV response. The FO + RO blend showed a response that was somewhere in between the other two responses. Other studies on the effect of rosemary extract on PV formation in vegetable and FOs did not show the zig-zag pattern observed for neat FO [24, 26, 37]. In the study by Frankel and Huang [31], two different types of rosemary extracts with different levels of carnosol and carnosic acid were evaluated in FO in concentrations from 300 to 1000 ppm during storage at 60°C. Both extracts significantly reduced formation of primary oxidation products measured as conjugated dienes compared to a control sample without antioxidants. The extract with the highest concentration of carnosic acid and carnosol was the most effective and for both extracts the largest antioxidative effect was observed when the extract was added in the highest concentration. The present data do not allow us to suggest an explanation for why there was no clear effect of increasing the rosemary extract concentration on PV in the present study. Rosemary extracts have also improved the oxidative stability during storage of cooked salmon and smoked trout determined as decreased PV [32, 33].

FO blends used in the storage experiment contained variable amounts of total tocopherols. The three oil blends with only mixed tocopherols added (AO1) contained an average of 2057 mg/kg total tocopherols (from 2024 to 2100 mg/kg) and the other six blends with AO2 and AO3 added contained an average of 672 mg/kg total tocopherols (from 620 to 714 mg/kg). Figure 3 shows that the tocopherols in the FO blend with AO1 were consumed very rapidly. In 9 days, 86% of the tocopherol in the product was used up and by day 14 all the tocopherol was gone. Consumption of the tocopherols was much less rapid in the oil blends with AO2 (2000 ppm of rosemary plus 600 ppm of tocopherol) added

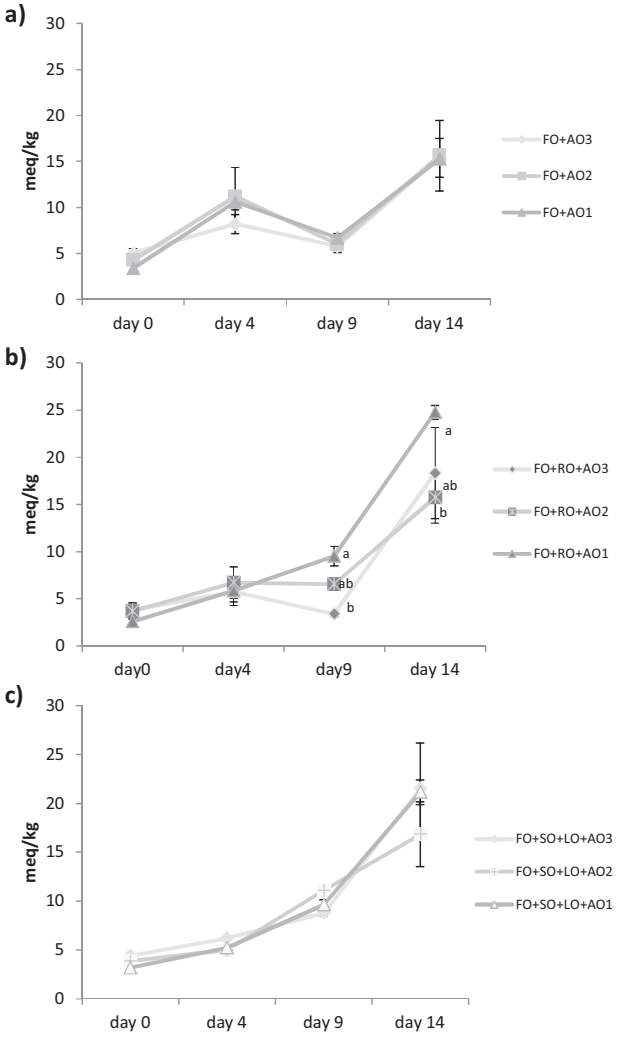

Figure 2. Effect of antioxidant addition on peroxide value in fish oil (a), 70% fish oil + 30% rapeseed oil (b) and 70% fish oil + 20 % sunflower oil and 10% linseed oil (c) during 14 days of storage at 40°C.

with only about one third used by day 9. During the last 5 days of the storage test, the tocopherols in AO2 oils were also completely consumed. In oil blends with AO3 added (5000 ppm rosemary extract plus 500 ppm tocopherol), the total tocopherol content remained stable throughout the experiment. Adding vegetable oil to FO did not have any appreciable effect on the tocopherol consumption. These findings clearly showed that compounds present in the rosemary extract were able to prevent the consumption of tocopherol. This could either be due to the ability of carnosol and carnosic acid to regenerate tocopherol or due to the fact that they were consumed at the expense of tocopherols. Interestingly, these findings are contradicting results reported by Hopia et al. [19]. They studied the effect of addition of α-tocopherol and carnosol or carnosic acid, all

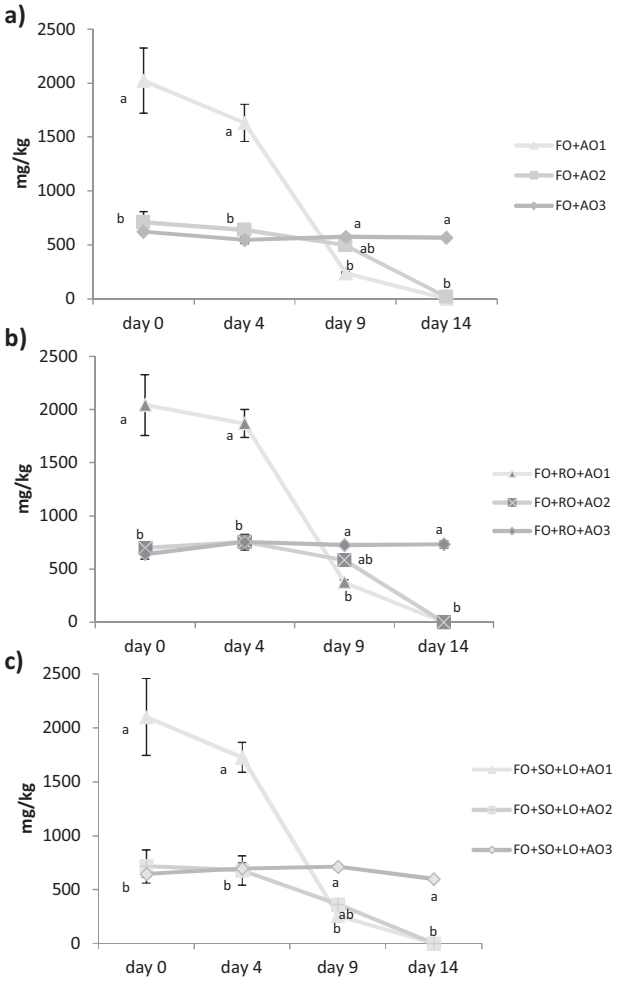

Figure 3. Effect of antioxidant addition on total tocopherol content in fish oil (a), 70% fish oil + 30% rapeseed oil (b) and 70% fish oil + 20% sunflower oil and 10% linseed oil (c) during 14 days of storage at 40°C.

added in a concentration of either 150 μM (= 50 ppm carnosol/carnosic acid, 65 ppm α-tocopherol) or 300 μM (= 100 ppm carnosol/carnosic acid, 130 ppm α-tocopherol) to SO either alone or in combination. The SO was oxidized at 60°C. They found that pure carnosol was more efficient in inhibiting hydroperoxide formation than a combination of carnosol and tocopherol. It was also observed that α-tocopherol decreased the oxidative stability of carnosol and that carnosol decreased the stability of α-tocopherol. Similar findings were observed when carnosic acid was used in combination with α-tocopherol. However, carnosic acid was able to delay the disappearance of tocopherol from 5 to 7 days. It was suggested that carnosic acid may be able regenerate tocopherol or that it had a sparing effect on the consumption of tocopherol by being oxidized at the expense of tocopherol.

Wada and Fang [34] studied the synergistic effect of α-tocopherol (500 ppm) plus rosemary extract (200 ppm) in oil extracted from sardine using the Bligh and Dyer extraction. In agreement with the findings from the present study, they observed that the rosemary extract delayed the disappearance of tocopherol from 10 to 15 days when the oil was stored at 30°C. They also found that rosemary extract and tocopherol acted synergistically when evaluated by PV. They suggested that compounds in the rosemary extract regenerated tocopherol. When storage temperature was increased to 60°C the synergistic effect of the rosemary extract weakened, perhaps due to a low heat stability of the antioxidant compounds in the rosemary extract.

A total of 12 different secondary volatile oxidation products were monitored during the storage experiment. Six of these were identified as products of omega-3 PUFA oxidation and included in the sum of omega-3 volatiles used when comparing the oxidation of different oil and antioxidant blends. The total n-3 volatiles was representative of the development of the individual volatiles originating from n-3 PUFA. These volatile omega-3 PUFA oxidation products included 2-ethylfuran, 1-penten-3-one, 1-penten-3-ol, 2-hexenal (E), 2,4-hexadienal, and 2,4-heptadienal (E,E) [35, 36].

Figure 4 with three panels shows the effect of added antioxidants in different FO + vegetable oil blends. In general, addition of 2000 ppm tocopherol (AO1) or 2000 ppm rosemary extract plus 600 ppm tocopherol (AO2) was associated with formation of rather high levels of omega-3 volatiles whereas the addition of 5000 ppm rosemary extract plus 500 ppm tocopherol (AO3) was associated with formation of very low levels of omega-3 volatiles. The increase in omega-3 volatiles in FO + AO1 followed a peculiar pattern: initial rapid increase in volatiles between day 4 and 9 was followed by an equally rapid decline in omega-3 volatiles formed. The broken stick response pattern suggests that the secondary volatile oxidation products were rapidly converted to tertiary oxidation products after day 9 and the balance of oxidation products formed had moved beyond the accumulation of the secondary volatiles. This finding indicates that in FO with AO1, secondary oxidation products decomposed faster into tertiary oxidation products than in FO with AO2 and AO3. The broken stick pattern was not observed for AO1 when mixtures of FO and vegetable oils were used (Fig. 4b and c). Comparison of concentrations of total omega-3 volatiles in oils with AO1 at day 9 clearly showed that the neat FO had the highest concentration followed by the mixture with SO and LO, whereas the lowest concentration was found in the mixture with RO.

Figure 4 showed that in all oils 5000 ppm rosemary extract plus 500 ppm tocopherol was the most efficient antioxidant mixture as it almost completely prevented formation of omega-3 volatile oxidation products during the entire storage period. This finding is in agreement with the results from the tocopherol measurements and confirmed

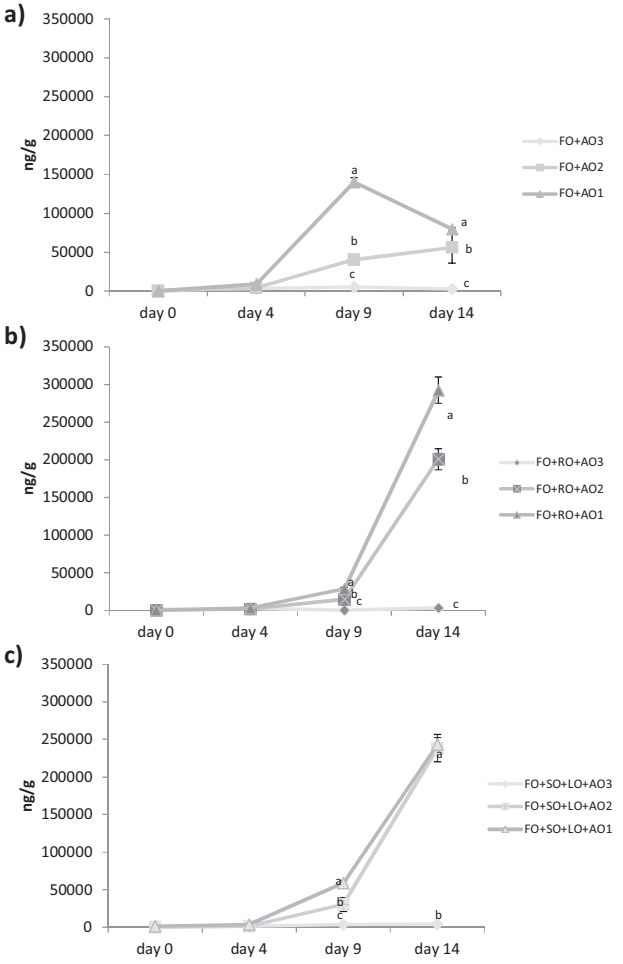

Figure 4. Effect of antioxidant addition on formation of secondary volatile oxidation products from omega-3 fatty acids in fish oil (a), fish oil + rapeseed oil blend (b) and in fish oil + sunflower oil + linseed oil blend (c) during 14 days of storage at 40°C.

that rosemary extract and tocopherol are strong antioxidants when added in high concentrations. The strong effect of the two antioxidants together could be due to the ability of the constituents in rosemary extract (primarily carnosol, carnosic acid) to regenerate tocopherol or because these compounds have a tocopherol sparing effect. Our data do not allow us to conclude which of the two mechanisms is the strongest.

In FO, addition of tocopherol (AO1) or addition of 2000 ppm rosemary extract plus 600 ppm tocopherol (AO2) delayed the onset of formation of omega-3 volatiles to day 4, but after day 4, the concentration of volatiles increased much faster in AO1 than in AO2. A similar finding was observed in the mixtures for FO with vegetable oils (Fig. 4b and c), but here the formation of volatiles was slower between day 4 and 9 than when pure FO was used. Surprisingly, the effect of AO1 and AO2 differed slightly between the oil mixtures at day 14. Thus, in the FO + RO, AO2 resulted in significantly lower levels of volatiles at day 14 than AO1, whereas this was not the case in the mixture with SO and LO. Furthermore, the RO + FO mixture with AO1 had a concentration of omega-3 volatiles of ca. 300 000 ng/g whereas the mixture with LO and SO with AO1 had a concentration of ca. 250 000 ng/g indicating a better protection by tocopherol when LO and SO was used. This is in accordance with the findings from the Oxipress experiments. The better performance of AO1 in the presence of LO and SO might be related to the different composition of the endogenous tocopherols in the different vegetable oils.

Taken together our data suggest that high concentrations of both rosemary extract and tocopherol are needed to obtain the strongest interaction between the two antioxidants and thereby the optimal antioxidant effect.

5 Storage stability at 30°C and experiment with commercial marine oil products

The second storage stability experiment was performed at 30°C instead of 40°C, because we anticipated on the basis of the results in Fig. 4a, that some of the commercial marine oils might be quite unstable and storing these oils for 2 weeks at 40°C would push the oxidation balance well beyond the phase were the secondary volatile oxidation products still accumulate as the main oxidation product.

Fortunately, we had tested one of the least stable commercial FOs at both temperatures and were able to compare the pattern of oxidation products formed. The FO that was tested twice was the concentrated FO containing high level of tocopherols (Table 2). Figure 5a shows that the tocopherols in the concentrated oil were rapidly consumed both at 30 and 40°C. Figure 5b shows that PV value was lower at 40°C compared to 30°C and followed the zig-zag pattern we had seen for the FO + AO1 blend in the first storage stability experiment. Figure 5c shows that the formation of the omega-3 volatiles produced the broken stick response in the oil incubated at 40°C as also seen in the first experiment for FO + AO1 blend, but not in the oil incubated at 30°C. This comparison of two incubation temperatures confirmed that the balance of oxidation may move beyond accumulation of the secondary volatile oxidation products in the case of the least stable FOs when the oils are incubated at 40°C for 2 weeks. Oils sold for pets are usually stored at room temperature. Experiments performed at 40°C may lead to results that would be quite different from those obtained at room temperature, whereas this is less likely to be the case for an experiment carried out at 30°C. It is well known that temperature can significantly influence the oxidation kinetics. Sullivan et al. [37] observed that increasing storage temperature of FO from 40 to 60°C produced lower PV and a similar zig-zag pattern as we observed when we increased the storage temperature of concentrated FO samples from 30 to 40°C. Hence, our data suggest that

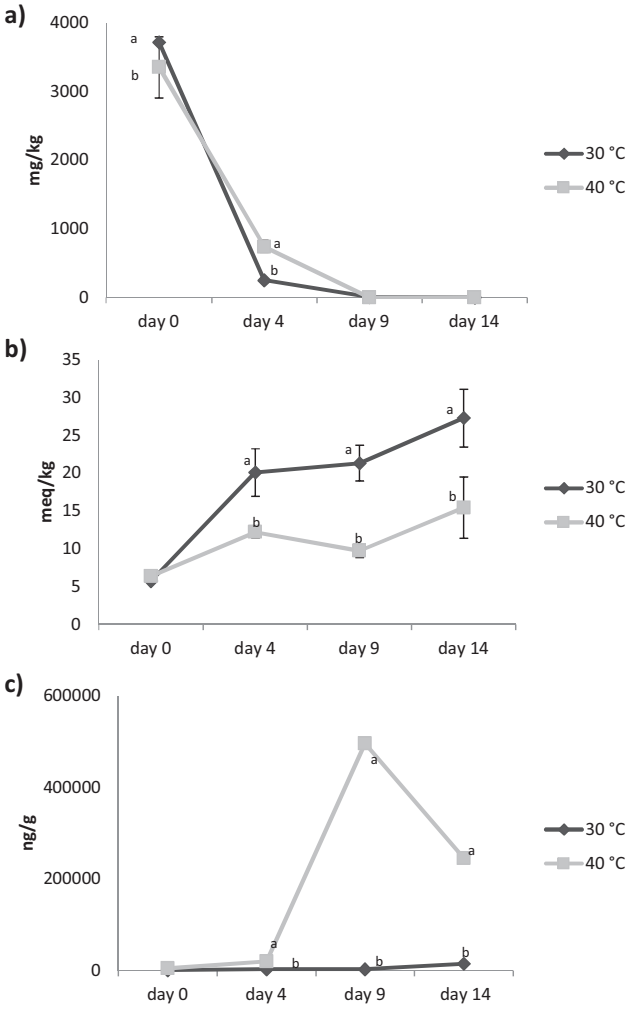

Figure 5. Total tocopherol content (a), peroxide value (b), and secondary volatile oxidation products from omega-3 fatty acids (c) in concentrated fish oil during 14 days of storage at 30 and 40°C.

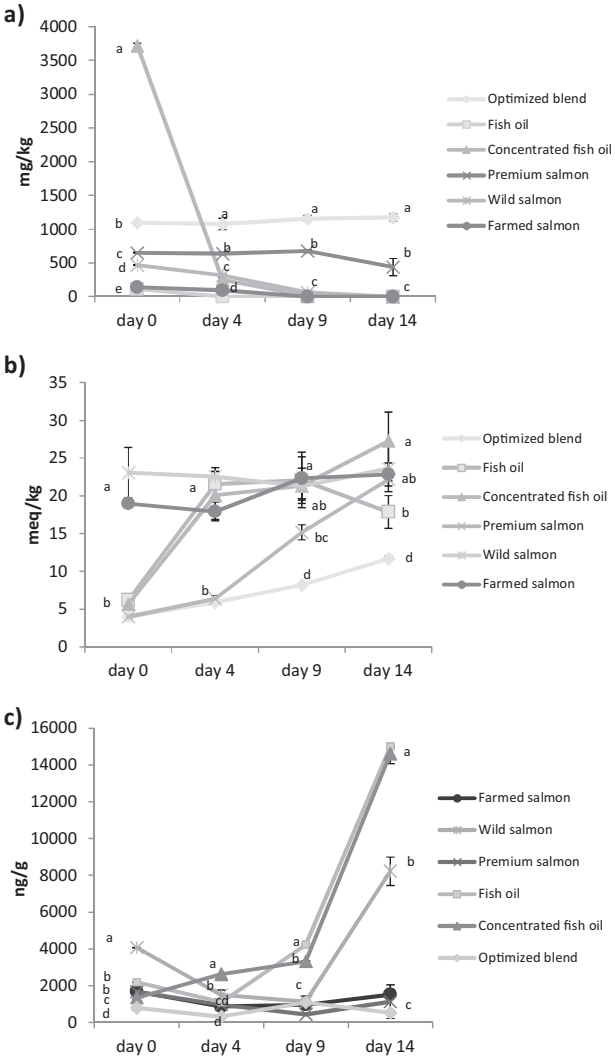

Figure 6. Changes in peroxide value (a), total tocopherol content (b) and secondary volatile oxidation products from omega-3 fatty acids of commercial marine oils during 14 days of storage at 30°C.

running storage stability tests even at 40°C may be a too high temperature for some types of FO.

Total tocopherol and omega-3 EPA and DHA content of the commercial marine oil products used in this experiment are presented in Table 2. One of the salmon oils was defined as farmed salmon oil. This oil was purchased from a supermarket. The other salmon oil was defined as premium salmon oil purchased from a specialty pet store. The pet store product sells typically for higher price and the consumers perceive the product to be of higher quality compared to the supermarket variety salmon oil. Omega-3 fatty acid content defined as EPA + DHA% of total fatty acids was markedly higher for the premium salmon oil compared to farmed salmon oil suggesting some difference in quality and maybe in the origins of the oil also. Wild salmon oil was declared to be of Alaskan origin and it had the highest omega-3 content of the salmon oils. The FO included in the test was of the standard 18/12 variety and the Concentrated FO was produced by molecular distillation. Optimized blend was a commercial product optimized for composition based on the results of the storage stability test performed at 40°C. This Optimized blend comprised FO, SO, and LO plus high concentrations of mixed tocopherols and rosemary extract.

Four out of six oils had PV of around 5 meq/kg on day 0 (Fig. 6a). Two oils, farmed salmon and wild salmon were quite oxidized already at the start with PV of 19.0 and 23.1 meq/kg, respectively. PV of these two oils remained high throughout the experiment. PV of FO and Concentrated FO increased on day 4 to around 20 meq/kg and remained high until the end of the experiment. PV of premium salmon oil and Optimized blend remained low until day 4 and increased

after that. The increase was slowest for the Optimized blend reaching 11.7 meq/kg by day 14.

Variable amounts of tocopherol had been added to oils (or remaining in the oils on day 0) ranging from 3717 mg/kg in concentrated FO (mostly α-tocopherol) to 115 mg/kg in FO and 137 mg/kg in farmed salmon oil both comprising a typical mixture of tocopherols (Fig. 6b). Total tocopherol content of the Concentrated FO dropped precipitously and was down to 7% of the initial concentration by day 4 of storage (Fig. 6b). Small amounts of tocopherol in FO and farmed salmon oil were consumed and decreased to zero by day 4 and 9, respectively. The tocopherols were consumed more gradually in the wild salmon oil and reached zero by day 14. Tocopherol content of the premium salmon oil was quite stable until day 9 and dropped slightly during the last 5 days of storage. Optimized blend was remarkably stable throughout the storage.

As expected, the changes in the secondary volatile oxidation products derived from omega-3 fatty acids varied among the oils tested. The rate of decomposition of the primary oxidation products was highest in FO and Concentrated FO (Fig. 6c). Pronounced increase in the secondary volatile oxidation products started when the tocopherols were consumed, at day 4 for FO and day 9 for Concentrated FO. The level of secondary volatiles was the highest for wild salmon oil at the start of the storage experiment, dropped to ¼ of the initial level at day 9 and increased again to a level, which was twice the initial level. The other three oils followed a similar pattern while remaining low throughout the 14 day storage period.

Taken together, the results showed that the optimized blend was the most oxidatively stable with a low formation of both peroxides and volatile oxidation products and with a stable level of tocopherol. These results further confirmed our previous observation that a high concentration of tocopherol in combination with a high concentration of rosemary extract is able to retard lipid oxidation and prevent decomposition of tocopherol.

6 Conclusions

Addition of RO to FO increased oxidative stability of the blend significantly. Addition of SO plus LO also increased the oxidative stability, but not to the same extent as RO. The stabilizing effect of RO was most likely due to its tocopherol content and favorable composition of tocopherols. Addition of a combination of rosemary extract and tocopherol increased the oxidative stability of FO. The relative increase in oxidative stability due to addition of antioxidants was highest in pure FO and lowest in a mixture of FO and RO. A high level of rosemary extract (6000 ppm) and tocopherol (600 ppm) was needed to obtain optimal protection and this combination of antioxidants performed better than addition of only tocopherol in high concentration (2000 ppm).

Comparison of results obtained at 30 and 40°C showed different patterns of development of PV and volatiles indicating that storage at 40°C may be too high to mimic oxidation at room temperature.

Evaluation of commercially available marine oils for pets demonstrated a pronounced variation in oxidative stability upon storage at 30°C. The observed variation is probably due, in most part, to a suboptimal level and composition of antioxidants used in oils. The Optimized blend consisting of 5000 ppm rosemary extract and 500 ppm tocopherol was formulated on the basis of the results in the first part of this study and it performed better than any of the other commercial oils tested.

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