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Exploring the possibility of 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. The main purpose is to find a potential initiator not to assess oxidation stability of the formulations.

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

1) Reaction kinetics using a Clark electrode (Oxygraph);
2) Effect of adding an initiator on the product's physical and oxidative stability in prototype skincare formulations by visual observation, peroxide value 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, peroxide value increased significantly when AMVN was added. However, the peroxide value 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.

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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

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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 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). Earlier studies showed that initiation by elevated temperature and exposure to light did not result in the same sample ranking as long term storage studies within one month (2). We hypothesised that the addition of such molecules to a prototype skincare formulation may be able to comparatively predict the level of resistance to oxidation in different prototype skincare formulations, and therefore help to identify lead prototype skincare formulation 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 prototype skincare formulations. 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 (1,3,4). 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 (4)**.**

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 (3).

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 (3,5). 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 (6,7). 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 (8).

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 prototype skincare formulation. Oxidative stability is in this case defined as the lag phase, i.e. the time to which the oxidation parameters measured starts to increase significantly. The effectiveness of the four initiators were explored in three steps: 1) reaction kinetics using a Clark electrode (Oxygraph) for electrochemical measurement of oxygen; 2) the effect of adding an initiator on the product's physical and oxidative stability when prototype skincare formulations were stored for one month at 20^{o}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 prototype skincare formulation (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 prototype skincare formulations and prototype creams 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 prototype skincare formulations (Table II) were produced by Glaxo Smith Kline (Brentford, UK):

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

These prototype skincare formulations 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, prototype skincare formulations with initiators added were used for comparison of the oxidation rate with neat prototype skincare formulations (no initiators added) after six months of storage at 20^{o}C.

A second storage experiment was conducted on a prototype cream 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.* (9). Poyato *et al.* (9) 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 prototype cream.

The experimental design is shown in Table III, where all samples (prototype skincare formulations and prototype creams) 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.* (10–12) 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 prototype skincare formulations, the evaluation of the four initiators was performed in diluted versions of the prototype skincare formulations (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 prototype skincare formulations

Initiators were added to freshly produced prototype skincare formulations (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 prototype skincare formulations without initiators were stored for 6 months at 20°C.

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

Initiators were added to freshly produced prototype creams (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 prototype creams 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. The reference is the formulation without initiator as the negative control. The physical stability was assessed at each sampling point but only mentioned in the result section when it was different from the control.

Peroxide value

Oil was extracted from the prototype skincare formulations prior to peroxide value analysis. Oil extraction was performed as described by Bligh and Dyer (13) but with a reduced amount of solvent as described by Iverson *et al*. (14). 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 (15). In brief, the hydroperoxides were quantified by colorimetric determination of iron thiocyanate. The peroxide value 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 prototype skincare formulations with initiators added was conducted by the method described by Thomsen *et al.* (16). 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 [%].

Statistical Analysis

The experimental design was not conducted in duplicate because it was an initial screening. Therefore, statistical analysis was not performed as it is not possible to conduct a proper analysis on singlet results

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 prototype skincare formulations without phase separation of the prototype skincare formulations. 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 prototype skincare formulation without initiator (Figure 1).

The radicals, AAPH and AMVN, could only be added in concentrations of 5-10 mM without phase separation occurred by injection into the cell in the Oxygraph. At concentrations above10 mM immediate physical instability occurred in the prototype skincare formulations (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 prototype skincare formulations in the Oxygraph experiment. However, the increases in oxygen consumption rates differed between the prototype skincare formulations with the added $FeCl_2/H_2O_2$. Hence, prototype skincare formulation C had the highest decrease in Δ followed by prototype skincare formulation B and lastly prototype skincare formulation A. In a Oxygraph study on liposome dispersions by Mozuraityte *et al.* (10), 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 prototype skincare formulations 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 prototype skincare formulation C may make it more sensitive to iron-catalysed oxidation than prototype skincare formulations A and B. However, the antioxidants can of course impact the oxygen consumption. All the formulations contained antioxidants specific for each product. Kristinova *et al*. (11) 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 prototype skincare formulation C is less stable at room temperature than prototype skincare formulations 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*. (11,12) 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: Storage experiment with prototype skincare formulations

Physical stability

The initiator addition must not affect the prototype skincare formulation's physical stability because physical instability can affect the oxidative stability significantly (17). The physical stability was assessed visually (Table IV).

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 prototype skincare formulations and still maintain physical stability of the product. The initiator system $FeCl_2/H_2O_2$ did not change viscosity initially and the prototype skincare formulations remained physically stable for 30 days of storage. However, it was not possible to add $FeCl_3$/ascorbic acid in prototype skincare formulation 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 prototype skincare formulations. 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 prototype skincare formulations 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, prototype skincare formulations with AMVN showed early signs of phase separation as oil appeared on prototype skincare formulation B surface after 16 days of storage. Prototype skincare formulation 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

The oxidative stability was using a combination of several analytical methods to follow the progress of lipid oxidation from primary oxidation products to secondary oxidation products. PV is an unspecific spectrophometric method often used as industry standard therefore the result from this study can be used by others scientists in future article for comparison. Headspace GC-MS is a specific method which can be used to follow the development of specific volatile compounds that can affect product safety and sensory (odour). This method was chosen instead of more simple methods such as determination of anisidine value (AV) or thiobarbituric acid reacting substances (TBARS) often applied in the industry, because these methods often do not correlate well with sensory data.

In the prototype skincare formulations with initiators added, lipid oxidation was measured by peroxide value and volatile compounds. Peroxide value was initially below 2 meq/kg oil and remained below 2 meq/kg in all prototype skincare formulations with $FeCl_2/H_2O_2$. Moreover, peroxide value was below 5 meq/kg oil in all prototype skincare formulations with AAPH. However, addition of AMVN significantly increased peroxide value in all three prototype skincare formulations to 21, 4 and 10 meq/kg oil after 30 days of storage, in prototype skincare formulations A, B and C, respectively. The neat prototype skincare formulations A, B and C had an initially low peroxide value below 2 meq/kg oil and it remained below this value for six months of storage. The higher peroxide value in prototype skincare formulations 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 (3,18).

The results from the peroxide value s 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 prototype skincare formulations stored for 6 months based on the peroxide value measurements.

Earlier studies have shown that butanal, pentanal and 3-methyl-1-butanol (from hydrolysis of UV

filter: Only prototype skincare formulation B) are good markers for lipid oxidation and degradation in the prototype skincare formulations used in the present study (2,19,20). The concentration of butanal increased significantly in all three prototype skincare formulations 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 prototype skincare formulations than on the type of formulation. The most efficient initiator system was $FeCl_2/H_2O_2$ in all three prototype skincare formulations.

No correlation was observed between the lipid content of prototype skincare formulations and peroxide value or the butanal concentration. prototype skincare formulation C had the highest lipid content at 22% compared with 12% and 2% in prototype skincare formulations B (omit UV filter) and A, respectively. There was no significant difference in concentrations of the oxidation products between prototype skincare formulations 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 prototype skincare formulation B) were calculated for neat prototype skincare formulations (no initiator) after six months of storage and after one month of storage with initiators. Both prototype skincare formulations 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 prototype skincare formulations after 6 months.

In prototype skincare formulation 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 prototype skincare formulation 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 prototype skincare formulation A (no initiator).

As observed for prototype skincare formulation A, $FeCl_2/H_2O_2$ was the best accelerator of lipid oxidation as evaluated by formation of butanal and pentanal in prototype skincare formulation B. In contrast, the radicals, AAPH and AMVN, only gave rise to low oxidation rates, which were lower than those of the neat prototype skincare formulation 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 prototype skincare formulation A (no initiator) (20).

Similar to the findings in prototype skincare formulation B, only $FeCl_2/H_2O_2$ was able to reach and

exceed the oxidation rates of butanal and pentanal of the neat prototype skincare formulation 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 prototype skincare formulations 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 prototype skincare formulations 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 prototype creams, with and without extracts, oxidation rates after 56 days of storage (9). 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 prototype creams with two different *Fucus vesiculosus* extracts (EE1, WE1) and to prototype cream without extract (REF) to evaluate the initiators' ability to accelerate oxidation and rapidly predict the ranking of the prototype cream' oxidative stability at 20°C. The study by Hermund *et al.* (21) showed that the oxidation rate was lowest in prototype cream with EE1 and WE1 extract for pentanal and heptanal, respectively. The prototype cream without initiator was stored at 20°C for 42 days because time did not allow a six-month storage experiment. Overall, the prototype cream REF had the highest oxidation rate for the prototype creams 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 prototype cream REF had the highest oxidation rate. Only $FeCl_2/H_2O_2$ resulted in the same ranking of prototype cream with antioxidants as prototype cream 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 prototype creams as no initiator.

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

Summary

The Oxygraph results may suggest that prototype skincare formulation C is less stable at room temperature than prototype skincare formulations 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 prototype skincare formulation B had the lowest stability followed by prototype skincare formulations 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 prototype skincare formulations 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 peroxide value but not a significantly higher concentration of the volatile compounds compared with the other initiators. The higher peroxide value 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 peroxide value and the ranking of prototype skincare formulations were not comparable to those obtained for prototype skincare formulations without initiator.

The hydrophilic initiators, $FeCl_2/H_2O_2$ and AAPH, were able to initiate peroxide value in the same pattern as prototype skincare formulations 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 peroxide value. AAPH resulted in a low peroxide value 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 peroxide value, but the lack of contribution to the redox reaction led to a lower decomposition to volatile oxidation products (11,12). 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 prototype skincare formulations with no initiators and prototype skincare formulations with added radicals, AAPH and AMVN, were not the same. The sample ranking of the butanal in prototype skincare formulations with no initiators was B>C>A, whereas it was B>A>C when AAPH or AMVN were added. However, the sample ranking in prototype skincare formulations with no initiators was the same as observed for prototype skincare formulations with added $FeCl_2/H_2O_2$.

A study by Baron *et al.* (18) 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.*(18) 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 prototype skincare formulations and prototype creams because it led

immediately to phase separation.

Moreover, Baron *et al.*(18) 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, prototype creams, where the addition of *Fucus* vesiculosus led to an increased oxidative stability. However, Baron *et al.*(18) 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 prototype creams 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 prototype skincare formulations.

In neat prototype skincare formulations, the physical stability was not affected by $FeCl_2/H_2O_2$ addition. However, $FeCl_3$/ascorbic acid resulted in phase separation in all prototype skincare formulations 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 peroxide value showed that the formulation with $FeCl_2/H_2O_2$ and AAPH both had a low and stable peroxide value for 30 days. However, the AMVN addition to all prototype skincare formulations resulted in an increased peroxide value. After six months of storage, the neat formulations without initiators also had a low and stable peroxide value. 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. This study showed that the initiator having the largest potential among the evaluated initiators was $FeCl_2/H_2O_2$. More studies in other types of cosmetic emulsions are needed to confirm this finding.

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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 prototype skincare formulations and (3) ability to differentiate unstable vs. stable prototype creams by initiator addition.

Experiment/ Formulation	**EX 1: Oxygraph**	**EX 2: Product stability: Physical stability, PV and GC-MS**	**EX 3: Ability to differentiate: GC-MS**
$FeCl_2/H_2O_2$	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. INCL list for prototype formulations used.

Product	Prototype night cream formulation (paper V)	Prototype serum formulation (papers IV and V)	Prototype skin cream formulation (papers III and V)
Product contained several other raw materials including the listed.	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	Glycerine Isostearyl isostearate Acetamide MEA Lecithin Palmitamide MEA	Rice bran wax Glycerine Isostearyl isostearate Palmitic acid monoethanolamine

Table III. 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 prototype skincare formulations (PFs) and (3) ability to differentiate unstable vs. stable prototype creams (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 IV. Visual changes observed in physical stability due to addition of initiators in prototype skincare formulations (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

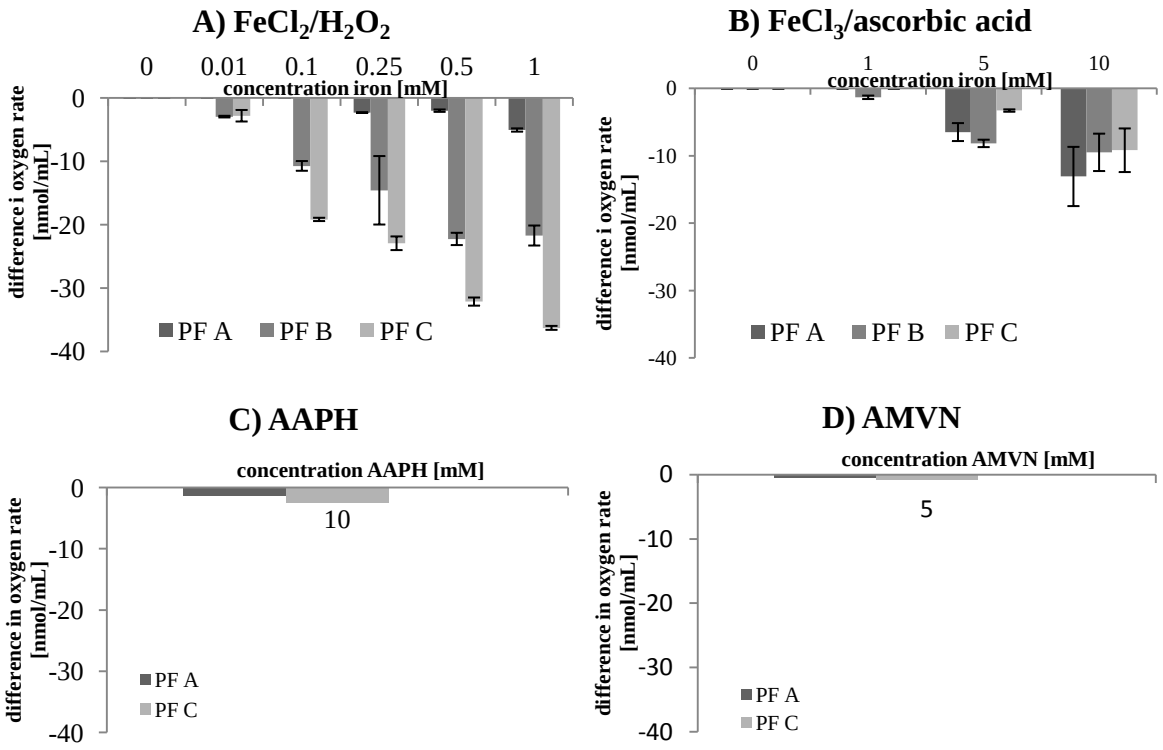


Figure 1. The difference in oxygen consumption rate compared with the no initiator [nmol/mL] in the prototype skincare formulation (PF) A, B and C due the initiator addition estimated using Oxygraph. A) $FeCl_2/H_2O_2$ and B) $FeCl_3$/Ascorbic acid initiator systems are used, and C) AAPH and D) AMVN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

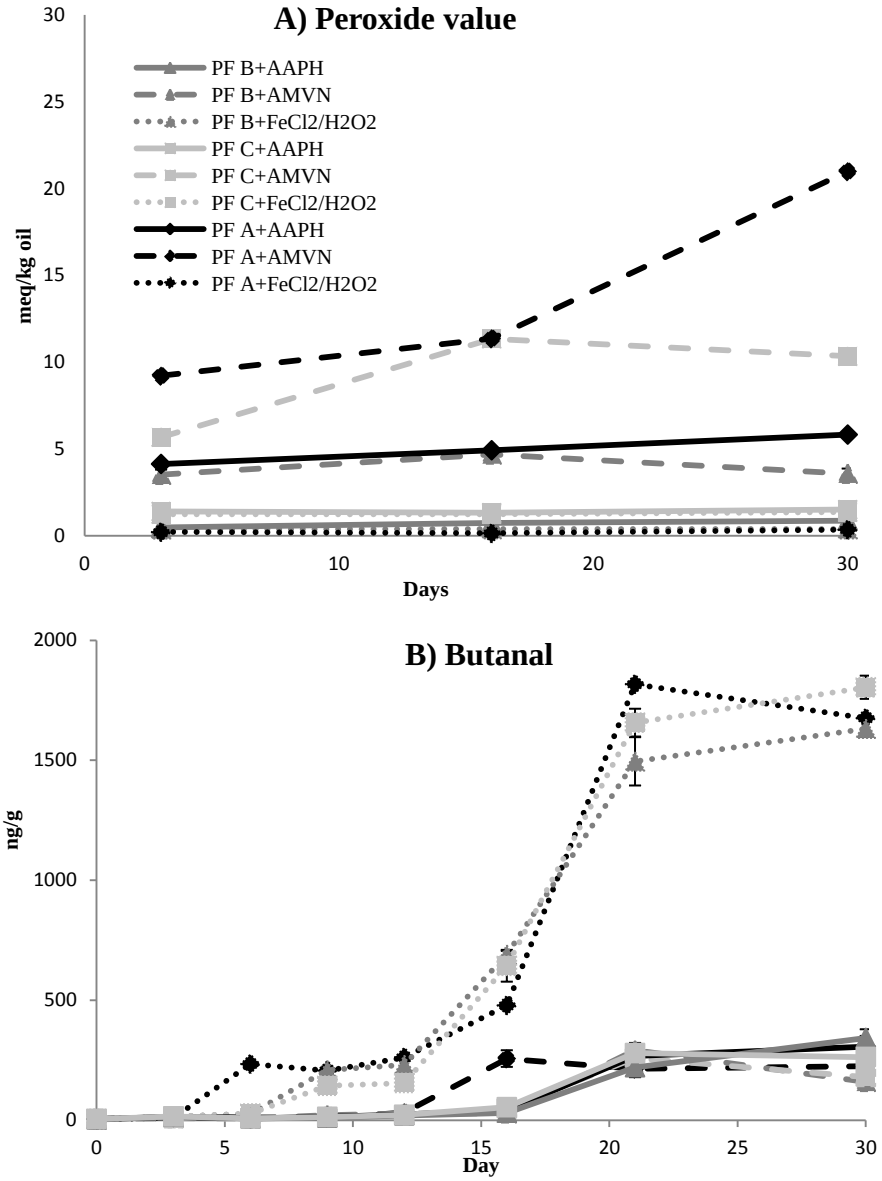
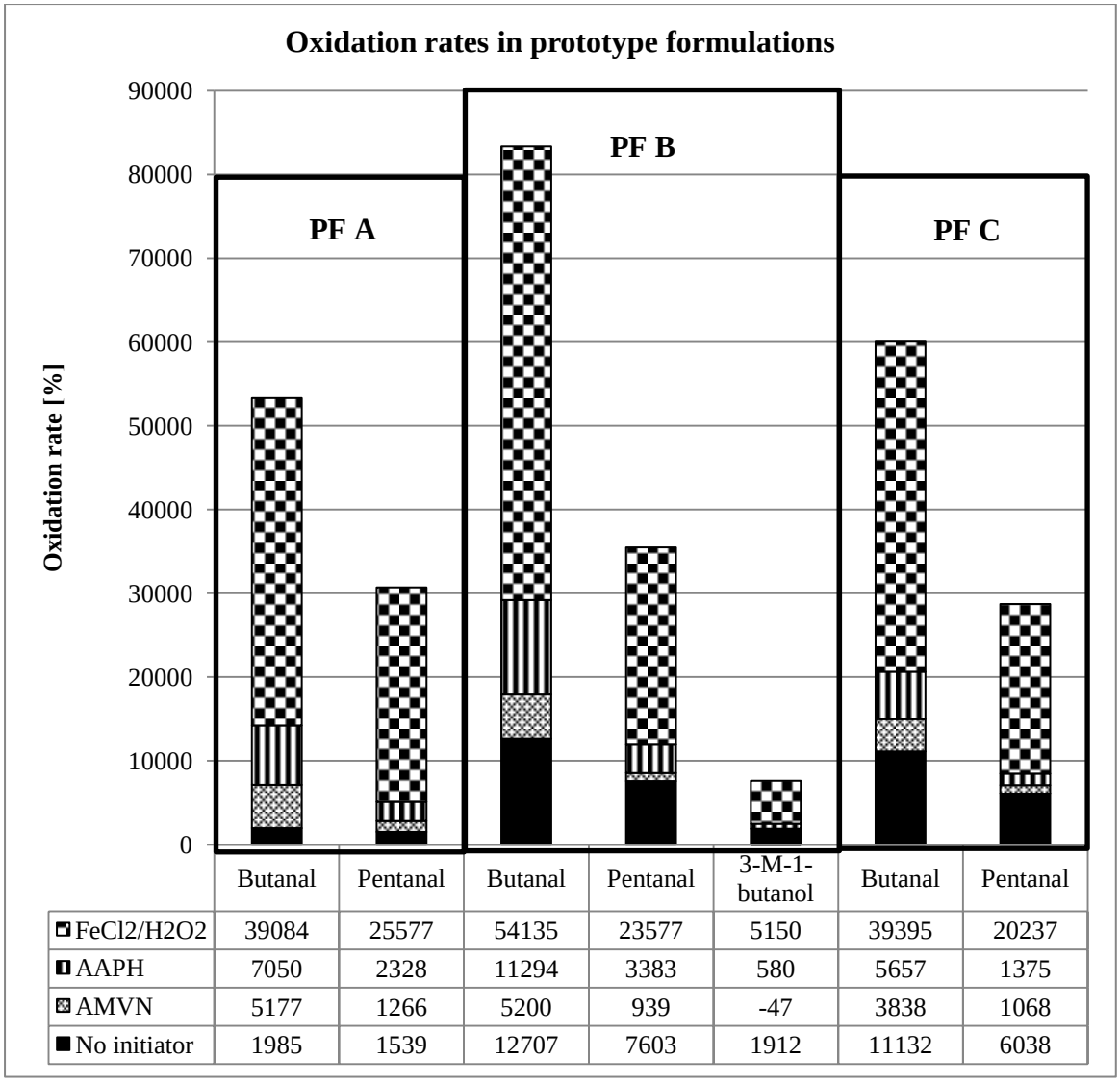
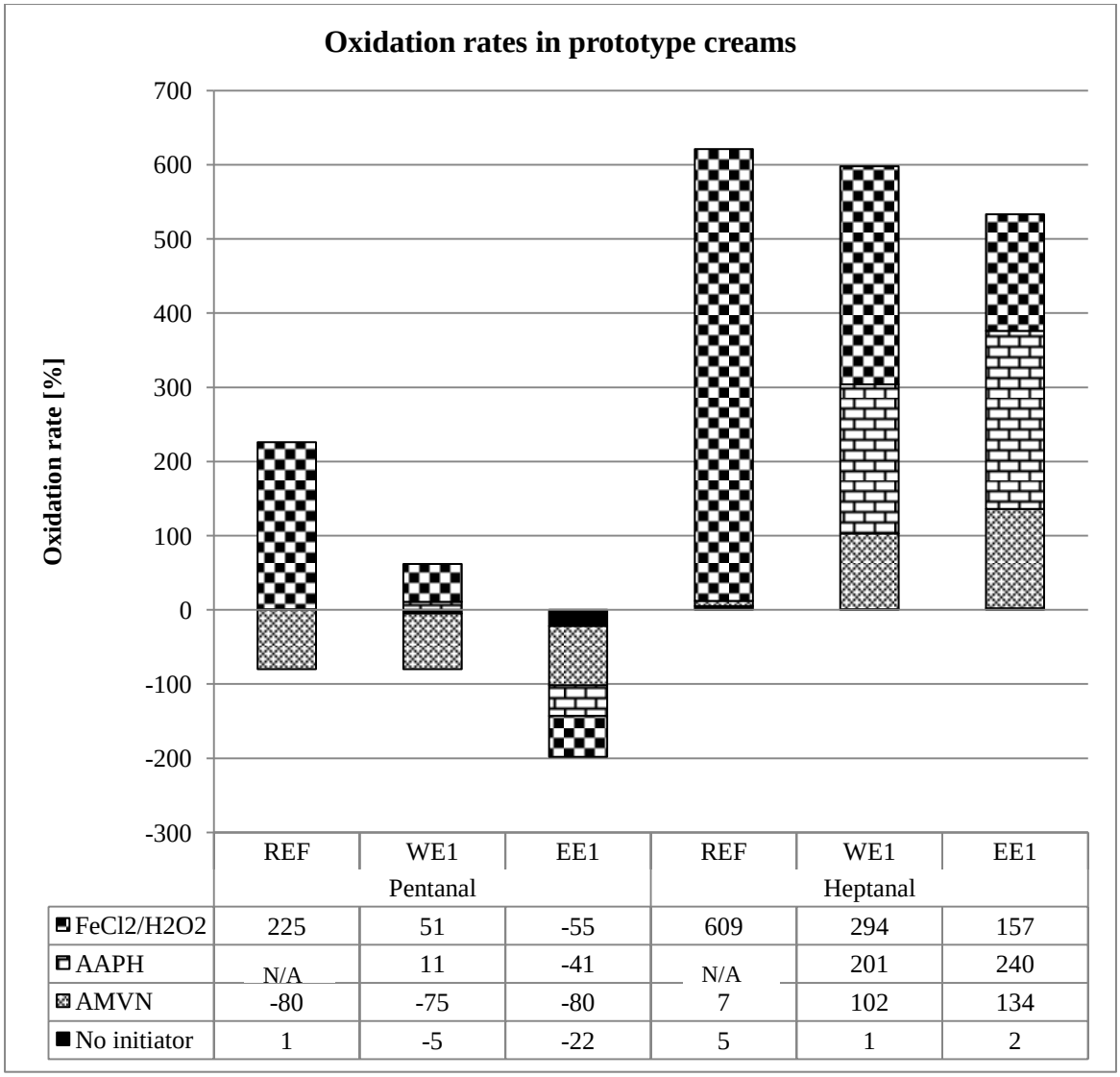


Figure 2. The development of A) peroxide value [meq/kg oil] and B) butanal [ng/g] in prototype skincare formulations (PFs) A, B and C with added initiators for 30 days of storage.



	Butanal	Pentanal	Butanal	Pentanal	3-M-1-butanol	Butanal	Pentanal
FeCl2/H2O2	39084	25577	54135	23577	5150	39395	20237
AAPH	7050	2328	11294	3383	580	5657	1375
AMVN	5177	1266	5200	939	-47	3838	1068
No initiator	1985	1539	12707	7603	1912	11132	6038

Figure 3. Oxidation rate [%] of butanal, pentanal and 3-methyl-1-butanol (3-m-1-butanol) in the neat prototype skincare formulations (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 prototype creams (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.