



Physical and oxidative stability of high fat fish oil-in-water emulsions stabilized with combinations of sodium caseinate and sodium alginate

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Abstract

A systematic study was carried out in order to evaluate the physical and oxidative stability of high fat omega-3 delivery fish oil-in-water emulsions stabilized with combinations of sodium caseinate (NaCas) and sodium alginate (NaAlg). The influence of 3 factors related to emulsion composition (fish oil content: 50, 60 and 70%; total amount of NaCas and NaAlg: 1.4, 2.1 and 2.8 %; and ratio NaCas:NaAlg: 0.4, 1.2 and 2) on physical (droplet size, viscosity and zeta potential) and oxidative (primary and secondary oxidation products) parameters was evaluated. It was possible to produce emulsions with a combination of NaCas and NaAlg, except when the ratio between NaAlg and aqueous phase was high (0.047 or 0.054). Viscosity of the emulsions significantly increased with increasing fish oil and total stabilizer content. Zeta potential was significantly affected by total stabilizer content. The content of primary oxidation products in the emulsions was very low (0.93 meq peroxides/kg oil). Secondary oxidation products were detected in small amounts (<60 ng/g emulsion). Even though the optimum formulation concerning physical parameters was suggested as 61.8% fish oil content, 1.4% total stabilizer and 1.2 ratio NaCas:NaAlg by Box-Behnken's design, the formulae 70%-1.4%-1.2 was decided due to high fish oil content's decreasing effect on droplet size and peroxide value.

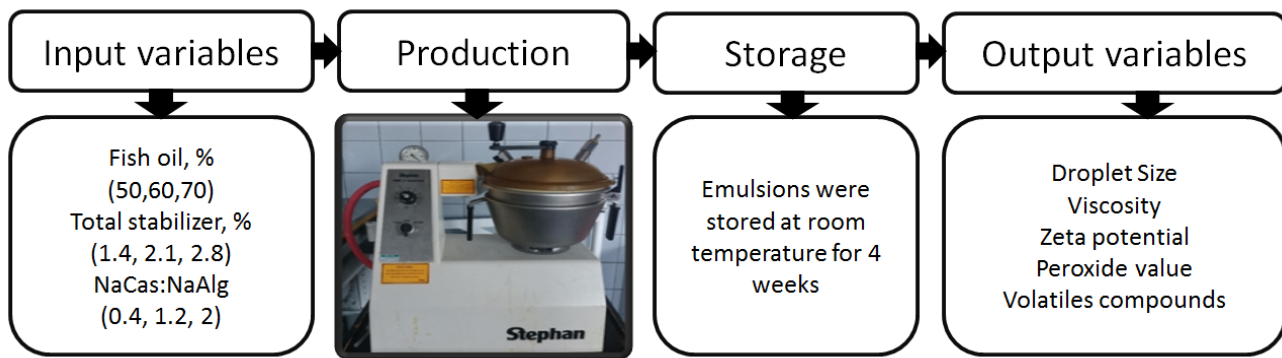
Practical applications

Physically and oxidatively stable high fat (50-70%) omega-3 delivery fish oil-in-water emulsions are of high interest to food industry for the production of omega-3 fortified products. Our results show the feasibility to stabilize high fat delivery fish oil-in-water emulsions using combinations of NaCas and NaAlg. As these emulsions had high amount of fish oil, food products can be enriched with smaller amounts of high fat emulsions when compared to low fat delivery emulsions. This results in minor changes of the product's original structure. Examples for enrichment of food products with omega-3 are dressings, cream cheese, yoghurt and mayonnaise.

Keywords: lipid oxidation, emulsifier, stabilizer, 50-70% o/w emulsion, omega-3

Abbreviations: NaCas, sodium caseinate, NaAlg, sodium alginate, PV, peroxide value, LC, long chain, PUFAs, polyunsaturated fatty acids, PCA, principal component analysis, RSM, response surface methodology, MRO, multiple response optimization

Graphical abstract



Optimization of emulsion composition which maximizes the physical and oxidative stabilities of high fat fish oil-in-water emulsions stabilized with combinations of sodium caseinate and sodium alginate.

1 Introduction

Long chain (LC) omega-3 polyunsaturated fatty acids (PUFAs) have been found to be beneficial to human health in several respects such as preventing cardiovascular diseases, improving immune system and mental health [1,2,3]. As a consequence and due to the low consumption of fish, food products enriched with omega-3 fatty acids have been developed (e.g. milk, mayonnaise, yoghurt, dressings and cream cheese). However, these LC omega-3 PUFAs are highly prone to oxidation due to the high content of bis-allylic hydrogens in their structure. The production of fish oil delivery emulsions could be an efficient strategy to protect omega-3 fatty acids against oxidation when incorporated into food matrices. In addition to preventing lipid oxidation, maintaining the physical stability of emulsions is another important parameter, which needs to be addressed due to its direct effect on overall quality and shelf life of the final product [4,5]. A large number of studies have been performed on physical and oxidative stability of low fat (5-30%) emulsions, however, only a few studies have been reported on high fat (50-70%) emulsions. The advantage of using a high fat emulsion for enrichment of food products is its lower water and higher fat content compared to low fat emulsions, which means that lower amounts of emulsions would be required for enrichment. Therefore, high fat omega-3 delivery emulsions are gaining increasing interest by the food industry, particularly for highly viscous food

products such as mayonnaise, dressings and cream cheese etc., which have similar structure as high fat emulsions. Challenges are to keep them physically and oxidatively stable.

Physical and oxidative stability of emulsions are affected by charge density, ionic strength, temperature, pH, emulsifier or surfactant concentration as well as homogenization techniques and conditions [6,7,8,9,10,11]. Particularly, the type of emulsifiers and thickening agents employed has a great influence on emulsion stability. They determine the properties of the interface, which is the place where oxidation is initiated and they increase the viscosity of the emulsions' aqueous phase slowing the creaming of oil droplets [12,13]. In this regard, protein and polysaccharide combinations have been widely used in food industry in order to stabilize low-fat emulsion based food systems [14].

There are two different methodologies to produce emulsions stabilized with combinations of proteins and polysaccharides, leading to the so called 'mixed emulsion' and 'bilayered emulsion' [15]. Mixed emulsion is prepared by dissolving both protein and polysaccharide in the buffer and then oil is emulsified within the aqueous phase. Bilayered emulsion is produced by emulsification only with protein followed by addition of polysaccharide into the emulsion. The polysaccharides are oppositely charged than the protein so that they may adsorb at the interface creating a second layer. It was stated in previous studies that bilayered emulsion has a major

problem with flocculation which promotes creaming instability; hence these authors found mixed emulsion to be the best alternative [6,15]. On the other hand, bilayered emulsions are more appropriate for nanoencapsulation of nutritious compounds inside the protein and polysaccharide layers.

Some studies have shown that the use of polysaccharide in protein stabilized mixed oil-in-water emulsions increased their physical stability [11,15,16,17,18]. The main functionality of polysaccharides in food emulsions is their ability to thicken the system, which reduces the creaming rate and enhances the textural properties of the emulsion [4]. In this study, mixed emulsion technique was applied and sodium alginate was used as a thickener. It was also reported that the amount of polysaccharides dissolved in the water phase in the emulsion has a critical importance due to promoting the occurrence of depletion or bridging flocculation and coalescence [5].

Previous studies on high fat fish oil-in-water emulsions employed whey protein isolate, NaCas, milk phospholipids, or soy lecithin as emulsifier [9]. These authors found that emulsions prepared at pH 7 and stabilized with NaCas were more oxidatively stable compared to the other emulsions and to neat fish oil. This is mainly explained by the metal chelating activity of NaCas and to its flexible structure, which allowed a better coverage of the droplets. Combinations of sodium caseinate and sodium alginate have already been studied for the stabilization of low fat emulsions. Pallandre et al. aimed at obtaining a double-interfacial layer by using caseinate and alginate for the stabilization of 1 wt% corn oil-in-water emulsions [19].

However, to the best of the authors' knowledge, stabilization of high fat fish-oil-in-water emulsions with combinations of NaCas and NaAlg has not yet been reported.

Therefore, this work aimed to investigate the physical and oxidative stability of high fat omega-3 delivery fish oil-in-water emulsions stabilized with combinations of NaCas and NaAlg. A systematic study was carried out in order to evaluate the influence of fish oil content, total content of NaCas plus NaAlg and ratio between NaCas and NaAlg on both physical (droplet size, zeta potential and

viscosity) and oxidation (peroxide value and volatiles content) parameters.

2 Materials and methods

2.1 Materials

Cod liver oil was provided by Maritex A/S, subsidiary of TINE, BA (Sortland, Norway), and stored at -40°C until use. Fatty acid composition, peroxide value and tocopherol composition of the cod liver oil was previously reported by García-Moreno et al [20]. Sodium caseinate (Miprodan 30) was donated by Arla Foods Ingredients amba (Viby J, Denmark). Arla reported a protein content of 92% in sodium caseinate for Miprodan 30. Sodium alginate (Grindsted® Alginate FD 170) was provided by DuPont (Brabrand, Denmark).

2.2 Emulsion preparation and sampling

Prior to emulsification, both NaCas and NaAlg were dissolved in distilled water and left for mixing on a magnetic stirrer over night at 4°C. All aqueous phases were adjusted to pH 7 with 2M HCl or 2M NaOH. Emulsions were produced in 500 g batches in a Stephan Universal mixer (Stephan, UMC5, 1995, Hameln, Germany) equipped with an emulsification blade as described by Horn et al [9]. The three experimental factors considered for this study were fish oil content, total content of NaCas plus NaAlg and ratio between NaCas and NaAlg since they are expected to affect the physical and oxidative stability of the emulsions by determining the composition of the interface. A Box-Behnken design including 3 central points was executed, in which each input variable was set at three levels: 50, 60 and 70% for fish oil content; 1.4, 2.1 and 2.8% for total amount of NaCas+NaAlg; and 0.4, 1.2 and 2 as ratio between NaCas and NaAlg (Table 1). Emulsions were stored in 100 mL blue cap bottles at room temperature for up to 4 weeks with sampling points at day 0 or 1, day 3, week 1, week 2, week 3 and week 4. The viscosity and droplet size were measured on day 1, week 1, 2, 3, 4 and zeta potential was measured on day 4. Peroxide value was measured at all sampling points. Volatile oxidation products (GC-MS) were measured at day 0 and week 4.

2.3 Characterization of emulsions

2.3.1 Droplet Size

Droplet size of the emulsions was measured by laser diffraction in a Mastersizer 2000 (Malvern Instruments, Ltd., Worcestershire, UK) using the method described by Let et al [21] and Horn et al [9]. Results were given as the volume weighted mean diameter $D[4,3]=\sum n_i d_i^4 / \sum n_i d_i^3$. Measurements were carried out in duplicate.

2.3.2 Zeta potential

Surface charge of the emulsion droplets was determined by the zeta potential measured with a Zetasizer Nano 2S (Malvern Instruments, Ltd.) at 25°C. Each sample (0.32 g sample) was diluted in distilled water (40 g of distilled water) before measuring and the zeta potential range was set to -100 to +50 mV. Measurements were carried out in duplicate.

2.3.3 Apparent viscosity measurements

Viscosity was measured using a stress-controlled rheometer (Stresstech, Reologica Instruments AB, Lund, Sweden) equipped with a CC25 standard bob cup system in a temperature vessel. Measurements were done at 25°C over a shear stress range from 0.0125 to 500 Pa. The apparent viscosity was obtained at a shear rate of 20 s⁻¹ and was expressed in Pa·s. Viscosities were measured twice on each emulsion.

2.4 Lipid oxidation measurements of emulsions

2.4.1 Primary oxidation products—peroxide value (PV)

For determination of primary oxidation products, a lipid extract was prepared according to the method described by Bligh and Dyer [22] using 5–10 g of emulsion for each extraction and a reduced amount of solvent (30 mL of methanol and chloroform, 1:1). PV was subsequently measured on the lipid extracts by colorimetric determination of iron thiocyanate at 500 nm, as described by Shantha and Decker [23]. Measurements were carried out in duplicate.

2.4.2 Secondary oxidation products—Dynamic Head Space GC-MS

Volatile secondary oxidation products were analyzed according to the method described by Jacobsen et al [24]. Approximately 1 g of emulsion was mixed with 1 mL antifoam and 5 mL distilled water in a 100 mL purge bottle. The bottle was

heated in a water bath at 60°C while purging with nitrogen (flow 150 mL/min, 30 min). Volatile compounds were trapped on Tenax GR tubes. The volatiles were separated in a gas chromatograph (Agilent Technologies, 6890N Network GC System) on a 30 m DB 1701 fused silica capillary column (0.25 mm i.d., 1 μm film thickness; Agilent Technologies, J&W GC Columns, USA). The oven program had an initial temperature of 45°C for 5 min, increasing with 1.5 °C/min until 55°C, with 2.5°C/min until 90°C, and with 12.0°C/min until 220°C, where the temperature was held for 4 min. The individual compounds were analysed by mass spectrometry (Agilent 5973 Network Mass Selective Detector, Agilent Technologies, electron ionization mode, 70 eV; mass to charge ratio scan between 30 and 250). The individual compounds were identified by both MS-library searches (Wiley 138 K, John Wiley and Sons, Hewlett-Packard) and quantified through calibration curves. The external standards employed were 2-ethylfuran, 1-penten-3-ol, 1-penten-3-one, 2-pentenal, hexanal, (E)-2-hexenal, heptanal, octanal and (E,E)-2,4-heptadienal and nonanal. Measurements were made in triplicate in each sample.

2.5 Statistical analysis

Statgraphics XVII (Statpoint Technologies, Inc., Virginia, USA) was used to generate the statistical analysis and the regression models for the physical parameters of the emulsions. Firstly, the output variables (Y: viscosity at week 4, droplet size at day 1 and zeta potential at day 4) were related to the input variables (X: total fish oil content, total content of NaCas plus NaAlg and ratio between NaCas and NaAlg) by second degree polynomials as follows, Eq. 1:

$$Y = b_0 + \sum_{i=1}^3 b_i X_i + \sum_{i=1}^3 b_{ii} X_i^2 + \sum_{i<j}^3 b_{ij} X_i X_j \quad (1)$$

where the coefficients b_i and b_{ii} are related to the linear and quadratic effects, respectively, of each input factor on the output variable and the cross-product coefficients b_{ij} represent the interactions between two input variables. Secondly, the analysis of variance (ANOVA) was carried out. The significance of all terms in the models was judged statistically by computing the p-value at a confidence level $1-\alpha = 95\%$. The regression coefficients were then used to generate contour

maps and to find the optimal values of the input variables, which maximize the quality of the emulsions in terms of physical stability, by using the response surface method (RSM) [25].

Multiple response optimization (MRO) was applied in order to determine the values of the experimental factors that led to desired characteristics for more than one output response simultaneously. Desirability function was constructed based on values of response variables and then maximized. Desirability function $d(y)$ expresses the desirability of a response value equal to y on a scale of 0 to 1. Parameter s defines the shape of the function. Response could be maximized, minimized or a targeted to a specific value. In this study, response variables were minimized, thus the function is defined by following equation, Eq.2;

$$d = \begin{cases} 1 \\ 0 \end{cases} \left(\frac{y - \text{high}}{\text{low} - \text{high}} \right)^s, \quad y < \text{low}; \text{low} \leq y \leq \text{high}; y > \text{high} \quad (2)$$

To be able to use MRO, RSM was applied first in Statgraphics XVII for each output variable and then the information was retrieved to perform multiple optimization.

Samples with the codes 70-2.8-1.2, 70-2.1-0.4 and 60-2.8-0.4 (Table 1) did not form an emulsion. Droplet size and zeta potential for these samples were still measured by shaking the samples before running the analysis. On the other hand, viscosity was not determined for these samples. Thus, in order to have all the data to create a model, values being 10% higher than the highest measured viscosity value were given to these samples since minimum viscosity was desired.

Principal component analysis (PCA) was done by Latentix 2.12 (LatentiX, Copenhagen, Denmark). The PCA was carried out with the emulsions as objects and viscosity, droplet size, zeta potential and peroxide values as variables. Data were mean centered to make the variables contribute equally to the model, and the PCA model was validated systematically segmented, according to replicates of the emulsions.

3 Results and discussion

3.1 Production of emulsions

Emulsions 70-2.8-1.2, 70-2.1-0.4 and 60-2.8-0.4 did not lead to formation of an emulsion, since the two phases were totally separated right after production. This might be due to the high ratio between the amount of NaAlg and water phase where both stabilizers were dissolved. This ratio can be calculated from the compositional data in Table 1. It was found that emulsions did not form when the ratio of NaAlg to water phase was 0.047 or 0.054. On the other hand, the remaining emulsions (12 out of 15) were successfully produced when the ratio of NaAlg to water was between 0.012 and 0.031. The reason behind these findings could be both depletion flocculation and bridging flocculation when high amounts of NaAlg were used. Depletion flocculation may have occurred since an osmotic attractive force between droplets was generated which removed the polymer molecules present around oil droplets. Bridging flocculation might have been caused due to a rearrangement of an alginate molecule which adsorbs at more than one oil droplet [5,19]. Besides, high amount of NaAlg led to a highly viscous water phase, which might have hampered the diffusion of both emulsifier and stabilizer to the droplet interface and thus affected the stabilization of the oil droplets [19,26,27]. High viscosity of the aqueous phase might make it harder to detach and adsorb at the water/oil interface and result in droplet aggregation during production.

The other emulsions were stable right after production. NaCas is known as a surface active emulsifier and NaAlg is mainly used as a thickening agent for increasing the viscosity of aqueous phase [28,29]. This explains that the use of both molecules together in the right concentrations help to produce physically stable emulsions.

3.2 Characterization of Emulsions

Droplet size, viscosity and zeta potential results are shown in Table 1 together with the experimental design.

3.2.1 Droplet size

Most of the emulsions were stable during 4 weeks of storage, without a significant increase in droplet size. Therefore, results at day 1 (Table 1) are representative for the rest of the sampling points. Emulsions showed monomodal distributions with

droplet sizes ($D[4,3]$) which ranged between 6.6 and 17.9 μm except for the emulsions with the following codes 70-2.8-1.2, 70-2.1-0.4 and 60-2.8-0.4, which did not form an emulsion structure (Table 1). The ones that did not form an emulsion structure had bimodal distributions with much larger droplet sizes up to 127 μm due to a severe coalescence of oil phase (data not shown). Emulsions with codes 50-1.4-1.2 and 50-2.1-2 had the highest droplet sizes values among all emulsions and were also creaming after 4 weeks of storage (20.7 and 20.9%, respectively). The rest of emulsions were physically stable in terms of creaming (data not shown).

A high coefficient of correlation ($R^2=88.88$) was obtained for the model obtained for droplet size, indicating a good correlation between predicted and measured values.

ANOVA analysis indicated that droplet size significantly decreased when the ratio of NaCas to NaAlg and the interaction between fish oil content and the ratio between NaCas and NaAlg increased. Total stabilizer content did not have a significant effect on droplet size. Fish oil content also had a significant effect on droplet size, with a positive correlation (Table 2). However, raw data show that droplet size of emulsions significantly decreased when increasing fish oil (see droplet sizes for emulsions 50-1.4-1.2 and 70-1.4-1.2 or 50-2.1-2 and 70-2.1-2 in Table 1). Likewise, Hadnedev et al [31] also reported that an increase in specific surface area occurred with the increasing oil concentration when the emulsions were stabilized with 20% of emulsifier content.

Since the model fitted the experimental data well, RSM was employed to obtain the experimental factors related to emulsion composition, which minimized droplet size. This optimum value is shown in Fig. 1a and corresponds to 68.3% fish oil content, 1.4% total stabilizer and 2 as the ratio of NaCas to NaAlg. A minimum droplet size was desired since it implies higher physical stability of the emulsion.

3.2.2 Apparent Viscosity

Viscosity was measured at different time points during the storage of the emulsions. However, only at week 4 (last time point) all emulsions were measured at the same shear rate (20 s^{-1}), whereas

at earlier time points the emulsions were measured at different shear rates. Thus, results at week 4, which show the rheological behavior of the stable and unstable emulsions at the end of the storage, are shown in Table 1 for comparison among emulsions. Apparent viscosities of the emulsions varied from 0.4 to 20.9 Pa·s (Table 1) and all the emulsions were non-newtonian and showed shear thinning behavior. The ANOVA analysis showed that the viscosity of emulsions increased significantly with increasing total fish oil and total stabilizer content, whereas the ratio NaCas:NaAlg was not significant (Table 2). Nonetheless, it was observed for 50% o/w emulsions with a total emulsifier content of 2.1% that increasing the ratio between NaCas and NaAlg from 0.4 to 2.0 led to a lower viscosity due to the thickening effect of NaAlg, as also pointed out by Antonov et al [29].

The model obtained for viscosity had a high coefficient of correlation ($R^2=95.09$). Using RSM a minimum for viscosity was found when fish oil content was 52.2%, total stabilizer was 1.4% and ratio of NaCas to NaAlg was 1.2 (Figure 1b). A minimum viscosity was pursued since the impact of delivery emulsion would be minimized on textural properties of the final product. When optimum values for viscosity were compared with droplet size, both have the same total stabilizer content as 1.4%; however, fish oil content and ratio between stabilizers differs.

3.2.3 Zeta potential

Both NaCas and NaAlg have negative charge at pH 7 [28,29]. Zeta potential values of emulsions were all negatively charged and ranged between -97.3 and -72.2 mV as shown in Table 1. Zeta potential became significantly less negative with increasing total fish oil and total stabilizer contents (Table 2). The interaction between fish oil content and the ratio between NaCas and NaAlg had a significant negative effect on zeta potential, which means that zeta potential became less negative when the interaction of these two input variables decreased. NaAlg was more negatively charged compared to NaCas, which confirms that the increase in the NaCas and NaAlg ratio decreased zeta potential (not significant) meaning that the charge will be less negative at the water/oil interface.

The model obtained had a high coefficient of correlation ($R^2=93.41$). In order to obtain more negative zeta potential, which denotes a higher repulsion within droplets, optimum values for fish oil content, total stabilizer and ratio of NaCas to NaAlg were calculated as 70%, 1.4% and 2, respectively (Figure 1c).

3.2.4 Multiple response optimization of physical parameters

Each output variable was set to have the equal impact and weight when the desirability function was calculated. MRO provided joint optimization results for all physical parameters including droplet size, viscosity and zeta potential as 61.8% for fish oil content, 1.4% for total stabilizer and 1.2 for the ratio of NaCas to NaAlg (Figure 1d). Optimum total stabilizer amount was found the same for the individual optimization of each physical parameter as 1.4%, which was also confirmed by MRO. Optimum fish oil content varied for each output variable and multiple optimization gave 61.8% as the optimum, which is close to the middle condition (60%). The optimum ratio between NaCas and NaAlg was 2 for droplet size and zeta potential, whereas it was 1.2 for viscosity. MRO also suggested 1.2 as optimum NaCas:NaAlg ratio for the simultaneous optimization of the three physical parameters.

3.3 Lipid oxidation in the emulsions

3.3.1 Peroxide value

Peroxide values (PV) were very low (<0.93 meq peroxides/kg oil) and reached their top values in week 2 for most of the emulsions, except emulsion 50-1.4-1.2 and 50-2.8-1.2 for which PV continued to increase during the 3rd and 4th weeks, respectively (Figure 2). The decrease after week 2 could be explained by formation of secondary oxidation products. Increase in fish oil content provided better oxidative stability when 70-1.4-1.2 (0.32 ± 0.05 meq peroxides/kg oil) and 50-1.4-1.2 (0.71 ± 0.07 meq peroxides /kg oil) or 70-2.1-2 (0.18 ± 0.01 meq peroxides /kg oil) and 50-2.1-2 (0.35 ± 0.09 meq peroxides/kg oil) were compared after their storage of 4 weeks (Figure 2). This might be due to the better physical stability observed when the fish oil content was increased which promoted smaller droplet size and higher viscosity. Number of lipid molecules per droplet decreases when the droplet size becomes smaller which

could limit propagation chain reactions [27]. For all emulsions, low PVs might be explained by the metal chelating properties of NaCas [10,11,25,32] and radical scavenging and metal chelating activities of NaAlg [30]. Horn et al [9] showed that emulsions stabilized with 2.8% of NaCas at pH 7.0 had 3.9 meq peroxides/kg oil after 42 days of storage. Comparison of the results by Horn et al [9] with the present data, showed that emulsions stabilized with different combinations of NaCas and NaAlg had a lower PV (<0.93 meq peroxides/kg oil) when compared to 70% fish oil-in-water emulsion stabilized only with NaCas (2.8%) after 28 days of storage (≈ 2 meq peroxides/kg oil). This might be explained with the synergistic effect of both stabilizers on improving oxidative stability as well as physical stability.

3.3.2 Volatile compounds

2-ethylfuran, 1-penten-3-one, 1-penten-3-ol, hexanal, heptanal, (E,E)-2,4-heptadienal and nonanal are volatile secondary oxidation products that were identified in emulsions 70-1.4-1.2, 50-2.8-1.2, 50-2.1-0.4, 70-2.1-2, 60-1.4-0.4, 60-1.4-2 and the 3 center points of the experimental design (60-2.1-1.2). These emulsions were selected since they were physically stable in terms of creaming until the end of the storage (data not shown). However, the amounts of these volatile oxidation products were quite low (data not shown), indicating together with the PV results that the emulsions were oxidatively stable. Figure 3 shows the volatile compounds (1-penten-3-ol, heptanal and nonanal), which were present in higher amounts compared to other volatile compounds in the emulsions after 4 weeks of storage at room temperature. Heptanal and nonanal contents were found as 43.0 and 50.6 ng/g emulsion, respectively, for the emulsion 50-2.1-0.4 (0.6% NaCas + 1.5% NaAlg) which had significantly higher values than the other emulsions. These findings are in agreement with Horn et al [9], who reported heptanal content between 40 and 55 ng/g emulsion for 70% fish oil-in-water emulsions stabilized with 2.8 and 1.4% of NaCas at pH 7 and stored during 42 days at room temperature. The formation of other volatiles such as pentanal, (E,Z)-2,4-heptadienal and (E,E)-2,4-heptadienal were also reported [9]. Even though the storage time was shorter for 50-2.1-0.4, heptanal formation was

higher compared to 2.8% NaCas and 70% fish oil-in water emulsion. This could be due to the lower fish oil and NaCas contents which caused less physical stability. Lower levels of NaCas could also have decreased the metal chelating ability of NaCas [10,11,25,27,32].

3.4 Principal component analysis

As seen in Figure 4, PCA was run excluding 70-2.8-1.2, 70-2.1-0.4 and 60-2.8-0.4 that did not form an emulsion structure. Peroxide values were very low (<0.93 meq peroxides/kg oil) for all emulsions and had no effect on differentiating the emulsion samples. Volatile compounds were not included in PCA as their concentrations were very low and would not make any difference on separating emulsion samples from each other. As expected, 3 emulsions with the code 60-2.1-1.2 were placed close to each other since they were the center points of the Box-Behnken's design and had the same formulation.

PCA confirmed that emulsion 50-1.4-1.2, 50-2.1-2 and 60-1.4-2 had large droplets and low viscosity. Thus, these emulsions were found to be less physically stable compared to the other emulsions. Emulsion 70-2.1-2 had the highest viscosity. As seen in the PCA, 70-1.4-1.2 performed as the optimum formulation among all emulsions by giving smaller droplet size, lower viscosity, low PV and highest negative droplet charge. This formulation was also supported by the MRO results for physical parameters, which was 61.8% for fish oil content, 1.4% for total stabilizer and 1.2 for the ratio of NaCas to NaAlg; except for the lower fish oil content. 70% of fish oil was selected as the optimum because it provided lower PV values and droplet sizes when total stabilizer and the ratio between NaCas and NaAlg were kept the same. Based on both RSM and PCA analyses, the optimum formulation (70-1.4-1.2) was selected which was part of the experimental design. Therefore, the formulation was validated by characterization of rheology, size distribution, zeta potential and oxidation.

4 Conclusions

The use of NaCas and NaAlg in combination allowed the production of emulsions, except when high ratio between NaAlg and aqueous phase was employed. The viscosity of the emulsions was significantly influenced by fish oil and total stabilizer content, whereas droplet size was mainly affected by fish oil content and the ratio between the two stabilizers. Moreover, zeta potential was affected by the interaction between fish oil content and both total stabilizer content and the ratio between the two stabilizers. Emulsions were found oxidatively stable during their storage of 4 weeks which confirms the protecting effect of NaCas and NaAlg as stabilizers. Different optimum formulations were obtained depending on which output variables was used for the modelling. Therefore, the optimum recipe was selected by looking at all the results together with PCA and RSM. The emulsion produced with 70% fish oil content, 1.4% total stabilizer and 1.2 as the ratio of NaCas to NaAlg would be the optimum for enrichment of food systems with omega-3 fatty acids.

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Tables ((including table captions))

Table 1. Experimental design, droplet size, viscosity and zeta potential results for the emulsions

Emulsion Code	Fish oil, %	NaCas+NaAlg, %	NaCas:NaAlg, Ratio	Droplet Size D[4,3] (μm) (Day 1)	Viscosity ($\text{Pa}\cdot\text{s}$ @ 20 s^{-1}) (Week 4)	Zeta Potential (mV) (Day 4)
50-1.4-1.2	50	1.4	1.2	17.9 ± 0.0	0.4 ± 0.0	-81.3 ± 1.7
50-2.1-0.4	50	2.1	0.4	7.8 ± 0.0	3.3 ± 0.1	-91.2 ± 1.6
50-2.1-2	50	2.1	2	14.7 ± 0.1	0.5 ± 0.2	-84.7 ± 2.2
50-2.8-1.2	50	2.8	1.2	9.4 ± 0.0	4.1 ± 0.6	-85.3 ± 2.8
60-1.4-0.4	60	1.4	0.4	9.7 ± 0.3	2.8 ± 0.0	-93.2 ± 3.9
60-1.4-2	60	1.4	2	14.5 ± 0.0	0.9 ± 0.4	-89.3 ± 2.2
60-2.1-1.2	60	2.1	1.2	7.7 ± 0.0	5.7 ± 0.2	-88.4 ± 2.3
60-2.1-1.2	60	2.1	1.2	8.9 ± 1.3	5.2 ± 0.2	-89.0 ± 1.3
60-2.1-1.2	60	2.1	1.2	7.9 ± 0.2	5.3 ± 0.1	-89.8 ± 1.7
60-2.8-0.4	60	2.8	0.4	28.8 ± 10.6	22.9*	-82.3 ± 2.3
60-2.8-2	60	2.8	2	6.6 ± 0.0	7.9 ± 0.6	-87.9 ± 3.2
70-1.4-1.2	70	1.4	1.2	7.5 ± 0.0	6.5 ± 0.1	-97.3 ± 1.5
70-2.1-0.4	70	2.1	0.4	82.8 ± 5.2	22.9*	-75.8 ± 1.6
70-2.1-2	70	2.1	2	7.5 ± 1.4	20.9 ± 0.6	-90.1 ± 0.6
70-2.8-1.2	70	2.8	1.2	39.7 ± 3.3	22.9*	-72.2 ± 1.1

All samples were analyzed in duplicates

*These samples did not form an emulsion structure, therefore 22.9 Pa·s was given as a value which is 10% higher than the highest measured value (20.9 Pa·s).

Table 2. Polynomial coefficients and p-values for the response variables (Droplet size, viscosity and zeta potential)

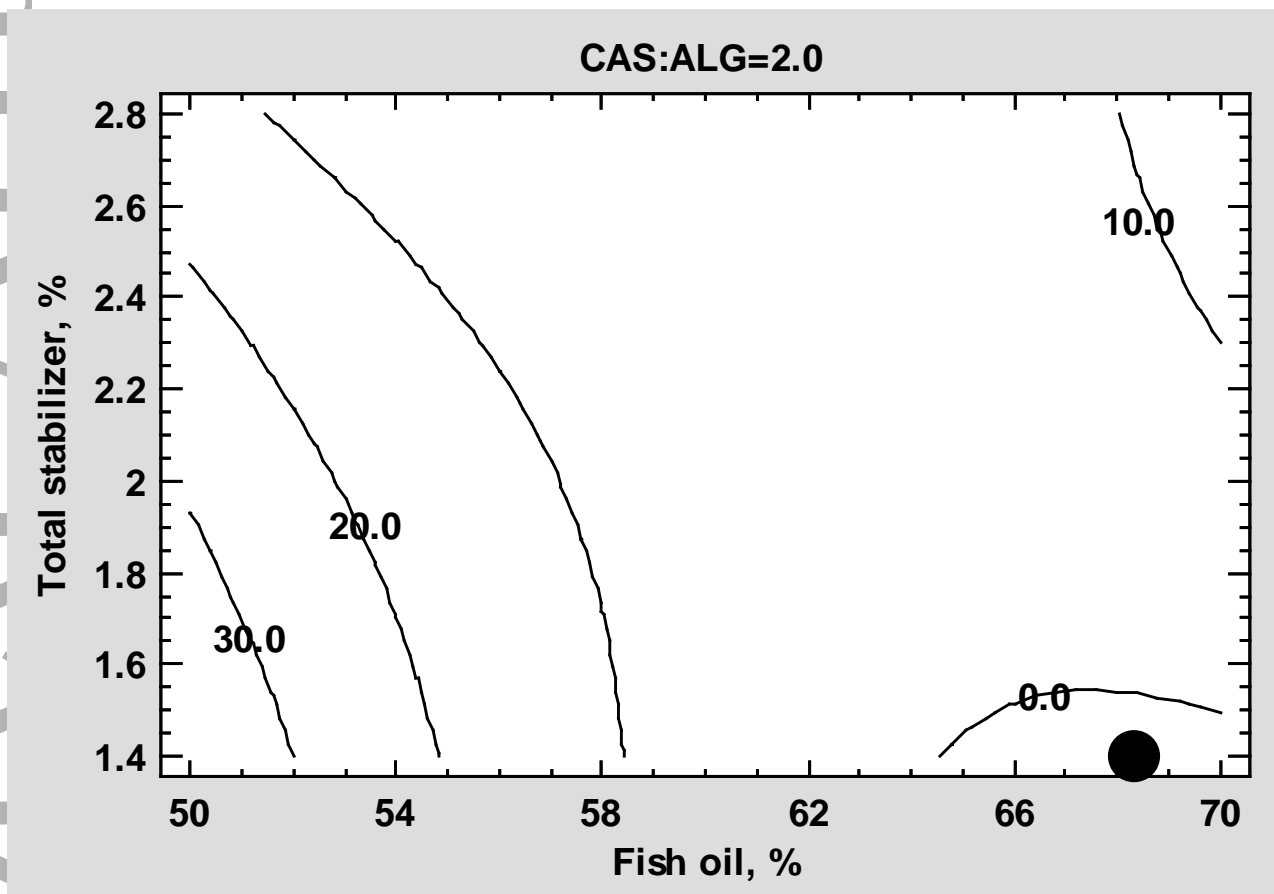
	Droplet size, D[4,3] (μm)			Viscosity (Pa-s)			Zeta potential (mV)		
	Coefficient	Correlation	p-value	Coefficient	Correlation	p-value	Coefficient	Correlation	p-value
Constant									
A: fish oil content, %	-13.1225	+	0.0403	-3.9689	+	0.0009	-5.2888	+	0.4081
B: NaCas+NaAlg, %	-54.4286	+	0.3218	-11.2089	+	0.0037	-61.0893	+	0.0065
C: ratio NaCas to NaAlg	135.4060	-	0.0431	-4.9922	-	0.0635	46.2969	-	0.3783
AA	0.1187	+	0.0987	0.0316	+	0.1214	0.0331	+	0.0708
AB	1.4571	+	0.1298	0.4582	+	0.1061	1.0393	+	0.0034
AC	-2.5687	-	0.0147	0.0222	+	0.9175	-0.6500	-	0.0134
BB	-2.9252	-	0.8165	-0.1913	-	0.9581	2.1684	+	0.4958
BC	-12.0536	-	0.2842	-5.8527	-	0.1005	-3.3929	-	0.2301
CC	12.7604	+	0.2222	5.2207	+	0.1059	-0.5664	-	0.8122
R ²			88.88			95.09			93.41

Statistical significance was considered at $p < 0.05$.

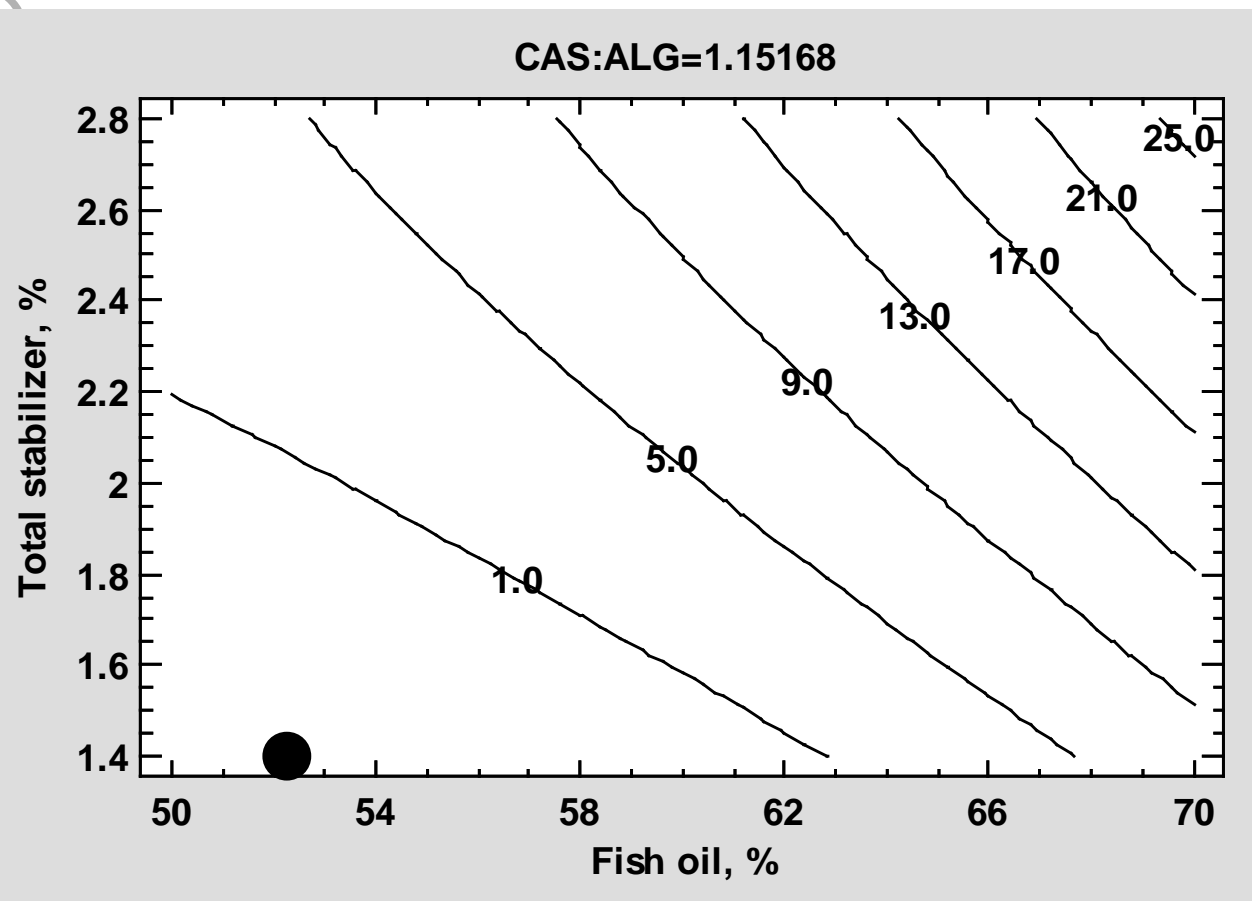
Figures ((please label the figures))

Figure 1. Contour plots of estimated response surface for output variables; (a) droplet size ($D_{4,3}$ in μm), (b) apparent viscosity at 20 s^{-1} in $\text{Pa}\cdot\text{s}$, (c) zeta potential in mV , and d) Desirability - estimated multiple response surface for viscosity, droplet size and zeta potential. Contour plots were created by fixing one of the factors, which had the least significant effect on the physical parameters. CAS:ALG ratio was fixed at its calculated optimum value which was suggested to obtain the most desired value for the specific physical parameter. Data are shown at the optimum values of CAS:ALG. Optimum recipes were shown with a black dot (●) on the graphs.

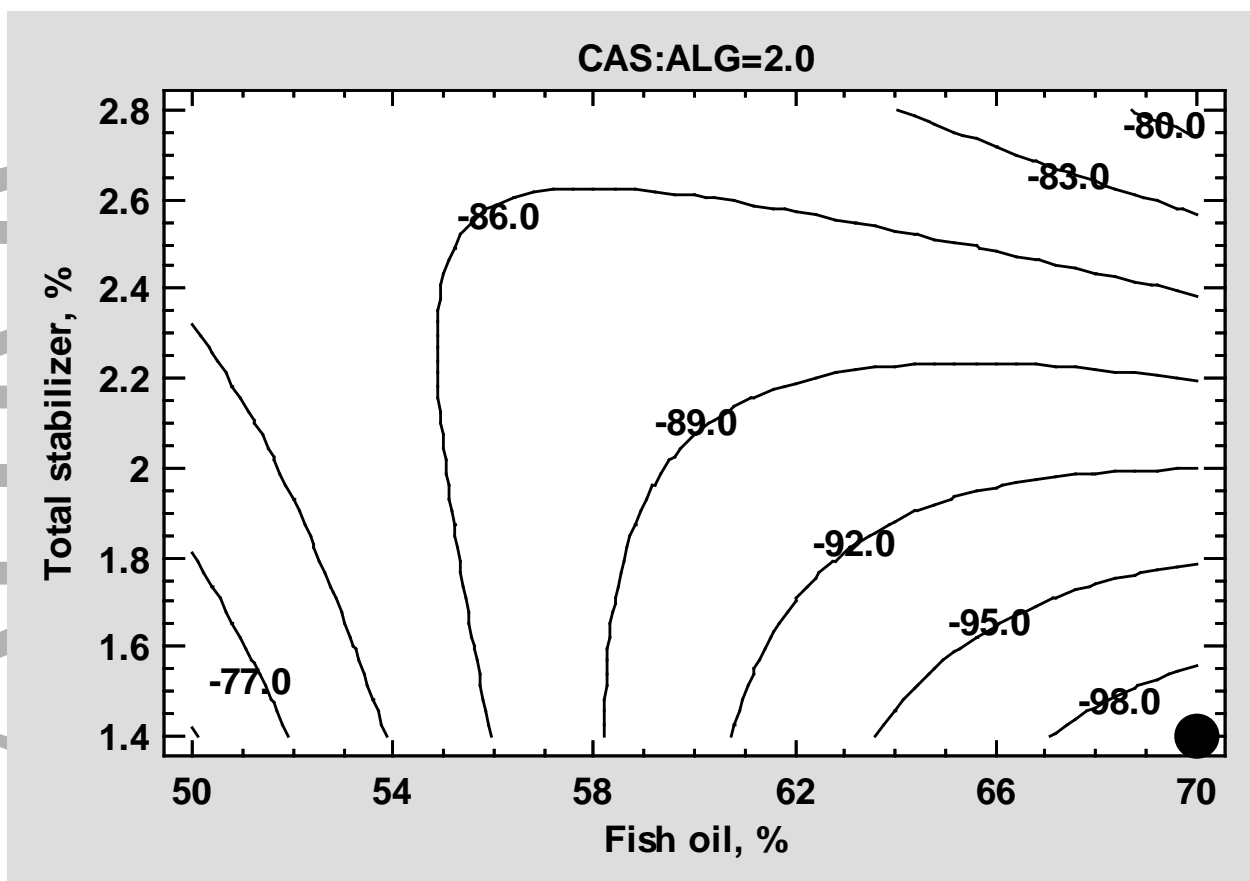
a)



b)



c)



d)

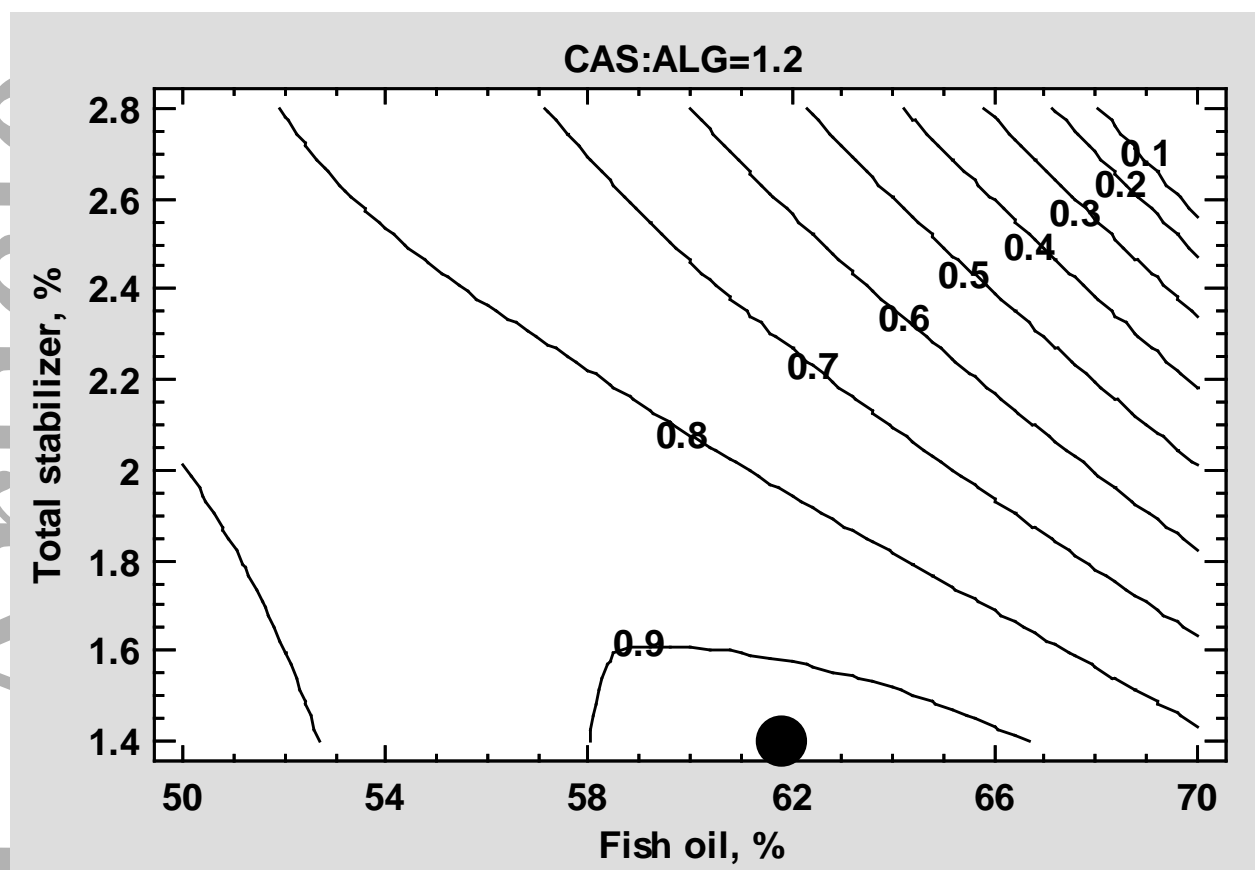


Figure 2. Peroxide value of emulsions during their storage time for 4 weeks

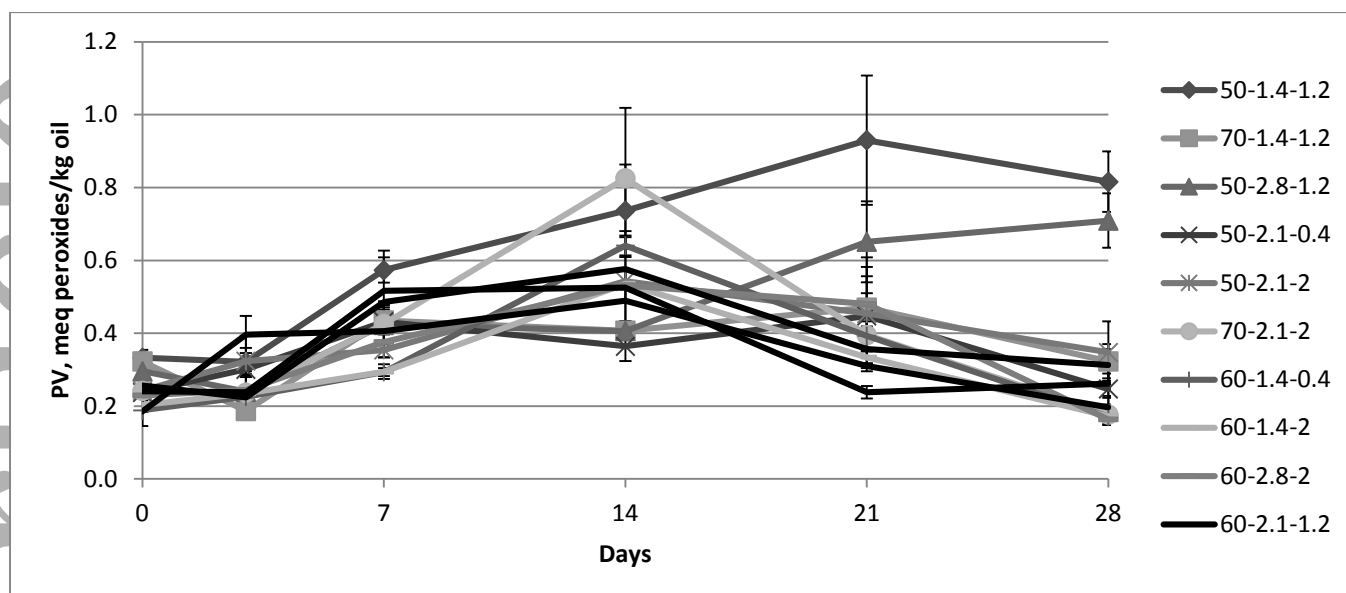


Figure 3. Volatile compounds (1-penten-3-ol, heptanal and nonanal) formed after 4 weeks of storage

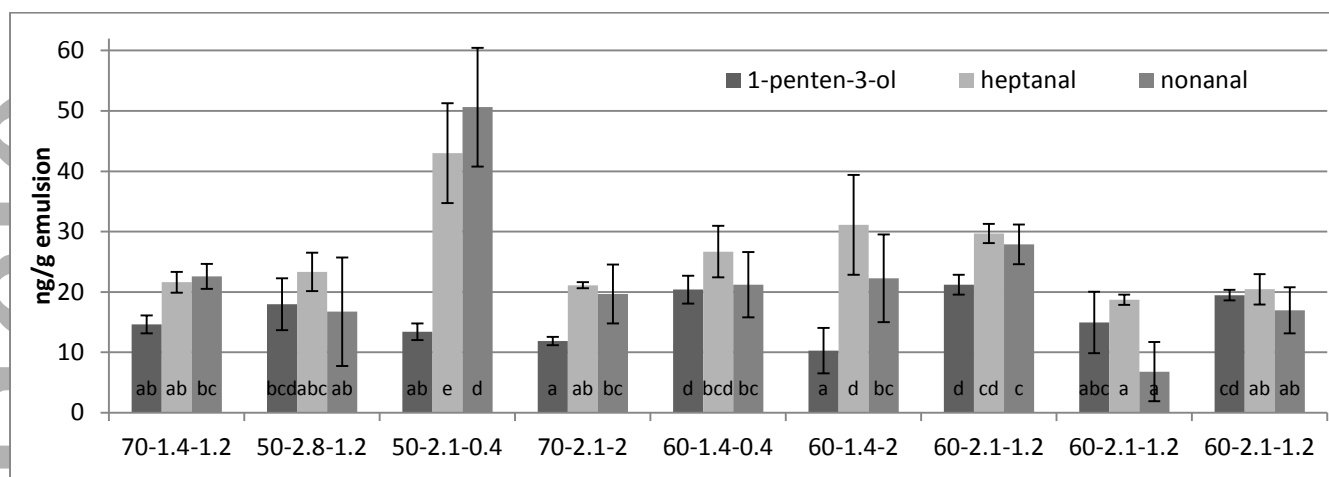
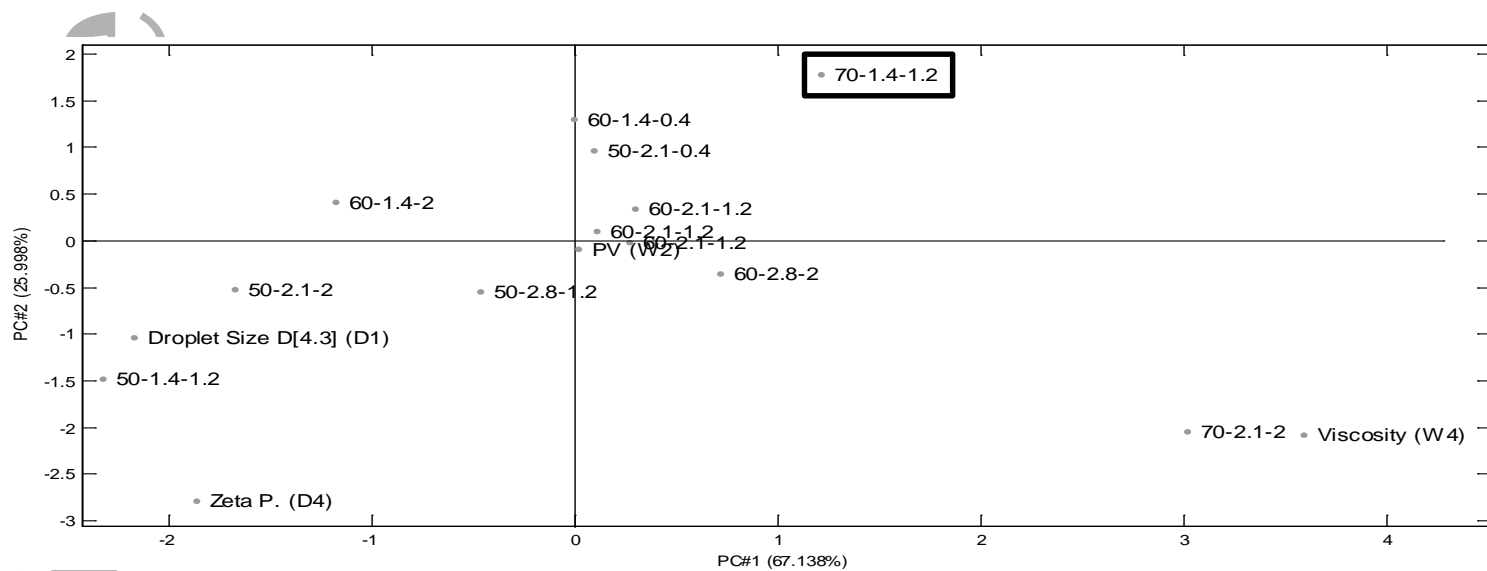


Figure 4. PCA Bi-pilot plotted using the results from Table 1.



Accepted A