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ARTICLE

Effects of Refining and Removal of Persistent Organic Pollutants by Short-Path Distillation on Nutritional Quality and Oxidative Stability of Fish Oil

Åge Oterhals*,† and Marc H. G. Berntssen§

†Nofima − Norwegian Institute of Food, Fisheries and Aquaculture Research, Kjerreidviken 16, N-5141 Fyllingsdalen, Norway, and §NIFES, Strandgaten 229, N-5004 Bergen, Norway

Food and feed legislations are implemented to control the level of unwanted persistent organic pollutants (POPs) below health risk concerns. Short-path distillation is established as the most effective industrial process to remove POPs in fish oil. However, the technology involves heating of the oil to high temperature levels (>200 °C) that possibly give unwanted heat-induced side reactions and coevaporation of minor compounds of importance for the nutritional quality of the oil. The effects on retention of vitamins, cholesterol, and unsaponifiable compounds, geometrical isomerization, loss of polyunsaturated fatty acids (PUFA), oxidation level, and oxidative stability have been studied on the basis of experiments designed to optimize and model the effect of process conditions (i.e., evaporator temperature, feed rate, and addition of working fluid) on the reduction of POPs. Loss of volatile nutrients was observed, but the extent will depend on the process conditions needed to obtain target decontamination level, as well as the concentration ratio and difference in vapor pressure between free and esterified forms of the studied compounds. Some reduction in oxidation level was documented with preservation of PUFA level and quality. Oxidative stability was influenced both positively and negatively depending on the applied process conditions. Generally, no adverse negative effects on the nutritional quality of the fish oil could be documented. Optimal process conditions were modeled that ensure removal of POPs to within legislation levels while retaining most of the vitamin levels in fish oil. A 76% reduction of the WHO-PCDD/F-PCB-TEQ level in the used feedstock was needed to be in accordance with the voluntary industrial monograph of GOED. This could be achieved on the basis of operation conditions giving <20% loss of vitamins. A 90% decontamination rate gave vitamin retentions in the 60−90% range.

KEYWORDS: Molecular distillation; dioxin; PCB; vitamins; cholesterol; unsaponifiables

INTRODUCTION

The health risk concern related to the content of potentially hazardous organic compounds in fish oil has been elucidated in several publications (*1*−*4*) and increased the industrial focus on optimization of process alternatives to reduce their levels, including activated carbon adsorption (*5*,*6*), steam deodorization (*7*,*8*), and short-path distillation (SPD) (*9*, *10*).

Activated carbon adsorption can be performed under vacuum at moderate temperature levels (< 90 °C), and no negative effects on oil quality have been documented (*5*, *6*). The use of vaporization technology, however, will involve heating of the oil to high temperature levels (> 200 °C) that possibly give unwanted heat-induced side reactions and coevaporation of minor compounds that are of importance for the nutritional quality of the oil. The residence time at operation temperature depends on technology and apparatus design and varies from hours in batch steam deodorizers to seconds in thin film evaporators. Steam deodorization has been extensively studied in edible fish and vegetable oil processing, and reported effects include removal of oxidation products (i.e., volatile off-flavors) (*11*), sterols (*12*), tocopherols (*12*), and free fatty acids (*13*) and polymerization (*14*) and isomerization of polyunsaturated fatty acids (*15*,*16*). The alternative SPD is established as a good manufacturing practice in the lipid-processing industry to separate heat labile substances (*17*). The process conditions are characterized by the combined use of high temperature and low vacuum level in a thin film evaporator to secure a short residence time and minimize heat-induced side reactions. Effects of SPD on oil quality have been less studied, but reports include reduction of cholesterol (*18*, *19*), tocopherol (*13*, *20*), and free fatty acids and oxidation products (*20*).

The best known bioactive components present in fish oil are the fat-soluble vitamins A, D, E, and K. The natural forms of these vitamins in fish are cholecalciferol (vitamin D_3) (*21*), *all-trans*-, 9-*cis*-, and 13-*cis*-retinol (vitamin A) (*22*), α-tocopherol (*23*), and phylloquinone (vitamin K_1) and menaquinones (vitamins MK-n; commonly known as K_2) (*24*, *25*). The fat-soluble vitamins act as essential factors in a wide range of biological functions in fish, for example, as antioxidants for protecting polyunsaturated fatty acids from oxidation (vitamin E) (*26*) or bone mineralization and/

*Corresponding author (phone +47 55 50 12 74; fax +47 55 50 12 99; e-mail aage.oterhals@nofima.no).

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or development (vitamins D and K) (*21*, *25*). Seafood, and in particular oily fish, is an important natural source for fat-soluble vitamin intake (e.g., vitamin D) in humans (*27*).

Fish oils are high in omega-3 polyunsaturated fatty acids (PUFA) and included in feed formulations to cover nutritional needs or in excess to give an edible product with the desired level of omega-3 PUFA (*28*). The health beneficiary effects of omega-3 PUFA in human nutrition include protective effects on cardiovascular disease, cancer, and inflammatory diseases. In addition, there is increasing documentation on positive effects on Alzheimer's disease, depression, and stress (*29*). The high PUFA content makes fish oil susceptible to oxidation with the resulting generation of hydroperoxides that can undergo further chemical transformations to yield a variety of rearranged and chain-cleavage products with possible negative implications on human health (*30*).

Modeling of the effect of process conditions (i.e., temperature, feed rate, and distillate flow rate) on the reduction of polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs), dioxin-like polychlorinated biphenyls (DL-PCBs), and polybrominated diphenyl ethers (PBDEs) has earlier been reported on the basis of the same SPD trials as used in this study (*10*). Use of SPD to remove POPs in fish oil will inevitably also remove other volatile minor compounds and thereby change the nutritional composition and oxidative stability of the processed oil. The main objectives of this study were (1) to quantify effects of alternative SPD process conditions on the nutritional composition and oxidative stability of fish oil and (2) to identify optimal process conditions combining decontamination effects in compliance with legislation levels and maximum retention of nutritional quality.

MATERIALS AND METHODS

Materials. Commercial crude fish oil was purchased from a Scandinavian fishmeal and oil producer. The fish oil was mainly produced from sprat (*Sprattus sprattus*) caught in the North Sea region and had a free fatty acid content of 46 g kg^{-1}. The oil was alkali refined and bleached as earlier described (*6*) before use as feedstock in the SPD experiments (*10*). The working fluid (WF), a light distillate byproduct fraction of fatty acid ethyl esters from the commercial production of EPA and DHA concentrate, was donated by Pronova Biopharma AS, Sandefjord, Norway. All solvents and reagents for the analyses were of analytical grade.

Process Conditions. The SPD process was performed on a stainless steel KD6 pilot plant (UIC GmbH, Alzenau-Hörstein, Germany) as earlier described (*10*). The evaporator was a falling film system (surface area = 0.06 m^2) equipped with a roller wiper (400 rpm) and a vacuum system consisting of a cold trap (−25 °C), oil diffusion, and rotary vane pump in series. The internal condenser temperature was 60 °C. The residue was collected in a residue cup (160 °C) and pumped through a cooler before sampling under nitrogen cover. Residue samples were collected in glass bottles and stored under nitrogen at −20 °C before analysis.

Oxidation Level. Peroxide value (PV) and anisidine value (AV) were determined according to AOCS methods Cd 8b-90 and Cd 18-90, respectively (*31*). Results given in the tables are averages of duplicate analyses.

Conjugated Double Bonds. Dienes and trienes were measured by UV spectrometry at 232 and 268 nm, respectively, and reported as specific absorbance in a solution of 10 g L^{-1} of fat in isooctane and a path length of 1 cm (DGF-method C-IV 6a (57)) (*32*). Results given in the tables are averages of duplicate analyses.

Fatty Acid Isomers. Trans isomers of polyunsaturated fatty acids were measured according to the method of Mjøs and Solvang (*15*). Fatty acid methyl esters in isooctane were analyzed on a HP-5890 gas chromatograph equipped with a HP-5972 mass spectrometric detector (Agilent, Palo Alto, CA) and a BPX-70 capillary column, L = 60 m, i.d. = 0.25 mm, d_f = 0.25 μm (SGE, Ringwood, Australia). Reported results are based on duplicate analyses.

Fatty Acid Composition. Fatty acid composition was analyzed using methods described by Lie and Lambertsen (*33*) and modified by Jordal et al. (*34*). Fish oil samples were saponified and methylated using 12% BF_3 in methanol and the methyl esters separated using a Trace gas chromatograph 2000 (Fison, Elmer, USA) ("cold on column" injection, 60 °C for 1 min, 25 °C/min, 160 °C for 28 min, 25 °C/min, 190 °C for 17 min, 25 °C/min, 220 °C for 10 min), equipped with a 50 m CP-sil 88 (Chromopack) fused silica capillary column (i.d. = 0.32 mm). The fatty acids were identified by retention time using standard mixtures of methyl esters (Nu-Chek, Elyian, MN). All samples were integrated using Totalchrom software (ver. 6.2, Perkin-Elmer) connected to the GLC. Amount of fatty acid per kilogram of sample was calculated using 19:0 methyl ester as internal standard. Reported results are based on single analyses.

Vitamins. Phylloquinone and menaquinones were measured in fish oil after liquid−liquid extraction and HPLC analysis with fluorometric detection after postcolumn reduction with metallic zinc, as described by Schurgers et al. (*24*). The chromatographic system consisted of a reverse-phase (Hypersil H5ODS 4.6 mm × 25 cm, HiCrom, Reading, U.K.) and steel reduction column, 4 mm × 12.5 cm (Grace Davidson Discovery Science, Shelton, CT), in series followed by fluorescence detection (Shimadzu RF-10AXL, Kyoto, Japan) using excitation and emission wavelengths of 243 and 430 nm, respectively. The mobile phase consisted of 1:9 dichloromethane/methanol with added 5% (v/v) zinc chloride acetate solution (13.7 g of zinc chloride, 4.1 g of sodium acetate, and 3.0 g of acetic acid in 50 mL of methanol). Vitamin D_3 was measured by HPLC using vitamin D_2 as internal standard according to the method of Horvli et al. (*35*). Samples where cleaned up on a silica column and analyzed using a C-18 column (Ace 5 C18, 5 μm, 4.6 × 250 mm) with UV detection (LaChrom, Merck Hitachi L-7420, Darmstadt, Germany). The mobile phase consisted of 12% (v/v) methanol and 6% (v/v) chloroform in acetonitrile. Vitamins A_1 and A_2 were measured using an HPLC procedure modified from that of Nôll (*36*). The HPLC system consisted of a Hichrom Hypersil column (4.6 × 150 mm, 3 μm particle size, Reading, U.K.) and UV detector (Shimadzu SPD-2 A) at 325 nm. The mobile phase was 90:10 hexane/isopropanol. Vitamin E (α- and γ-tocopherol) was analyzed by HPLC as described by Lie et al. (*37*). The chromatographic system consisted of a LiChroCART column (4.6 × 125 mm, Purospher STAR Si, 3 μm, Darmstadt, Germany) and a fluorescence detector (TSP, Spectra system, FL3000, Riviera Beach, FL), with a mobile phase of 5% (v/v) tetrahydrofuran in hexane. Reported results are based on duplicate analyses.

Lipid Classes. The quantification of lipid class composition was performed using high-performance thin-layer chromatography (HPTLC) as described by Bell et al. (*38*) and modified by Jordal et al. (*34*). Ten micrograms of fish oil was applied to a 10 × 20 cm HPTLC plate (silica gel 60, Merck) that had been prerun in hexane/diethyl ether (1:1 v/v) and activated at 110 °C for 30 min. The plates were developed at 5.5 cm in methyl acetate/isopropanol/chloroform/methanol/0.25% (w/v) aqueous KCl (25:25:25:10:9, by volume) to separate phospholipid classes with neutral lipids running at the solvent front (*39*). After drying, the plates were developed fully in hexane/diethyl ether/acetic acid (80:20:2, v/v/v) to separate neutral lipids and cholesterol. Lipid classes were visualized by charring at 160 °C for 15 min after spraying with 3% copper acetate (w/v) in 8% (v/v) phosphoric acid and identified by comparison with commercially available standards. Lipid classes were quantified by scanning densitometry using a CAMAG TLC Scanner 3 and calculated using an integrator (WinCATS-Planar Chromatography, version 1.2.0). Furthermore, quantitative determination (mg of lipid class/g of sample) of lipid classes was performed by establishing standard curves for each lipid class within a linear area, in addition to including a standard mix of all the lipid classes at each HPTLC plate for corrections of between-plate variations. Reported results are based on duplicate analyses.

Unsaponifiable Matter. The samples were saponified with KOH, and the unsaponifiable fraction was quantified gravimetrically according to AOCS method Ca 6b-53 (*31*). Reported results are based on duplicate analyses.

Oxidative Stability. The oxidative stability of the oil samples where assessed based on a weight gain method modified after Olcott and Einset (*40*). Ten gram (±0.01 g) of oil was transferred to a polystyrene Petri dish (i.d. = 8.6 cm; Heger AS, Rjukan, Norway) and stored in the dark in a convection oven at 36 °C. The percent weight gain, WG, given by

$$WG = [(W_t - W_0)/W_0] \times 100 \quad (1)$$

where W_t and W_0 are the weights at a given time and at time zero, respectively, was used to follow the oxidation process in pure oil samples and after the addition of 150 ppm BHT. The induction period (IP) was defined as the time needed to obtain a WG of 0.5% and reported on the basis of the measurement of four parallel samples.

Experimental Design. The factorial design consisted of a 2-factorial rotatable central composite design (CCD) based on the variables evaporator temperature (ET) and feed rate (FR) extended to test the effect of 4% working fluid (WF) with one center point (**Table 1**) (*10*). The responses were the different measurements of nutritional composition and oxidation parameters of the fish oil.

Statistical Analyses. The experimental data were fitted to a second-order polynomial (eq 2) by means of multiple linear regression (MLR) (*41*) with assistance of Statistica v.7.0 (StatSoft, Inc., Tulsa, OK). In the models y is the estimated response, b_0 is the intercept, b_i, b_{ij}, and b_{ii} are the regression coefficients of each factor, of the interaction term between them, and of each squared term, respectively, x is the predictor variable, and ε is the residual (error).

$$y = b_0 + \Sigma b_i x_i + \Sigma b_{ij} x_i x_j + \Sigma b_{ii} x_i^2 + \varepsilon \quad (2)$$

The predictor variables were mean centered and standardized before performance of the regression analysis. The best subset model was identified on the bais of backward removal of insignificant regressors (p remove > 0.05). The quality of the fitted models was evaluated on the basis of ANOVA, F statistics, and the coefficient of multiple determinations (R^2). Comparison between oxidation parameters in AB oil and the mean value after SPD treatment was based on a single-sample t test. Trend curves given in **Figure 3b** are based on second-order polynomial models in Excel 2002 (Microsoft Corp.).

RESULTS AND DISCUSSION

Polyunsaturated Fatty Acids. A small reduction of the EPA + DHA level in the crude oil from 209 to 204 g kg^{-1} fatty acids after the alkali refining and bleaching process could be observed (**Table 2**). However, after SPD treatment no significant alterations in the EPA + DHA content were observed, with the highest levels above the crude oil (**Table 2**). Possible thermally induced geometrical isomerization was studied in the crude oil, after alkali refining and bleaching and at three SPD process conditions to cover the span in operation temperature from 172 to 228 °C at a feed rate of 5 kg/h (i.e., expts 5, 7, and 8; **Table 1**). No trans isomerization of EPA and DHA could be detected (i.e., < 0.1%; **Table 2**) in any of the analyzed samples. Geometrical isomerization of fish oil during deodorization has been documented in commercial samples (*16*) and after experimental heat treatment for 2 h at 180 °C (*15*). The preservation of polyunsaturated fatty acids at all tested operation conditions up to 228 °C in this study confirms the gentle conditions obtainable with SPD compared to steam deodorization.

Table 1. Coded and Actual Levels for the Experimental Design Variables (ET, FR, and WF)[a]

expt	coded value			ET (°C)	FR (kg/h)	WF (%)
1	−1	1	−1	180	7.04	0
2	0	−1.41	−1	200	2.30	0
3	0	0	−1	200	5.06	0
4	−1	−1	−1	180	3.10	0
5	−1.41	0	−1	172	5.01	0
6	0	1.41	−1	200	7.83	0
7	0	0	−1	200	5.11	0
8	1.41	0	−1	228	5.03	0
9	1	−1	−1	220	3.06	0
10	0	0	−1	200	5.04	0
11	1	1	−1	220	7.05	0
12	0	0	0	200	5.10	2.0
13	−1	1	1	180	7.05	4.0
14	1	1	1	220	6.99	4.0
15	1	−1	1	220	2.93	4.0
16	−1	−1	1	180	3.03	4.0

[a] ET, evaporator temperature; FR, feed rate; WF, working fluid level in feedstock.

Minor Nutrients Retention. Retention of semivolatile compounds such as vitamins, cholesterol, and other unsaponifiable compounds in the residue flow after SPD depends on the vapor pressure and molecular weight of the individual compounds. The evaporation rate (j = mol m^{-2} s^{-1}) can be described by the Langmuir−Knudsen equation (*10*):

$$j_i = \gamma_i x_i P_i^0(T) \sqrt{\frac{1}{2\pi R M_i T}} \quad (3)$$

γ_i is the activity coefficient, x_i the mole fraction, $P_i^0(T)$ the saturation vapor pressure in Pa at absolute temperature T in Kelvin, R the gas constant, and M_i the molecular weight. Vitamins A, E, and D and cholesterol contain a hydroxyl group and can theoretically be found in both free and esterified forms. The chemical nature of the individual compounds will strongly influence the chemical and physical properties and thereby the evaporation rate under high vacuum according to eq 3. For the studied system the amount of distillate relative to feedstock (WF not included) was very low (i.e., < 0.9%) (*10*), and evaporation of the more volatile compounds will have an insignificant influence on the mole fractions of other compounds in the residue. Assuming the system obeys Raolt's law, eq 3 can be simplified to calculate the relative volatility of two compounds:

$$\alpha = j_1/j_2 = \frac{x_1 P_1^0}{x_2 P_2^0} \times \frac{\sqrt{M_2}}{\sqrt{M_1}} \quad (4)$$

Analysis of the chemical nature (i.e., free and esterified forms) of the studied compounds was outside the scope of this study but will have a significant impact on the relative retention of individual nutritional compounds found in fish oil. Vapor pressure data in the studied temperature range are in addition available for only a few of the possible candidate compounds, making a direct comparison based on such criteria difficult. Hickman (*42*) has studied the state and elimination of vitamins A and D in cod liver

Table 2. Content and Retention of EPA + DHA, Trans Isomers, and Minor Compounds in Crude, AB, and SPD Oils (Experiments 1−16)[a]

	EPA + DHA (g kg^{-1} FA)	trans isomers EPA/DHA (g kg^{-1})	vitamin A (mg kg^{-1})	vitamin D (μg kg^{-1})	vitamin E (mg kg^{-1})	vitamin K (μg kg^{-1})	cholesterol (g kg^{-1})	unsaponifiables (g kg^{-1})
crude oil	209	ND	16.1	790	75.2	296	27.1	10.1
AB oil	204	ND	ND	670	78.2	357	22.7	10.3
SPD oil (range)	199−215	ND[d]	ND	119−774	28.0−84.0	208−335	6.9−25.3	5.5−10.2
retention (%)								
AB oil[b]	98		0	85	104	121	84	102
SPD oil[c] (range)	97−105			18−115	36−107	58−97	30−111	54−99

[a] FA, fatty acids; ND, not detected; AB, alkali refined and bleached; SPD, short-path distilled. [b] Relative to crude oil. [c] Relative to AB oil. [d] Analyzed in expts 5, 7, and 8.

oil by use of molecular distillation. The experimental procedure was based on repeated distillation of an oil sample in a cyclic still employing constant time and 10 °C temperature intervals (*43*). The obtained elimination curves for vitamins A and D confirmed the existence of both free and esterified forms of the vitamins. In cod liver oil vitamin A was predominantly found in esterified form with elimination maxima around 120 and 200−210 °C, respectively (*42*). Vitamin D was partly observed in free form with elimination maxima around 160 °C and as a mixture of ester forms, giving several temperature maxima on the elimination curve in the 210−250 °C range (*42*).

Vitamin A in the fish oil was removed after the alkali refining and bleaching process (**Table 2**). On the basis of the elimination curves published by Hickman (*42*) vitamin A in the unesterified form will be substantially reduced by the applied SPD conditions (**Table 1**). However, if esterified, retention somewhat lower than that of vitamin D can be expected depending on the composition of the fatty acid moiety.

The vitamin D level was increased by 21% after alkali refining and bleaching. This increase is difficult to explain, but falls within the analytical uncertainty. Retention of vitamin D after SPD was at lowest 58% (**Table 2**), which implies that a large part of this vitamin must be present in esterified form (*42*). Müller-Mulot et al. (*44*) have documented that 40−70% of the vitamin D_3 in cod liver oil is present as fatty acid esters, covering the range from C16:0 to C22:6. However, we are not aware of any published studies on this partitioning in whole-body fish oils. The obtained response surface (**Figure 1a**) shows significant effects of all main variables (**Table 3**). The squared FR effect is, however, difficult to explain and is also, to a lesser degree, found in the cholesterol response surface (**Figure 1g**). Addition of WF has a limited effect in the low-temperature range but is reinforced at higher temperature levels due to a negative ET × WF interaction.

Vitamin E (α-tocopherol) exists in free form (*23*), and the crude oil level was retained after alkali refining and bleaching followed by a reduction, at lowest down to 36% retention, after SPD (**Table 2**). The response surface shows a significant effect of all main variables with a positive ET × FR interaction (**Table 3**; **Figure 1c**). Tocopherol retention and concentration have been studied by several research groups. Xu et al. (*20*) reported retention levels of 47% at an evaporator temperature of 200 °C and a feed rate of 4.6 kg/h in a similar KD6 apparatus. This is significantly lower than the 90% retention observed in this study at comparable conditions (i.e., expt 12, **Table 1**). Martinello et al. (*45*) have studied the retention of tocopherols in wheat germ oil after SPD and observed retention levels of 97 and 90% at evaporator temperatures of 200 and 220 °C, respectively. This is more consistent with the results observed in this study, in which only the two high-temperature−low-flow rate conditions (expts 9 and 15) gave retention levels below 72%.

Vitamin K was reduced after alkali refining and bleaching (**Table 2**). The higher levels observed after SPD indicate that this is within the analytical uncertainty. The reported vitamin K level in **Table 2** reflects a mixture of phylloquinone (vitamin K_1) and menaquinones (vitamins MK-n) with the following levels observed in the alkali refining and bleached oil: K_1, 53.1 μg/kg; MK-4, 67.6 μg/kg; MK-6, 13.1 μg/kg; MK-7, 39.2 μg/kg; MK-8, 182.9 μg/kg. The vapor pressure and molecular weight of the K vitamins are decreasing and increasing, respectively, in the listed order. The influence on the evaporation rate of the individual K vitamins according to eqs 3 and 4 is reflected by the observed order of retention relative to the reduction of WHO-PCDD/F-PCB-TEQ: K_1 < MK-4 < MK-7 < MK-8 (**Figure 2**). The high retention observed for MK-6 (**Figure 2**) is probably a combined effect of a low initial level and analytical uncertainty. The vitamin K group consists of a homologous series of molecules, and a common model based on combined process parameters and quantitative structure properties relationships (QSPR) might be developed as earlier reported for modeling of PCDD/F, PCB, and PBDE reductions (*10*). However, this has not been further pursued in this study.

Lipid class analysis of the crude oil showed the presence of 1,2- and 1,3-diacylglycerols (17 and 26 g kg^{-1}, respectively) with levels unchanged after alkali refining and SPD. The free fatty acid level was reduced from 93 to 17 g kg^{-1} after alkali refining and bleaching with some reduction after SPD (range of 10−18 g kg^{-1}). The higher levels observed relative to titration of free fatty acids based on AOCS-method Ca 5-40 (46 and 0.7 g kg^{-1}, respectively) (*10*) may be explained by different quantification methods. Phospholipids were not detected in the crude oil. Cholesterol was found only in free form and reduced after the refining and bleaching process (**Table 2**). This effect has been studied by Verleyen et al. (*12*) and attributed to the formation and transfer of free sterol containing micelles into the soapstock. Retention after SPD was at lowest down to 31% (**Table 2**) and is consistent with data reported by Sondbø and Thorstad (*19*). The response model shows a negative WF effect on the retention level, but this is partly reduced by a positive WF × WF effect (**Table 3**). A negative FR × FR is also observed but less influential than in the vitamin D model. The resulting odd-shaped response surface (**Figure 1g**) may be explained by crystallization of evaporated cholesterol on the internal condenser when no WF was added (*10*). This phase transition reduced the re-evaporation rate and also the effect of WF addition. The ratio between free and esterified cholesterol in fish oil is reported to be related to the freshness of the processed fish (*46*). Studies performed by IFFO (*47*) have confirmed this observation and demonstrated a significant correlation between the FFA level and the amount of cholesterol ester found in commercial fish oil. Levels of free cholesterol ranged from 0.18 to 1.17% and those of esterified cholesterol from 0.01 to 0.60% with mean values of 0.59 and 0.17%, respectively.

A significant correlation between the level of cholesterol and unsaponifiable compounds ($R^2 = 0.84$) could be observed in this study. The unsaponifiable fraction of fish oil includes cholesterol, glyceryl ethers, hydrocarbons, fatty alcohols, vitamins, and POPs (*48*). It is based on gravimetric analysis of diethyl ether extractable compounds after KOH saponification of the oil sample. The observed cholesterol level based on HPTLC analysis is, however, significantly higher than that of unsaponifiable compounds (**Table 2**) with a ratio between 1.3 and 2.5. Cholesterol level analyzed in the feedstock based on HPLC and mass detector (*10*) has given a level of 0.5%, well below and more consistent with the analyzed level of unsaponifiables and other reported levels of cholesterol in fish oils (*47*). The reported cholesterol levels in **Table 2** are probably not accurate but considered to be precise on the basis of the high correlation to unsaponifiable compounds. Correspondingly, the obtained high correlation between observed and predicted levels for the cholesterol retention model (**Table 3**; **Figure 1h**) supports this view. Cholesterol is also removed to roughly the same extent as vitamin E (**Figure 1c**,**g**). This is consistent with comparable vapor pressure levels in the studied temperature range (*49*).

The level of unsaponifiable compounds was almost unchanged after alkali refining and bleaching (**Table 2**). Unsaponifiables comprise a complex mixture of compounds with different physicochemical properties. The observed retention level (**Table 2**) represents the combined mean of cholesterol and several unknown constituents. The response surface still follows the same pattern with significant main variables, squared ET, and ET × FR interaction (**Table 3**; **Figure 1i**).

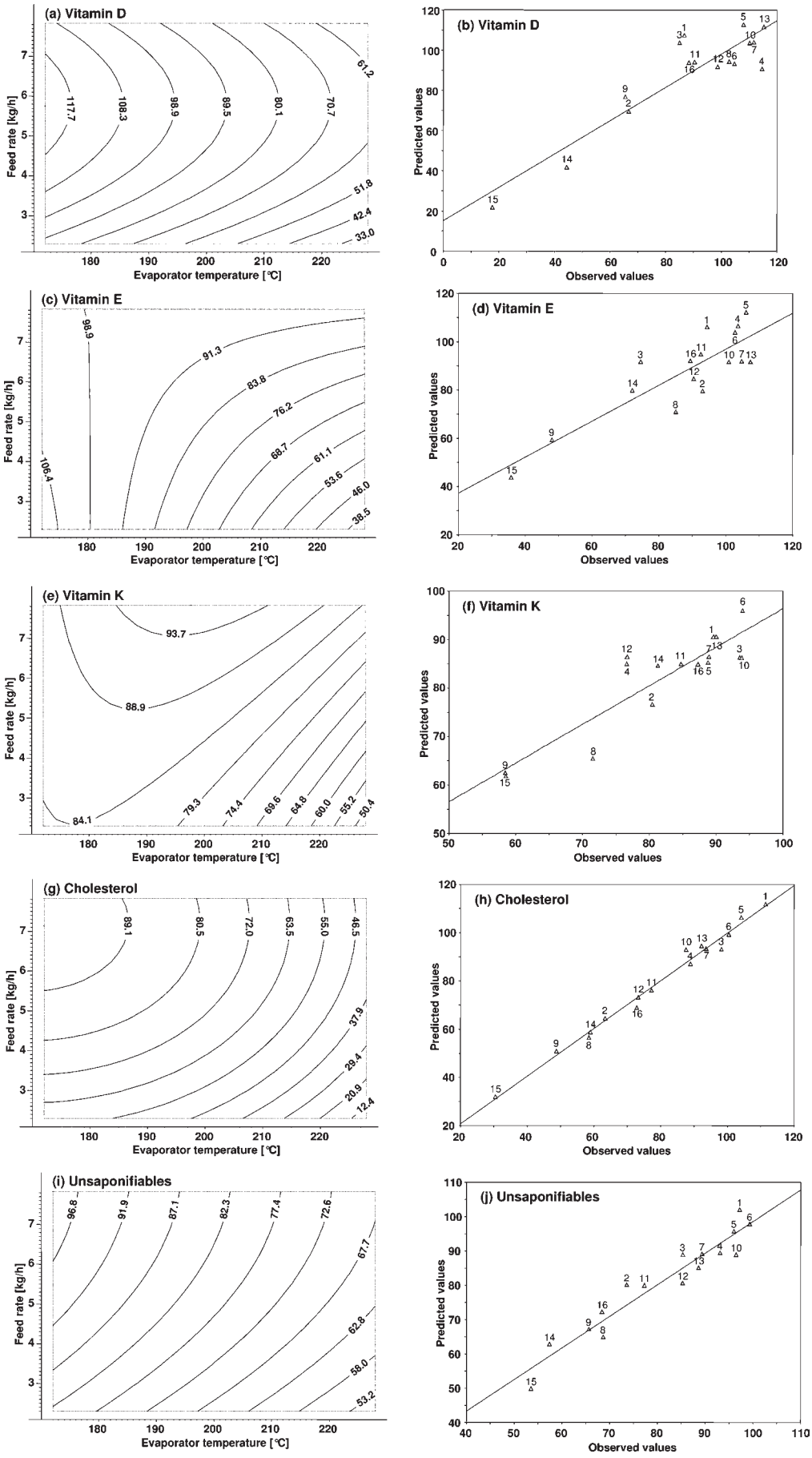

Figure 1. Contour plots and observed versus predicted values for the retention after SPD treatment (expts 1−16, WF addition 2%) of vitamins D, E, and K, cholesterol, and unsaponifiables.

Table 3. Response Models after Backward Elimination of Nonsignificant (NS) Regression Coefficients Representing Retention of Minor Compounds (Vitamins, Cholesterol, and Unsaponifiables) and Alteration of Oxidation Parameters (PV, AV, and IP) after Short-Path Distillation Relative to AB Oil[a]

variable	intercept	ET	ET × ET	FR	FR × FR	WF	WF × WF	ET × FR	ET × WF	FR × WF	R^2
vitamin D	96.88	−13.03	NS	7.82[b]	−9.22[c]	−10.81	NS	NS	−11.35	NS	0.828
vitamin E	87.64	−13.04	NS	7.79	NS	−6.43[d]	NS	7.12[e]	NS	NS	0.745
vitamin K	86.27	−6.25	−4.38	6.23	NS	NS	NS	3.32[f]	NS	NS	0.796
cholesterol	78.92	−15.89	−4.64	11.27	−4.63	−16.64	9.10	NS	NS	NS	0.987
unsaponifiables	84.20	−9.83	−3.43	5.68	NS	−7.54	NS	84.20	NS	NS	0.919
PV	−49.58	NS	−11.23[g]	NS	NS			NS			0.332
AV	−9.42	−1.84[h]	NS	NS	NS			NS			0.427
IP	4.85	−8.77	−3.83	6.13	NS			NS			0.928

[a] ET, temperature; FR, feed rate; WF, working fluid; PV, peroxide value; AV, anisidine value; IP, induction period; AB, alkali refined. [b] $p = 5.5 \times 10^{-2}$. [c] $p = 6.0 \times 10^{-2}$. [d] $p = 6.7 \times 10^{-2}$. [e] $p = 6.2 \times 10^{-2}$. [f] $p = 7.4 \times 10^{-2}$. [g] $p = 9.4 \times 10^{-2}$. [h] $p = 6.4 \times 10^{-2}$.

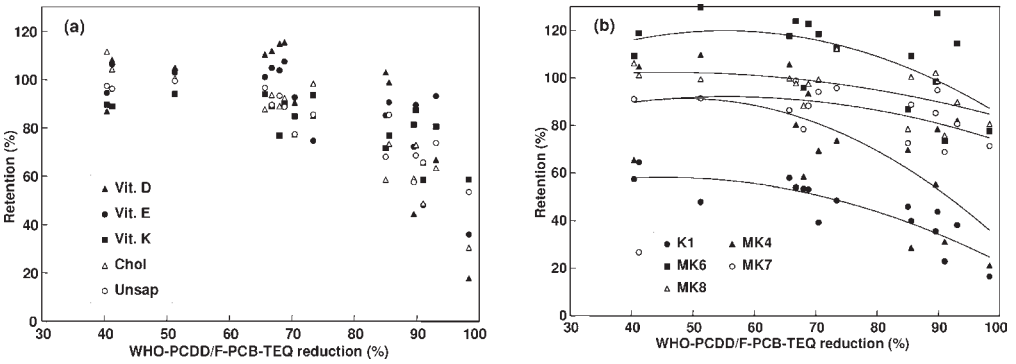

Figure 2. Retention of nutrients depending on the obtained WHO-PCDD/F-PCB-TEQ reduction (*10*): (**a**) vitamins E, D, and K, cholesterol, and unsaponifiables; (**b**) vitamins K_1 and MK-n.

With the exception of cholesterol and unsaponifiables, the plotting of observed versus predicted values in **Figure 1** reveals a clustering of most of the observed responses at retention levels >80%. This lack of spread in the observations has inevitably made it difficult to establish good response models as reflected by the lower R^2 levels in **Table 3**. However, the analysis of variance shows no lack of fit for any of the models representing vitamin, cholesterol, and unsaponifiables retention (**Table 5**). This indicates that the established MLR models (**Table 3**) represent a correct relationship between the responses and process variables. For vitamins D and E a large discrepancy between the observed level in expt 3 and the two other center points, expts 7 and 10, was registered (**Figure 1b**,**d**). Removal of expt 3 in the modeling improved R^2 and the root mean squared error of calibration (RMSEC). However, the respective experimental observation was not identified as an outlier on the basis of studentized residuals and therefore included in the reported regression models.

Oxidation Parameters. Oxidation level and oxidative stability are critical quality parameters for the application of fish oil in food and feed formulations. Alkali refining and bleaching of the crude oil reduced the level of all measured oxidation parameters, that is, peroxide value (PV), anisidine value (AV), Totox = 2 × PV + AV, and conjugated double bonds (**Table 4**). PV, AV, and Totox were further reduced after SPD. The reduced PV can be explained by thermal decomposition of hydroperoxides at elevated temperature levels, especially on the evaporator surface. Decomposition of hydroperoxides gives rise to a complex mixture of monomeric, polymeric, and small molecular weight volatile compounds. The reaction products will, depending on volatility, contribute to the final AV after SPD. Hydroperoxides may also react with tocopherol or cholesterol in the fish oil. Cholesterol oxidation products (COPs) have been reported in refined and deodorized fish oil but at very low levels (*50*). MLR gave rather poor response models for PV and AV with only ET × ET and ET, respectively, as significant variables (**Table 3**). For PV the observed levels can be divided into high and low groups (**Table 4**), and ANOVA shows a significant lack of fit (**Table 5**). This skewed distribution might indicate uncontrolled experimental or analytical variables influencing the results. Clear conclusions based on the results should be made with care, but the observed lack of FR influence indicates that the residence time on the evaporator surface might be of less importance for the thermal decomposition of hydroperoxides and the removal of anisidine reactive substances (i.e., volatile aldehydes). The lowest obtained PV values (i.e., 0.2−0.3 mequiv/kg) were observed at both low and high ET levels (**Table 4**) and demonstrate the possible almost complete decomposition of hydroperoxides at the applied SPD conditions.

Conjugated double bonds measured as the absorbance at 232 nm (dienes) and 268 nm (trienes) (**Table 4**) were reduced after the alkali refining and bleaching process. However, no significant models could be established for the two responses after SPD. The diene levels were to a small, but significant, extent ($p = 0.030$) further reduced on the basis of the mean value after SPD (**Table 4**). Experiment 4 fell outside the normal distribution and was excluded from the statistical analysis. The mean triene level increased significantly ($p = 0.0002$), but was still below the level observed in the crude oil.

The obtained mean PV, AV, diene, and triene values are all lower compared to levels observed after activated carbon treatment of the same feedstock fish oil (*6*). This comparison underscores the quality-retaining conditions possible to obtain by use of SPD technology to reduce the level of POPs in fish oil.

The oxidative stability of the fish oil was considerably reduced after alkali refining and bleaching (**Table 4**). Wanasundara et al. (*51*) measured the oxidative stability of crude, alkali refined, bleached, and deodorized cod liver and seal blubber oil and found the crude oil to be most stable and its alkali refined and bleached

Table 4. Oxidation Parameters and Oxidative Stability (± SD) in Crude, AB, and SPD Oils (Experiments 1−11)[a]

sample	PV (mequiv/kg)	AV	Totox	A_{232}[b]	A_{268}[b]	IP (h)	IP_{BHT} (h)
crude oil	1.8	10.4	14.0	8.91	0.55	311.8 ± 2.6	427.7 ± 4.5
AB oil	1.7	6.0	9.4	7.64	0.32	163.1 ± 0.8	323.6 ± 4.9
expt 1	0.9	5.6	7.4	7.55	0.48	183.2 ± 1.6	344.4 ± 4.9
expt 2	1.1	5.4	7.6	7.70	0.50	156.0 ± 1.0	328.6 ± 9.3
expt 3	0.9	5.3	7.1	7.59	0.53	179.9 ± 1.1	340.1 ± 2.7
expt 4	0.9	5.7	7.5	7.19	0.44	165.7 ± 0.9	344.6 ± 0.3
expt 5	0.2	5.5	5.9	7.49	0.56	181.5 ± 1.1	339.1 ± 2.9
expt 6	0.3	5.9	6.5	7.65	0.40	180.7 ± 1.4	347.5 ± 9.3
expt 7	0.9	5.6	7.4	7.57	0.45	173.0 ± 1.4	356.2 ± 9.6
expt 8	0.2	5.2	5.6	7.56	0.44	135.8 ± 0.4	340.5 ± 7.1
expt 9	0.3	5.3	5.9	7.52	0.26	130.2 ± 1.5	318.1 ± 2.0
expt 10	0.9	5.8	7.6	7.63	0.47	169.6 ± 0.2	331.9 ± 6.3
expt 11	1.0	5.4	7.4	7.64	0.47	160.6 ± 0.9	322.7 ± 7.8
mean value[c]	0.7 ± 0.4	5.5 ± 0.2	6.9 ± 0.8	7.55 ± 0.13	0.45 ± 0.08	165.1 ± 18.2	337.6 ± 11.3

[a] AB, alkali refined; SPD, short-path distillation; PV, peroxide value; AV, anisidine value; Totox, 2 × PV + AV; IP, induction period; IP_{BHT}, induction period after addition of 150 ppm BHT. [b] Absorbance of 10 g L^{-1} of fat in isooctane at 232 and 268 nm, respectively. [c] Mean value of expts 1−11 ± SD.

Table 5. ANOVA for the Fitted Models

source of variation	model	sum of squares	DF	mean square	*F* ratio	*p* value
regression	vitamin E	4.82×10^3	4	1.20×10^3	7.97[a]	2.9×10^{-3}
	vitamin D	9.41×10^3	5	1.88×10^3	9.61[a]	1.4×10^{-3}
	vitamin K	1.55×10^3	4	3.88×10^2	1.10×10^{1a}	7.8×10^{-4}
	cholesterol	7.39×10^3	6	1.23×10^3	1.11×10^{2a}	6.4×10^{-8}
	unsaponifiables	3.04×10^3	4	7.60×10^2	3.11×10^{1a}	6.1×10^{-6}
	IP	1.29×10^3	3	4.30×10^2	2.98×10^{1a}	2.3×10^{-4}
	PV	1.20×10^3	1	1.20×10^3	3.52[a]	9.4×10^{-2}
	AV	3.38×10^1	1	3.38×10^1	4.44[a]	6.4×10^{-2}
residual	vitamin E	1.66×10^3	11	1.51×10^2		
	vitamin D	1.96×10^3	10	1.96×10^2		
	vitamin K	3.89×10^2	11	3.54×10^1		
	cholesterol	9.98×10^1	9	1.11×10^1		
	unsaponifiables	2.69×10^2	11	2.44×10^1		
	IP	1.01×10^2	7	1.44×10^1		
	PV	3.08×10^3	9	3.42×10^2		
	AV	6.86×10^1	9	7.62		
lack of fit	vitamin E	1.12×10^3	9	1.24×10^2	4.60×10^{-1b}	8.3×10^{-1}
	vitamin D	1.51×10^3	8	1.88×10^2	8.37×10^{-1b}	6.5×10^{-1}
	vitamin K	3.74×10^2	9	4.16×10^1	5.48[b]	1.6×10^{-1}
	cholesterol	4.34×10^1	7	6.19	2.19×10^{-1c}	9.5×10^{-1}
	unsaponifiables	2.05×10^2	9	2.28×10^1	7.14×10^{-1b}	7.0×10^{-1}
	IP	8.10×10^1	5	1.62×10^1	1.64[b]	4.2×10^{-1}
	PV	3.07×10^3	7	4.39×10^2	2.34×10^{2b}	4.3×10^{-3}
	AV	2.17×10^1	7	3.10	1.32×10^{-1b}	9.8×10^{-1}
pure error	vitamin E	5.41×10^2	2	2.71×10^2		
	vitamin D	4.50×10^2	2	2.25×10^2		
	vitamin K	1.52×10^1	2	7.58		
	cholesterol	5.64×10^1	2	2.82×10^1		
	unsaponifiables	6.38×10^1	2	3.19×10^1		
	IP	1.98×10^1	2	9.89		
	PV	3.75	2	1.88		
	AV	4.69×10^1	2	2.34×10^1		
total	vitamin E	6.48×10^3	15			
	vitamin D	1.14×10^4	15			
	vitamin K	1.94×10^3	15			
	cuolesterol	7.49×10^3	15			
	unsaponifiables	3.31×10^3	15			
	IP	1.39×10^3	10			
	PV	4.28×10^3	10			
	AV	1.02×10^2	10			

[a] *F* ratio (regression/residual). [b] *F* ratio (lack of fit/pure error).

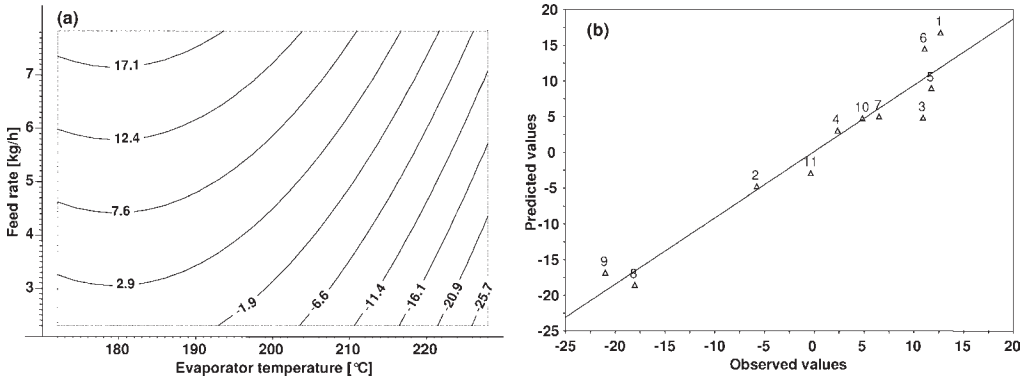

Figure 3. Alteration (%) of oxidative stability of SPD-treated oil (expts 1−11) relative to feedstock: (**a**) contour plot; (**b**) observed versus predicted values.

counterpart least stable. The findings were explained by removal of antioxidants (i.e., α-tocopherol), including phospholipid synergists, during bleaching. Phospholipid content was not assessed in this study but is generally low in crude fish oils (i.e., 5−100 ppm P) (*48*) compared to crude vegetable oils. Combined with the high retention of tocopherol documented in this study (**Table 2**), such mechanisms are less likely to fully explain the observed reduction in oxidative stability. Other compounds found in marine oils, such as retinol (*52*), peptides, peptide−lipid oxidation products (*53*), and polyamines (*54*) should be considered as possibly contributing and synergistic candidates. Especially the complete removal of vitamin A after alkali refining and bleaching (**Table 2**) may be an important contributing factor. However, we are not aware of any published study on the antioxidant effect of retinol in fish oil.

After SPD both further reduction and improved oxidative stability could be observed. The obtained response surface shows significant main and ET × ET effects (**Table 3**; **Figure 3**) and no lack of fit based on ANOVA (**Table 5**). The reduced oxidative stability at high temperature and low feed rate might be attributed to the increased loss of tocopherol at these operation conditions (**Figure 2**). Correspondingly, the combined high retention of tocopherol and reduced PV at low temperature and high feed rate conditions might explain the improved oxidative stability in the opposite operation region. Butylated hydroxytoluene (BHT) is the antioxidant normally applied to stabilize fish oil used in fish feed formulations with a maximum permitted level of 150 ppm. Addition of 150 ppm BHT to the fish oil samples improved the oxidative stability (**Table 4**). The observed improvement was, however, lower in the crude oil (116 h) compared to the AB oil (165 h) and the mean value after SPD (176 h). This observation indicates the presence of constituents in the crude oil giving negative interaction effects combined with BHT. Addition of BHT also increased the variance of the measured induction periods (**Table 4**), and it was not possible to establish a significant response surface on the basis of this data set.

Optimal Process Conditions. To identify the best operation conditions for decontamination of fish oil by SPD, the documented loss of nutrients and reduced oxidative stability must be combined with knowledge of effects on PCDD/F-PCB-TEQ reduction. The feedstock used in this study contained 20.5 ng of WHO-PCDD/F-PCB-TEQ kg^{-1} (*10*). This is below the present EC legislation (*55*) on the maximum permitted level (MPL) in fish oil for use in animal feed on a 12% moisture basis (24 ng of WHO-TEQ kg^{-1}) but above the present combined MPLs in fish oil for food applications (*56*) (12 ng of WHO-TEQ kg^{-1}). Modeling of PCDD/F and DL-PCB reduction based on the same experimental oil samples has been published earlier by Oterhals et al. (*10*), and TEQ reduction of >90% can easily be achieved by SPD, bringing the residual levels down to 2.1 ng of WHO-PCDD/F-PCB-TEQ kg^{-1}, that is, considerably below present feed and food MPLs. On the basis of the voluntary industrial monograph of GOED (*57*) a maximum level of 5 ng of WHO-PCDD/F-PCB-TEQ kg^{-1} is desirable, corresponding to 76% reduction in the used feedstock. At such target levels a satisfactory decontamination rate combined with <20% loss of the measured nutrients can be achieved (**Figure 2a**). The more volatile vitamin K_1 showed a lower degree of retention compared to the other K vitamins (**Figure 2b**), and around 50% retention can be expected at these operation conditions. If more effective SPD conditions must be applied, the loss of nutrients will increase, and at 90% TEQ reduction the retention levels are in the 60−90% range (**Figure 2a**). The corresponding retentions of vitamins K_1 and MK-4 will be around 30 and 50%, respectively (**Figure 2b**). On the basis of a feed rate of 6 kg h^{-1} (i.e., 100 kg h^{-1} m^{-2} evaporator surface) and 4% WF addition, 76 and 90% reductions of the WHO-PCDD/F-PCB-TEQ level in the used feedstock correspond to the use of an evaporator surface temperature of around 180 and 206 °C, respectively (*10*). This operation region gave a small positive effect on the oxidative stability of the oil (**Figure 3**).

Fish oil is an important source of the fat-soluble vitamins retinol (vitamin A) and cholecalcipherol (vitamin D_3), and liver oils have formerly been used to produce vitamin concentrates based on SPD (*58*). In refining of cod liver oil a high retention of native vitamins A and D_3 is desirable as the pharmacopoeia monograph (*59*) sets minimum levels for these compounds. However, the industry also has the possibility to fortify the oil with vitamins if the fully refined product contains levels below target specifications. Fish oil is an important ingredient in feed for Atlantic salmon, and if replaced with vegetable oils a significant reduction in EPA and DHA content of edible parts is observed (*28*). Studies have been requested by the feed and fish farming industry to confirm that decontamination of the fish oil by SPD does not remove nutrients of importance for growth rate and feed utilization. Feed compounders are adding a vitamin mixture to the feed formulations to ensure that the product contains adequate levels of vitamins for optimal feed performance at different life stages. Due to large variation in the levels of vitamins A and D in commercial fish oil parcels, it has not been industry practice to rely on a contribution from fish oil to cover the nutritional needs. However, an exception has been vitamin A due to the high level found in fish oil and fishmeal (*48*). The dietary requirement of vitamin A for rainbow trout is 0.75 mg/kg (*60*), that is, covered by use of the studied crude oil (**Table 2**) at the 5% inclusion level. To avoid toxic levels with negative effects on fish performance, vitamin A has been taken out of many vitamin mixtures (i.e., not declared as added in the fish feed). Vitamin A was quantitatively removed after the alkali refining

and bleaching step in this study, but also SPD treatment of crude fish oil will to some extent reduce the level. If such oils are used in feed formulations, the industry practice of not including vitamin A should be reconsidered. However, if the major part is in esterified form as observed in cod liver oil (*42*), the present industry practice most probably can be continued.

Cholesterol is a nonessential ingredient in fish and human nutrition, and the reduced level after SPD treatment (**Table 2**) may in food applications be perceived as favorable. However, crustaceans cannot synthesize cholesterol and require it to be supplied in the diet. The recommended cholesterol level in shrimp feed formulations is 2.5−4.0 g kg^{-1} (*61*). Cholesterol is one of the most expensive ingredients in shrimp diets with the addition of 2 g kg^{-1} representing >10% of total ingredient costs (*60*). In least-cost formulations the contribution of endogenous cholesterol from feed ingredients will be of economical importance and the observed reduction after SPD (**Table 2**) of disadvantage in this context.

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