Flavors' Decreasing Contribution to p-Anisidine Value Over Shelf Life May Invalidate the GOED Recommended Protocol for Flavored Fish Oils

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Abstract

American Oil Chemists' Society (AOCS)'s Official Method Cd 18-90 for p-Anisidine Value (pAV) is commonly used to evaluate secondary oxidation in fish oils. Flavoring agents in fish oil products may interfere with pAV and lead to inaccurate results. The Global Organization for EPA and DHA (GOED) recommends a protocol for calculating pAV of flavored fish oils, based on the assumption that flavors' contribution to the pAV does not change over the course of oxidation. The objective of this study was to test this assumption. All fourteen flavors evaluated increased the pAV when added to fresh fish oil; chocolate-vanilla and lemon flavors generated the largest increase. Under accelerated oxidation conditions, both chocolate-vanilla and lemon flavors had a similar effect; oxidized flavored fish oils had lower pAV than oxidized fish oils with newly added flavors. This was due to either an antioxidant effect of the flavor or degradation of the flavor during oxidation. Following the GOED recommendation, we would have underestimated the oxidation in the flavored oils. For this reason, pAV of flavored fish oils should be considered with caution and used in combination with other secondary oxidation markers when possible.

1 Introduction

Marine oil products are valued for their high eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) content. These fatty acids have been shown to exert positive health effects, such as reducing inflammation (Allaire et al., 2016) and lowering blood triglycerides (Zulyniak et al., 2016). However, the highly unsaturated structure of these fatty acids makes them susceptible to lipid oxidation, a deteriorative process that leads to off-flavors and off-odors. If consumed, the oxidized products may have negative health consequences (Esterbauer, 1993). For regulatory and quality control purposes, it is common practice to monitor the level of oxidation in marine oils. This is typically achieved by measuring multiple oxidation markers, such as peroxides and carbonyls. Peroxide value, as a primary oxidation indicator, can be measured using the American Oil Chemists' Society (AOCS) titration-based method (Cd 8b-90). Carbonyls, generated during hydroperoxide decomposition, are often used as secondary oxidation markers. Volatile carbonyls, such as hexanal, are commonly quantified using gas chromatography methods. Non-volatile carbonyls can be measured using the 2,4-Dinitrophenylhydrazine (DNPH) assay, the*p* -Anisidine Value (pAV), or the thiobarbituric acid reactive substances (TBARS) test.

Concentrations of non-volatile carbonyls, primarily unsaturated aldehydes such as 2-alkenals and 2,4-alkadienals, are often assessed using AOCS Official Method for pAV (Cd 18-90). This is a spectrophotometric method based on the absorbance of light by an imine chromophore that forms from the reaction of aldehydes with the*p* -anisidine reagent. Flavoring agents added to marine oils interfere with the test, as aldehydes in flavors also react with the*p* -ansidine reagent and form additional imine chromophores, leading to inaccurate results (The Global Organization for EPA and DHA (GOED), 2018; Jackowski et al., 2015; Semb, 2012) and

incorrect conclusions about the shelf-life and quality of the oil products. Unexpectedly high pAV have been reported in previous studies in flavored marine oil products (Albert et al., 2015; Jackowski et al., 2015).

To compensate for the increased pAV caused by flavors, GOED (2018) recommends a protocol to assess the pAV of flavored marine oils. There are four major steps: 1) measure pAV of an unflavored base oil (pAV); 2) determine the maximum allowable pAV increase in this oil (ΔpAV); 3) add flavor to the base oil and measure the pAV of this flavored oil (pAV*); 4) determine the maximum allowable pAV of the flavored oil (pAV^*_{max}), which constitutes a useful reference value for the shelf-life testing. ΔpAV is calculated by subtracting the pAV of the fresh unflavored base oil from the maximum acceptable pAV suggested by GOED. pAV^*_{max} is calculated by adding ΔpAV to pAV*. This recommendation is based on the assumption that the flavors themselves do not degrade or in any way change influence on the measured pAV over time (GOED, 2018). If this assumption is violated, estimates of the oxidation level will be inaccurate. However, this is very likely to occur; several common flavor compounds, such as citral (Djordjevic et al., 2008; Liang et al., 2004; Schieberle & Grosch, 1988) and vanillin (Mourtzinos et al., 2009), have been shown to degrade under a variety of conditions.

In this study, we tested the null hypothesis that flavors do not change their contribution to the measured pAV over the course of oxidation. To do this, we first evaluated 14 flavors to identify those with the greatest contribution to the measured pAV and would thus be most likely to change in observable ways over the course of oxidation. Following this, we performed a series of accelerated stability studies to compare oil samples to which flavor had been added either before or after oxidation. The pAV of all samples was evaluated at several sampling points during oxidation. We anticipated finding differences between the measured pAV of the two sample types, which would suggest the GOED recommendation may not apply to these flavored marine oils, and that an alternative protocol is needed to evaluate the aldehydes that result from lipid oxidation.

2 Materials and methods

2.1 Fish Oil and Flavors

Fish oil was manufactured by DSM Nutritional Products (Dartmouth, NS, Canada) and donated by Nature's Way of Canada Ltd. The fresh base oil contained approximately 18% EPA and 12% DHA and was stabilized with 3 mg g^{-1} natural mixed tocopherols. Flavoring agents were sourced from FONA International (Geneva, IL, USA). The flavors consisted of medium-chain triacylglycerols and natural flavor compounds. They were not stabilized with any additional antioxidants.*p* -Anisidine crystals and solvents used in this study were purchased from Sigma Aldrich (Oakville, ON, Canada)

2.2 Screening Flavored Fish Oils

The fourteen flavors screened were apple, blackcurrant, bubblegum, chocolate-vanilla, citrus punch, coconut, cranberry, grapefruit-tangerine, lemon, mandarin, mango, meat, orange, and peanut butter. Fourteen 4 mL screw top vials were loaded with accurately weighed fish oil (about 2.5 g per vial). As a common industrial practice, 2% (w/w) flavor was added to each vial. All vials were purged with nitrogen, capped, and vortexed for 30 seconds. pAV of these flavored oils and the unflavored base oil were measured in duplicate, following AOCS Official Method Cd 18-90 (AOCS, 2011).

2.3 Identifying Major Flavor Compounds Using GC-MS

After determining that chocolate-vanilla and lemon flavors had the largest influence on the measured pAV, GC-MS was used to identify major flavor components in these flavors. A Thermo Trace 1310 GC was coupled with a Thermo ISQ 7000 single quadrupole mass spectrometer equipped with an autosampler. Flavors were diluted 100 times in hexane with 50 ppm internal standard (tribenzylamine). Half a microliter of each diluted flavor was injected in split mode with a split ratio of 150:1 at 250 °C. Separation of compounds was achieved on a Zebron ZB-5 capillary column (5% phenyl, 95% dimethylpolysiloxane; 5 m GUARDIAN; 30 m length, 0.25 mm I.D., 0.25 μm film thickness). The oven temperature was: 1) held at 60 °C for 2 min, 2) increased to 80 °C at a rate of 7 °C min^{-1}, 3) increased again to 150 °C at a rate of 3 °C min^{-1}, 4) further increased to 260 °C at 7 °C min^{-1}, 5) increased to 300 °C at 3 °C min^{-1}, and 6) held for 20 min. Total run time was 78

min. This long temperature program ensured large molecules, including the triacylglycerol carrier oil, had eluted from the column at the end of each run. The MS was operated in TIC mode, with a scanning range from 60 to 600 amu and dwell time of 0.2 sec. Transfer line and ion source were both set at 300 °C. MS data were processed by Chromeleon Chromatography Studio. Peaks with area larger than 1.0 % of internal standard were tentatively identified through the NIST library search and confirmed using an MS processing method with three confirmation ions that had been developed and employed for routine in-house analysis of flavors. The retention indices of all the identified compounds were compared to further confirm the peaks' identities.

2.4 Oxidation of Chocolate-Vanilla and Lemon Flavored Fish Oils

The following experiment and subsequent analyses were performed twice, once with chocolate-vanilla flavor and once with lemon flavor. Accurately weighed aliquots of fish oil (˜3.5 g) were added to fifty-one 4 mL screw top vials. To eighteen of these vials, 2% (w/w) flavor was added. All vials were then purged with nitrogen, capped, and vortexed for 30 seconds. Following vortexing, three vials without flavor and three with flavor added were set aside to serve as unoxidized baseline samples. The remainder of the samples were placed uncapped in a 40 ± 2°C oven for oxidation. Every four days for a period of 20 days, nine oil samples (two triplicate sets of unflavored oils, and one triplicate set of oil to which flavor had been added prior to incubation) were taken out of the oven and cooled to room temperature. To one set of triplicate unflavored oil samples, 2% (w/w) flavor was added, thus creating three streams of oxidized oil: unflavored oil (UFO), oil to which flavor had been added *before* oxidation (FBO), and oil to which flavor was added *after* oxidation (FAO). All three triplicate sets of oil samples were then purged with nitrogen, capped, and vortexed for 30 seconds. pAV of oils was measured following the protocol described above.

2.5 Statistical Analyses

The following equation was used to model (SPSS, IBM, New York) the pAV growth data for each treatment:

$$Y_f(t) = A + Bexp^{\mathrm{kt}} \quad Equation\ 1$$

where $Y_f(t)$ is the pAV on a given day, Arepresents contribution from stable interfering components that respond to the pAV reagent but do not change over time, B represents the aldehydes that respond to pAV reagent and change over the course of oxidation either due to lipid oxidation or aldehyde degradation,k is the rate constant for aldehyde formation, and t is the time. Pooled-variance t-tests (df=30, α=0.05) were used to compare$k$, A , and B between different treatments. On each testing day, treatment effect was evaluated with one-way analysis of variance (ANOVA), followed by a post-hoc Tukey test with a significance level of 0.01.

3 Results

3.1 Selection of Favors

The pAV of flavored fish oils were consistently higher than the pAV of the unflavored control oil. However, the magnitude of this pAV difference varied (Fig. 1). Among all the flavors screened, chocolate-vanilla and lemon flavors caused the greatest change in pAV. Therefore, these flavors were selected for further analysis.

3.2 Compound Identification in Chocolate-Vanilla and Lemon Flavors

Although there were only six compounds identified in chocolate-vanilla flavor with peak areas > 1% of the internal standard (Table 1), three of them (p -anisaldehyde, piperonal, and vanillin) were aldehydes that have a great potential to condense with the p -anisidine reagent and increase the pAV. Among the six identified compounds, the three aldehydes also had the highest area relative to the internal standard (ARIS), with p -anisaldehyde, piperonal, and vanillin at 4.7%, 24.5%, and 90.7%, respectively. A small amount of trimethylpyrazine, ethyl caprylate, and ethyl caprate were also identified. On the other hand, 15 compounds were identified in lemon flavor, but there were still only three aldehydes (decanal, β-citral, and α-citral) that

had potential to react with$p$ -anisidine. Decanal, β-citral, and α-citral had ARIS of 2.1%, 19.2%, and 29.6%, respectively. Total ARIS of the three aldehydes in the chocolate-vanilla flavor was much larger than the three aldehydes in lemon flavor. Besides aldehydes, a large amount of terpenes was also identified in the lemon flavor. Limonene and β-pinene had ARIS of 824.5% and 123.0% respectively.

3.3 Oxidation of Chocolate-Vanilla and Lemon Flavored Fish Oils

The pAV of all the fish oil samples increased over the course of the 20-day study at 40°C (Fig. 2). In chocolate-vanilla flavored oils, a significant difference in pAV was found between FBO and FAO samples on Day 20. In lemon flavored oils, a significant difference in pAV occurred between FBO and FAO since Day 12. According to regression analysis, an exponential model was best fitted to the pAV data of each treatment (R^2 = 0.961 - 0.975). In both chocolate-vanilla and lemon flavored oils, A (stable interfering aldehydes) was significantly higher in FBO and FAO than in UFO (Table 2). No differences were detected in B (changing aldehydes). In the chocolate-vanilla flavored fish oils, FBO samples had a significantly lower k (rate constant) than UFO. In lemon flavored oils, no significant difference was detected in the k of UFO, FBO, and FAO.

4 Discussion

The AOCS pAV test (Cd 18-90), as a simple colorimetric method to measure non-volatile carbonyls, primarily aldehydes, has limitations due to its non-specificity. The imine chromophore that forms between an aldehyde and the p -anisidine reagent differs considerably among aldehydes (Szabó et al., 2010), with the method being more sensitive to unsaturated than saturated aldehydes (Gordon, 2004). So, pAV results provide only relative aldehyde concentrations. Although pAV is expected to reflect the amount of aldehydes arising from hydroperoxide decomposition during oxidation, it is known to respond to extraneous aldehydes as well, such as those from flavors. The interference by flavors is dose and type-dependent. Jackowski et al. (2015) found that marine oil products with citrus flavors, as well as products marketed towards children (e.g., bubblegum-flavored products) often had higher pAV than other flavored products. This was also confirmed in this study; the pAV increase caused by 14 different flavors varied from 1.1 to 46.5, with chocolate-vanilla, citrus, and bubblegum flavors having the largest impact (Fig. 1). Despite these limitations, pAV is still one of the most common methods to measure marine oil oxidation, due to its simple procedure and low cost compared to sensory evaluation or volatile analysis using GCMS.

GOED recognized these limitations and provided advice for those who want to use the AOCS pAV method for fish oil testing (GOED, 2018). In the guidance document, GOED recommended a protocol for flavored fish oils, based on an assumption that flavors have a constant contribution to the measured pAV throughout shelf life studies. However, in this study we found chocolate vanilla and lemon flavors had a diminishing effect on the pAV (Fig. 2), indicating that the current GOED recommendation may not apply to all the flavored oils. For both flavors, the significant difference in interfering components (A) in FBO and FAO compared to UFO was expected since the flavors introduced a large amount of stable interfering aldehydes. Based on the observed differences in pAV of FBO and FAO, we had also expected to find a significant difference in$B$ (changing aldehydes) and k (rate constant for aldehyde formation) for both flavors. However, the uncertainty introduced by the 4-day sampling frequency limited our ability to identify statistical differences. A more frequent sampling plan would have given us a greater confidence in estimating the model parameters. Despite this, we did find that chocolate-vanilla FBO samples had a significantly lower rate constant (k) than UFO. This might be caused by flavor components with antioxidant properties or by flavor aldehydes that degraded over time.

With the current experimental design, it is difficult to determine if this diminishing effect was due to anti-oxidant activity, degradation of flavor aldehydes, or both. It is not concerning if the diminishing effect on measured pAV was solely due to any antioxidant activity because in this case, the lower pAV truly reflected the slower oxidation in the flavored oil. It is concerning if the decreasing contribution to the pAV was caused by flavor loss. In this case, more oxidation would have occurred before the maximum pAV allowed by the GOED recommendation is reached. In other words, following the GOED recommended protocol, we would underestimate the amount of oxidation that was occurring in the oil. This underestimation of the extent

of oxidation may lead to overly optimistic inferences about the quality and shelf-life of the flavored oil in question.

In the chocolate-vanilla flavor used in this study, vanillin had the highest ARIS (90.7%). Antioxidant activity of vanillin has been demonstrated in antioxidant capacity assays (Tai et al., 2011) and in food systems containing polyunsaturated fatty acids (Burri et al., 1989). Vanillin may be oxidized to vanillic acid, which is 3.3 times more effective as an antioxidant than vanillin in bulk oils (Mourtzinos et al., 2009). Vanillic acid has also been shown to exert antioxidant effects in corn oil subjected to deep-frying conditions (Naz et al., 2005) and in fish oil at temperatures between 35 to 55°C (Farhoosh et al., 2016). Thus, vanillin is very likely to have a real influence on the rate constant for aldehyde formation (k) in the FBO samples, through an antioxidant effect.

In the lemon flavored oils, although pAV of FBO and FAO samples were significantly different on days 12, 16, and 20, no significant differences were observed in the rate constant (k) between FBO and other samples. In the lemon flavor, citral had the highest ARIS (total of α- and β-citral at 48.8 %). Citral does not exert strong antioxidant effects in edible fruit coatings (Guerreiro et al., 2015, 2016) and in essential lemon oils (Misharina et al., 2011). In fact, an antioxidant is often added to inhibit citral degradation. GC analysis of cold-pressed lemon oil stored in the dark for two months at 30°C showed significant losses of citral (Nguyen et al., 2009). This effect is exacerbated at increased temperatures (Djordjevic et al., 2008; Nguyen et al., 2009). Compounding this, citral is quite volatile. In the open vials in this study, citral might have been evaporating throughout the study. Thus, it is very likely that loss of citral caused the lower pAV in FBO compared to FAO samples, without significantly changing the rate constant (k).

As a small study to estimate the influence of some common flavors on pAV testing in fish oil products, the current results do not allow us to calculate a pAV that solely reflects the aldehydes generated through lipid oxidation. Future studies should include a systematic experimental design that tracks multiple complementary oxidation products, such as peroxides and volatile aldehydes, and monitors matrix changes, such as tocopherol and flavor loss. Such a study would demonstrate the overall oxidative status, elucidate the correlation between various oxidation indicators, and clarify the source of the diminishing pAV inflation. This would also allow us to define the maximum pAV through its correlation with other oxidation markers and their respective limits. When pAV is the only applicable method for routine analysis for quality control purpose, this predefined pAV can be used as the control limit. For example, if we are confident about the correlation between 2,4-heptadienal and pAV after systematic studies on a flavored fish oil product under normal storage conditions, and if we have established a standard for 2,4-heptadienal levels, we can predefine our maximum allowable pAV for this product. For daily quality control, pAV can be measured and compared with this maximum pAV.

5 Conclusion

In this study, all fourteen flavor enhancers influenced the pAV test, among which chocolate-vanilla and lemon had the largest impact. During a shelf-life study of chocolate-vanilla and lemon flavored fish oils, the flavor aldehydes had a diminishing contribution to the measured pAV over time, indicating either an antioxidant activity or degradation of the flavor compounds. This may have ramifications for GOED's current recommendation for measuring the pAV of flavored oils; flavor degradation would cause the extent of oxidation to be underestimated in the flavored products, which in turn may lead to incorrect judgements on the quality and shelf-life. Before alternative solutions are found, the GOED recommended protocol should be followed with caution.

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Tables

Table 1. Identified peaks in chocolate-vanilla and lemon flavors. "*" indicates aldehydes.

Chocolate-Vanilla Flavor	**Chocolate-Vanilla Flavor**	**Chocolate-Vanilla Flavor**	**Chocolate-Vanilla Flavor**	**Chocolate-Vanilla Flavor**
Compound	**Retention Time (min)**	**Retention Index (iu)**	**Relative Area to Internal Standard (%)**	**Relative Area to Internal Standard (%)**
Trimethylpyrazine	8.70	1004	1.6	1.6
Ethyl caprylate	15.36	1196	6.1	6.1
p-anisaldehyde*	17.76	1251	4.7	4.7
Piperonal*	20.89	1330	24.5	24.5
Ethyl caprate	23.28	1396	4.0	4.0
Vanillin*	23.47	1404	90.7	90.7
Lemon Flavor Compound	**Lemon Flavor Retention Time (min)**	**Lemon Flavor Retention Index (iu)**	**Lemon Flavor Relative Area to Internal Standard (%)**	**Lemon Flavor Relative Area to Internal Standard (%)**
α-Thujene	6.79	929	3.7	3.7
α-Pinene	6.93	937	20.8	20.8
Sabinene	7.91	974	20.0	20.0
β-Pinene	8.02	979	123.0	123.0
β-Myrcene	8.31	991	18.1	18.1
α-Terpinene	9.12	1017	2.0	2.0
p-Cymene	9.36	1025	12.3	12.3
D-Limonene	9.52	1030	824.5	824.5
γ-Terpinene	10.43	1060	97.0	97.0
Terpinolene	11.44	1088	4.1	4.1
Linalool	11.77	1099	1.7	1.7
Decanal*	15.67	1206	2.1	2.1
β-Citral*	17.09	1240	19.2	19.2
α-Citral*	18.27	1270	29.6	29.6
Caryophyllene	24.38	1419	1.3	1.3

Table 2. Estimates of A (stable interfering components),B (changing aldehydes), and k (rate constant for aldehyde formation) of UFO (unflavored fish oil), FBO (flavor added before oxidation), and FAO (flavor added after oxidation), derived from the non-linear regression on the data obtained during 20 days of oxidation at 40°C.

Sample treatment	**Sample treatment**	R^2	A	A	B	B	k	k
			Estimate	*Std. Error*	*Estimate*	*Std. Error*	*Estimate*	*Std. Er*
Chocolate-vanilla	**UFO**	0.961	4.925[a]	1.951	0.759[a]	0.474	0.211[a]	0.030

Sample treatment	Sample treatment	R^2	A	A	B	B	k	k
	FBO	0.971	52.311^{b}	1.832	2.336^{a}	1.003	0.136^{b}	0.020
	FAO	0.975	54.418^{b}	1.532	1.166^{a}	0.522	0.182^{ab}	0.022
Lemon	**UFO**	0.975	6.032^{a}	0.666	0.352^{a}	0.171	0.207^{a}	0.024
	FBO	0.966	33.158^{b}	0.696	0.254^{a}	0.152	0.219^{a}	0.029
	FAO	0.975	32.056^{b}	0.982	0.992^{a}	0.415	0.163^{a}	0.020

Different letters in the same column (A , B , or k) and in the same experiment group (chocolate-vanilla or lemon) indicate a significant difference following pooled-variance t-test (df=30, α=0.05).

Figure legends

Figure 1. The change in pAV caused by the addition of 2% (w/w) flavor. The values reported are the means of duplicate measurements with error bars indicating the propagated standard deviation.

Figure 2. The pAV of UFO (unflavored fish oil), FBO (flavor added before oxidation), and FAO (flavor added after oxidation) during oxidation at 40°C for 20 days in (a) chocolate-vanilla flavored fish oil and (b) lemon flavored fish oil. Curves represent the exponential interpolation, while the error bars indicate the standard deviation.