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Analysis of Volatile Compounds as Spoilage Indicators in Fresh King Salmon (*Oncorhynchus tshawytscha*) During Storage Using SPME−GC−MS

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A method was developed for the analysis of salmon volatiles using solid-phase microextraction and gas chromatography−mass spectrometry. This method was used to monitor the volatiles of fresh king salmon (*Oncorhynchus tshawytscha*) stored in ambient air or in a 40:60 (v/v) mixture of CO_2:N_2 over time. The levels of several of the volatile compounds were found to change during storage, with some showing a clear difference between storage in air and storage in CO_2:N_2. Of these, several alcohols (cyclopentanol, *Z*-2-penten-1-ol, 1-penten-3-ol, and 1-octen-3-ol) and aldehydes (hexanal, octanal, *E*-2-pentenal, and *E*-2-hexenal) were identified as potential markers for salmon freshness. Several other volatiles (acetoin, ethyl benzene, propyl benzene, styrene, 3-methyl butanoic acid, and acetic acid) were identified as potential markers for salmon spoilage. A comparison of salmon harvested with and without the "rested harvesting" technique showed that *E*- and *Z*-isoeugenol levels were increased by the use of the isoeugenol based anesthetic. The use of the anesthetic did not affect the levels of any of the other compounds identified.

KEYWORDS: King salmon (*Oncorhynchus tshawytscha*); modified atmosphere packaging; SPME; fish spoilage marker compounds

INTRODUCTION

The demand for fresh quality salmon for the export market (which pays a premium price for the fish) necessitates the development of treatments that can extend the shelf life of fresh salmon fillets. Modified atmosphere packaging is widely used to extend the shelf life of chilled food products, and a few studies have focused on its potential for extending the shelf life of seafood products (*1*−*4*). It is well-known that certain combinations of gases will inhibit the growth of micro-organisms (for example, inclusion of CO_2 will retard the growth of typical spoilage bacteria for seafood) and limiting exposure to oxygen will reduce the rate of oxidation. It has also been suggested that deterioration resulting from microbial fermentation is the determining factor in fish spoilage (reduction of quality below the sensory acceptance level) while autolysis is responsible for initial quality loss (*5*). The right combination of gases should therefore be able to extend the shelf life of fresh salmon fillets.

In order to develop methods to increase the shelf life of fresh salmon, it is necessary to be able to monitor the quality of the fish throughout shelf life trials. Commonly used approaches for monitoring the quality of fresh fish include sensory, microbiological, texture, and chemical analyses (*6*). Of these approaches, the most comprehensive measure of quality (in relation to acceptability by the consumer) is sensory analysis, which requires the use of a trained sensory panel for quality assessment of fish appearance, odor, taste, and texture. This is not only time-consuming but also costly to set up. The other analytical methods measure specific attributes that impact on fish quality, for example microbiological testing will give an indication of microbial spoilage and various chemical tests will target the products of specific chemical or enzymatic reactions (for example oxidation) or microbial metabolism. A need therefore exists for an instrumental method that can be used to monitor the quality of salmon and is related to the sensory changes reported by a trained panel.

Solid-phase microextraction (SPME) is a proven tool in volatile analysis (*7*, *8*) and has previously been used for the analysis of flavor volatiles in seafood (*9*−*14*) as well as in a variety of other foods (*7*, *15*−*17*). In general, SPME is both simple and cost-effective to use and can be used to analyze the levels of a wide range of volatile compounds.

The main objectives of this research were (i) to develop a method to monitor the quality of fresh chilled salmon stored under different atmospheres (air and 40:60 (v/v) CO_2:N_2 mix) using SPME headspace analysis and gas chromatography−mass spectrometry (GC−MS) and (ii) to identify volatile compounds that can be used as markers for freshness or spoilage. The

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influence of anesthetic-mediated harvesting on the volatile profile of the fish was also assessed. This research is an extension of previously published work on the effect of different storage atmospheres on the spoilage of fresh King salmon (*4*).

MATERIALS AND METHODS

Fish Sampling and Preparation. King salmon ($n = 12$) were supplied by the New Zealand King Salmon Co. Limited (Nelson, New Zealand). The salmon were starved for 3 days then commercially harvested by either "rested harvesting" procedures using the anesthetic AQUI-S (AQUI-S New Zealand Limited, Lower Hutt, New Zealand) (6 fish) or dip netted and killed directly by a blow to head (without AQUI-S) (6 fish), and then packed in ice for overnight transfer to the Crop & Food Research laboratory in Auckland (anal temperature on arrival ranged from 2.8 to 4.7 °C) where they were placed in a chiller (0.5 ± 1 °C) until packaging that day. The whole fish were weighed, and the fork length of the fish was measured. The belly cavity of the fish was opened to determine sex, and the gonads and liver were removed and weighed. The 12 fish consisted of 10 females and 2 males with mean biological status as follows (standard deviations in parentheses): weight 2883.6 (676.2) g, length 54.8 (3.9) cm, liver 23.6 (6.3) g, and gonad 10.3 (4.5) g. The fish were then filleted and the fillet weights recorded. The pH of anterior dorsal flesh was measured by using a flat electrode (Sensorex 450C, Garden Grove, CA) placed onto a freshly cut end of the fillet. The fillets had mean weights of 743.6 g (standard deviation 187.1 g, $n = 23$). The mean pH of the fillets was 6.5 (standard deviation 0.14). Both left and right-hand fillets were further trimmed to loins at least 2 cm thick. The fillets were cut and trimmed in portions to fit into a 2 × 2 × 4 cm^3 potting box (Boss Industrial Mouldings Ltd, Bury St Edmunds, U.K.), and numbered according to their position on the fillet, as described by Fletcher et al. (*4*).

Storage Conditions of Fish Portions. Each portion (with potting box) was vacuum packaged in a pouch (20 × 9 cm^2) of double metallized laminate (oxygen transmission rate of less than 0.1 cm^3/m^2/24 h at 23 °C and 0.03 cm^3/m^2/24 h at 5 °C, at atmospheric pressure, and 75% relative humidity) using a SecurePack 2 machine (SecureFresh Pacific Limited, Auckland, New Zealand). Two storage treatments were applied: ambient air (AIR) and modified atmosphere (MA) which consisted of a 40:60 (v/v) CO_2:N_2 mix. All samples were stored in melting ice (0 ± 0.1 °C). The air or CO_2:N_2 mix (100 mL) was added to the sealed pouches through a septum as described previously by Fletcher et al. (*18*). This resulted in a total of 4 sample treatments: AQUI-S harvested fish stored in ambient air (AQAIR), AQUI-S harvested fish stored in CO_2:N_2 mix (AQMA), non-AQUI-S harvested fish stored in ambient air (AIR), and non-AQUI-S harvested fish stored in CO_2:N_2 mix (MA). Gases (CO_2, N_2) were supplied by BOC Gases Limited (Auckland, New Zealand), and certified as containing less than 10 ppm oxygen. Sampling for analysis occurred at time zero (before packaging) and on the following days for the different treatments: AQAIR and AIR: 9, 19, and 26 days; AQMA and MA: 19, 40, 61, and 91 days. On each sampling day, 5 portions (each from a different fish) were sampled for each of the relevant treatments (AQAIR, AIR, AQMA, or MA). Portions from positions 1, 2, 3, or 4 were used for chemical, physical, and microbiological tests (for comparison with data from Fletcher et al. (*4*)). The ventral strip portions (positions 11−14) were air blast frozen to −60 °C and stored at −85 °C before being used for volatile analysis (SPME headspace analysis).

Chemical and Physical Analysis of the Fish Portions. Nucleotides and derivatives (adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), inosine 5′-monophosphate (IMP), inosine (INO), and hypoxanthine (HX)) were determined for each treatment. Fish muscle tissue was homogenized with chilled (0 °C) perchloric acid (0.6 M) at a 1:6 g/mL ratio, allowed to stand (30 min, 0 °C), and then centrifuged (3500−4000 rpm, 15 min). The supernatant was neutralized to pH 6.5−6.8 using potassium hydroxide (0.1−1.0 M), filtered to remove the precipitate, made into a suitable dilution, and stored at −85 °C for subsequent testing by HPLC (*19*). *K*-values (*20*) (the percentage of HX + INO over the total pool of ATP derivatives) and *H*-values (*21*) (the percentage of HX over the sum of IMP, INO, and HX) were calculated. Total volatile basic nitrogen (TVBN) and trimethylamine nitrogen (TMA-N) were determined by the microdiffusion method of Pearson (*22*). Drip loss (expressed as a percentage of the initial loin weight) was calculated as the weight loss between packaging and sampling (measured before portioning and freezing). The pH was measured on the sample surface using a surface electrode (Sensorex 450C; Garden Grove, CA).

Microbiological Analysis. Samples (10 g) were homogenized (2 min, Stomacher 400, Seward Limited, London, U.K.) in salt−peptone water (1% NaCl, 0.1% peptone (Difco, Detroit, MI)) at a ratio of 1:10. Further decimal dilutions were made in salt−peptone water, and the drop plate method using 5 drops (10 μL) from each dilution (*23*) was used for total plate aerobic counts on two agars (plate count agar (Difco) with 1% NaCl (sPCA) and modified Long and Hammer's medium after van Spreekens (*24*)). Plates were incubated aerobically at two temperatures (3 days at 20 °C and 5 days at 15 °C) and colonies counted and recorded as colony forming units (cfu).

Solid-Phase Microextraction (SPME) Headspace Analysis. *Sample Preparation.* The portions were defrosted at 4 °C overnight, and then removed from the pouches and wrapped in aluminum foil (foil size 16 cm × 16 cm) for oven cooking. Four samples were cooked at once, with samples being placed 6 cm apart and each sample being 3 cm from the center of the oven tray. This ensured central positioning in the oven, resulting in exposure to a stable temperature. Samples were baked at 180 °C (fan bake) for 14 min. The cooked samples were transferred to new double metallized laminate pouches, vacuum-sealed, and stored at −80 °C.

Headspace vials were baked at 250 °C for 2 h, and then cooled to room temperature before use. Frozen cooked salmon portions were cut into smaller pieces using a sharp knife on a clean stainless steel tray. The sample pieces were placed back into the pouch, and then dipped in liquid N_2 until it stopped bubbling (temperature of sample equilibrated with liquid N_2 temperature). The sample was then placed in a precooled (with liquid N_2) 250 mL stainless steel mini container on a 2 speed Waring blender base (Waring Products Inc., Torrington, CT) with some liquid N_2 and blended into a fine powder (20−30 s on high speed). Salmon powder (8 g per vial) was poured into 2 headspace vials (20 mL) through a funnel (precooled with liquid N_2). Headspace vials were sealed with an aluminum seal (with a 10 mm center hole) fitted with a 20 mm chlorobutyl septum (Chromacol, Herts, U.K.) and stored at −80 °C until analysis. The stainless steel Waring blender container and funnel were washed thoroughly and dried between samples.

Sample Analysis. There were 16 carboxen-polydimethylsiloxane (CAR-PDMS) (75 μm) coated SPME fibers (Supelco, Bellefonte, PA) (lot number: P307759D) used for this study so that each fiber was used no more than 10 times. The first set of GC−MS analysis was completed on the first headspace vial from each sample (5 portions per day/treatment, $n = 20$), analyzed in the following sequence: day 0 samples, day 91 samples, day 9 samples, day 19 samples, day 26 samples, day 40 samples, and day 61 samples (see Storage Conditions of Fish Portions subsection for sample details). A new SPME fiber was used for each lot of 10 analyses (for example: all day 0 samples (fiber 1), all day 19 AIR/AQAIR samples (fiber 2), all day 19 MA/AQMA samples (fiber 3), and so forth). The second set of GC−MS analysis was completed using the second headspace vial from each sample, and the analysis was randomized to reduce the potential influence of using different SPME fibers, SPME fiber aging, and sampling order. The samples were held at room temperature for 30 min before incubating with the SPME fiber exposed to the sample headspace for 90 min at 60 °C (*25*).

Gas Chromatography−Mass Spectrometry (GC−MS). Gas chromatography−mass spectrometry analysis was carried out using a Fisons 8000 Top GC (Carlo Erba Instruments, Milan, Italy) coupled to a Finnigan MAT MD 1000 mass detector (Finnigan Instruments, Manchester, U.K.). Separation was achieved on a ZB-Wax column (60 m, 0.32 mm i.d., 0.5 μm film thickness) (Phenomenex, Torrance, CA, U.S.A.) using helium (constant flow: 31 cm/s at 40 °C) as the carrier gas. Samples were desorbed from the SPME fiber in the injector (250 °C, splitless 2 min) for 5 min with cryofocusing (−70 °C for 2 min, then heated to 250 °C) using a Micro Cryo-trap (Scientific Instrumental Services Inc, Ringoes, NJ) with liquid CO_2 as the coolant (BOC Gases

Ltd, Dunedin, New Zealand). The oven temperature was held at 40 °C for 2 min, then increased to 250 °C at 3 °C/min, and held at this temperature for 10 min. The mass spectrometer was operated in the electron impact ionization mode (70 eV). Source and interface temperatures were 195 and 250 °C, respectively. Detector voltage was 250 V, mass range was from 35 to 400 amu and scan rate was 0.9 scan/s.

Compound Identification. Compounds were tentatively identified by matching mass spectral data with the Wiley (seventh ed., 2000) and NIST (1998) reference libraries of standard compounds. Finnigan Masslab software version 1.4 (Finnigan Instruments, Manchester, U.K.) was used for data analysis. A series of alkanes were analyzed to obtain retention indices. The mixture of 5−16 carbon atom alkanes (C5−13: 5 μL each; C14−16: 10 μL each) in a 10 mL headspace vial sealed with an aluminum seal (with 10 mm center hole) fitted with a 20 mm chlorobutyl septum (Chromacol, Herts, U.K.) was incubated with a 30 μm polydimethylsiloxane (PDMS) fiber (Supelco, Bellefonte, PA) exposed to the headspace for 5 s at 20 °C. The mixture of 17−30 (excluding C19, C28) carbon atom alkanes (C17, C18, and C20: 20 μL each; C21−27 and C29−30: 15 μL each) in a 10 mL headspace vial sealed with an aluminum seal (with 10 mm center hole) fitted with a 20 mm chlorobutyl septum (Chromacol, Herts, U.K.) was incubated with a 7 μm PDMS fiber (Supelco, Bellefonte, PA) exposed to the headspace for 2 min at 70 °C. The alkane samples were desorbed from the SPME fiber and analyzed in the same way as the salmon samples (described above).

The identification was confirmed by comparison of the retention indices and mass spectra with authentic standards for the following compounds (purity in parentheses): $C_{10}-C_{15}$ alkanes (Alltech Associates Inc., Deerfield, IL); hexanal (98%), heptanal (95%), *E*-2-hexenal (98%), benzoic acid (>99.5%), 3-methyl butanoic acid (99%), phenol (>99.5%), styrene (99%), 1-butanol (>99%), 1-pentanol (>99%), 1-heptanol (99%) (Sigma, St. Louis, MO); octanal (99%), *E*,*E*-2,4-hexadienal (95%), *E*,*Z*-2,6-nonadienal (95%), acetone (>99%), acetophenone (99%), isoeugenol (mixture of *Z*- and *E*-, 98%), 1-octen-3-ol (98%), 1-penten-3-ol (99%), 2-ethyl-1-hexanol (>99%), *E*-2-pentenal (95%), 4-ethyl benzaldehyde (Aldrich Chemical Co., Milwaukee, WI); 2,3-pentanedione (97%), acetoin (>97%), 1-hexanol (>98%) (Fluka Chemie AG, Buchs, Switzerland); acetic acid, butanoic acid, pentanoic acid, hexanoic acid, heptanoic acid, octanoic acid (>99%, Nu-Check Prep Inc., Elysian, MN); *Z*-2-penten-1-ol (Bedoukian Research Inc., Danbury, CT); ethyl benzene, cyclopentanol (Penta Manufacturing Co., Livingston, NJ).

Statistical Analysis. The GenStat statistical package (GenStat, 2001) was used for the statistical analyses on the chemical, physical, and microbiological data. Analysis of variance (ANOVA) was applied to log transformations of the microbiological counts and to the untransformed results of the remaining physical and chemical analyses as the residual plots from the latter showed a random pattern with constant variance. The calculated least significant difference at $p = 0.05$ was used to compare mean results. Residual maximum likelihood (REML) variance analysis was used to compare the effect of incubation medium and temperature on plate counts. The SPSS (version 14.0) statistical package (SPSS, 2005) was used for statistical analysis on the data for volatile compounds identified by GC−MS. Analysis of variance was performed with Tamhane's T2 test ($p = 0.05$).

RESULTS AND DISCUSSION

Chemical, Physical, and Microbiological Data. The mean cut-surface pH was 6.6 (AQUI-S harvested) and 6.4 (non-AQUI-S harvested) on arrival, which decreased slightly (AQUI-S 6.5; non-AQUI-S 6.3) after processing. During storage, the pH for AQUI-S harvested salmon decreased further (AIR and MA 6.3) while non-AQUI-S harvested salmon showed no further change in pH. The pH of the salmon used in this study was higher on arrival than the salmon used in the previous study by Fletcher et al. (*4*), which reported a pH of 6.1 on arrival. While a decrease in pH was observed for this study, Fletcher et al. (*4*) reported an increase in pH; however, the final pH for both studies was 6.3 for both AIR and MA stored salmon.

Figure 1 summarizes the results for drip loss, nucleotide derivatives data (IMP, inosine, hypoxanthine, *H*-values, and *K*-values), TMA-N, and microbiological data (aerobic plate counts). Error bars depict the least significant difference ($p = 0.05$). The mean drip loss increased over the storage time for all treatments (**Figure 1A**). After an initial sharp increase in drip loss from 0 to 19 days, the rate of drip loss appeared to slow with storage. Overall, there were no significant differences between the AQUI-S and non-AQUI-S harvested samples or between the AIR and MA treatments ($p < 0.05$). The initial increase in drip loss (day 0−19) was similar to that reported by Fletcher et al. (*4*); however, subsequent drip loss (day 19 onward) was lower, resulting in a lower total drip loss for all treatments. TMA-N increased only slightly in all treatments (**Figure 1B**). There was a rapid loss of IMP to form inosine in the first 19 days of storage and a linear increase in hypoxanthine over the duration of the trial (**Figure 1C**−**E**). Because of the rapid conversion of IMP, *K*-values quickly reached a maximum of around 96% by day 19 (**Figure 1F**). The *H*-values, however, showed a linear increase for all treatments over the duration of the trial (**Figure 1G**). Similar results were observed by Fletcher et al. (*4*); however, the conversion of IMP to form inosine appeared to occur more rapidly (11 days).

The REML variance analysis showed that, on average, incubation at 20 °C gave higher microbial counts than at 15 °C, and plate count agar with added salt gave higher counts than modified Long and Hammer's medium ($p < 0.05$) (data not shown). Therefore, only the aerobic plate count results from sPCA incubated at 20 °C are reported here (**Figure 1H**). Logarithmic increases in total aerobic plate counts were recorded for all treatments with lag phases of less than 9 days for samples stored under air and 19 days for samples stored under MA. There was no difference between samples from salmon harvested with or without AQUI-S. Microbial numbers increased rapidly from 3 log cfu/g (day 9) to 7 log cfu/g (day 26) in samples stored under air, while a much slower increase is observed for samples stored under MA (2.7 log cfu/g day 19 to 5 log cfu/g day 61). The salmon in the current study had a lower initial microbial load and subsequently lower levels throughout storage (especially for samples stored under MA) compared to the salmon studied by Fletcher et al. (*4*).

Compounds Identified by SPME−GC−MS. The analysis of the fresh and stored salmon samples using the SPME method allowed for the identification of more than 100 volatile compounds, including short-chain saturated aldehydes, *E*-2-unsaturated aldehydes, alcohols, sulfur compounds, and short chain fatty acids. Several of these compounds were only detected at trace levels while others were only identified in specific stored salmon samples (for example, 5-methyl hydantoin, tentatively identified by mass spectra, was only found in stored samples from 2 fish that had a higher microbial load than the other fish used) (data not shown).

Influence of AQUI-S Anesthetic on Salmon Volatiles During Storage. Data for AQUI-S treated salmon and salmon harvested without anesthetic were initially analyzed separately to determine the effect, if any, of AQUI-S on the profile and levels of volatile compounds in salmon. With the exception of two compounds (see below), AQUI-S was not found to influence the initial levels nor the evolution of salmon volatile compounds during storage. This is illustrated in **Figure 2** for acetoin and acetone by the almost identical nature of the data for AQUI-S and non-AQUI-S samples. Consequently, the data for AQUI-S and non-AQUI-S samples were combined for the rest of the data analysis. Salmon stored under AIR was not studied beyond

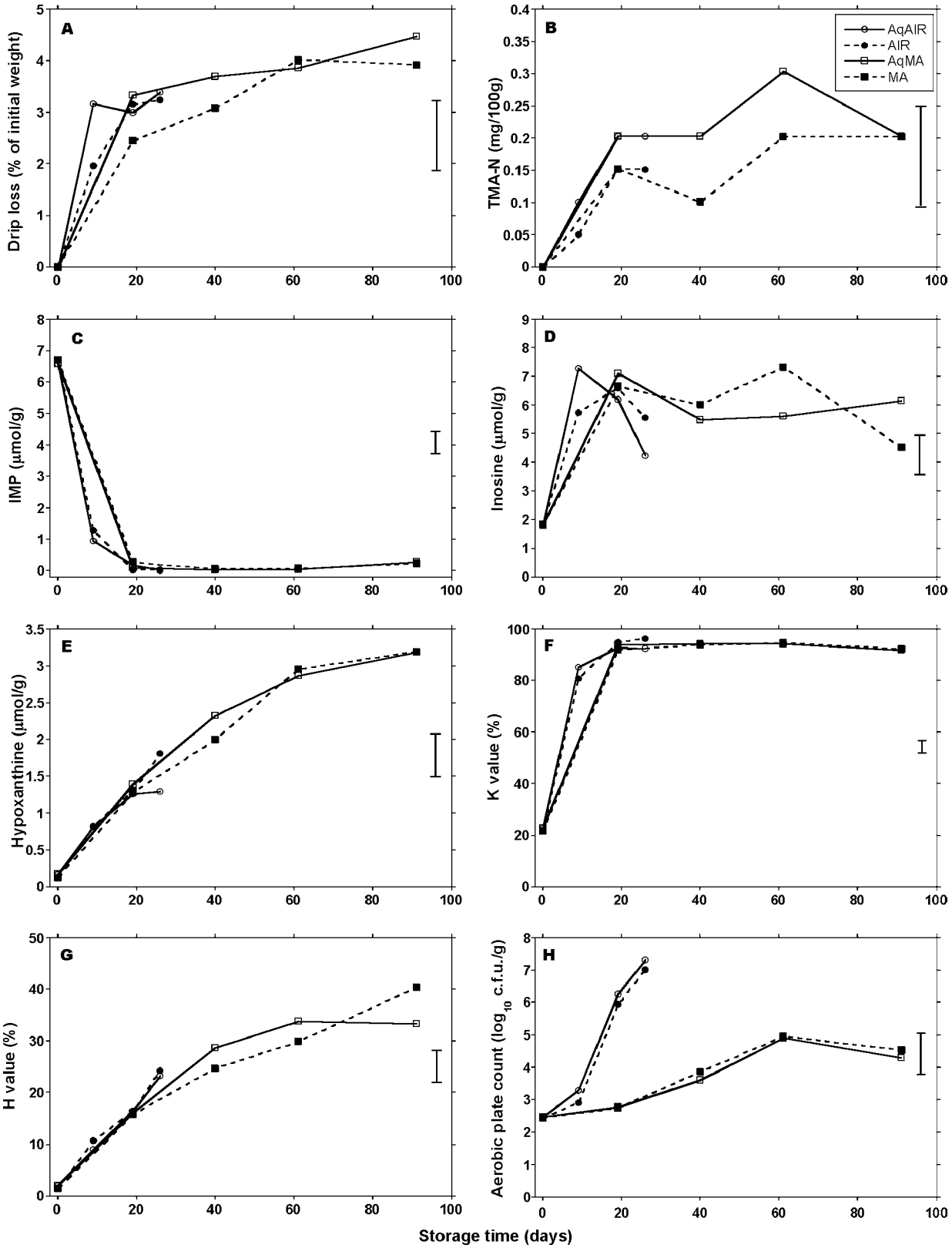

Figure 1. Physical, chemical, and microbiological changes in salmon during storage (**A**, drip loss %; **B**, TMA-N; **C**, IMP; **D**, inosine; **E**, hypoxanthine; **F**, *K*-values; **G**, *H*-values; **H**, aerobic plate count).

26 days of storage as microbial numbers reached unacceptable levels at this point (**Figure 1H**).

Two compounds were found to have differing levels in AQUI-S treated salmon compared to salmon harvested without anesthetic. These were the isomers, *E*- and *Z*-isoeugenol. Their levels were significantly higher in salmon treated with AQUI-S (see **Table 1**). The level of *Z*-isoeugenol was only detected at trace levels in salmon without AQUI-S treatment. Levels of both isomers remained constant over the storage period (data not shown). The increased levels of isoeugenol in AQUI-S treated salmon can be explained by the fact that AQUI-S is an isoeugenol based anesthetic (*26*), which enters the system of

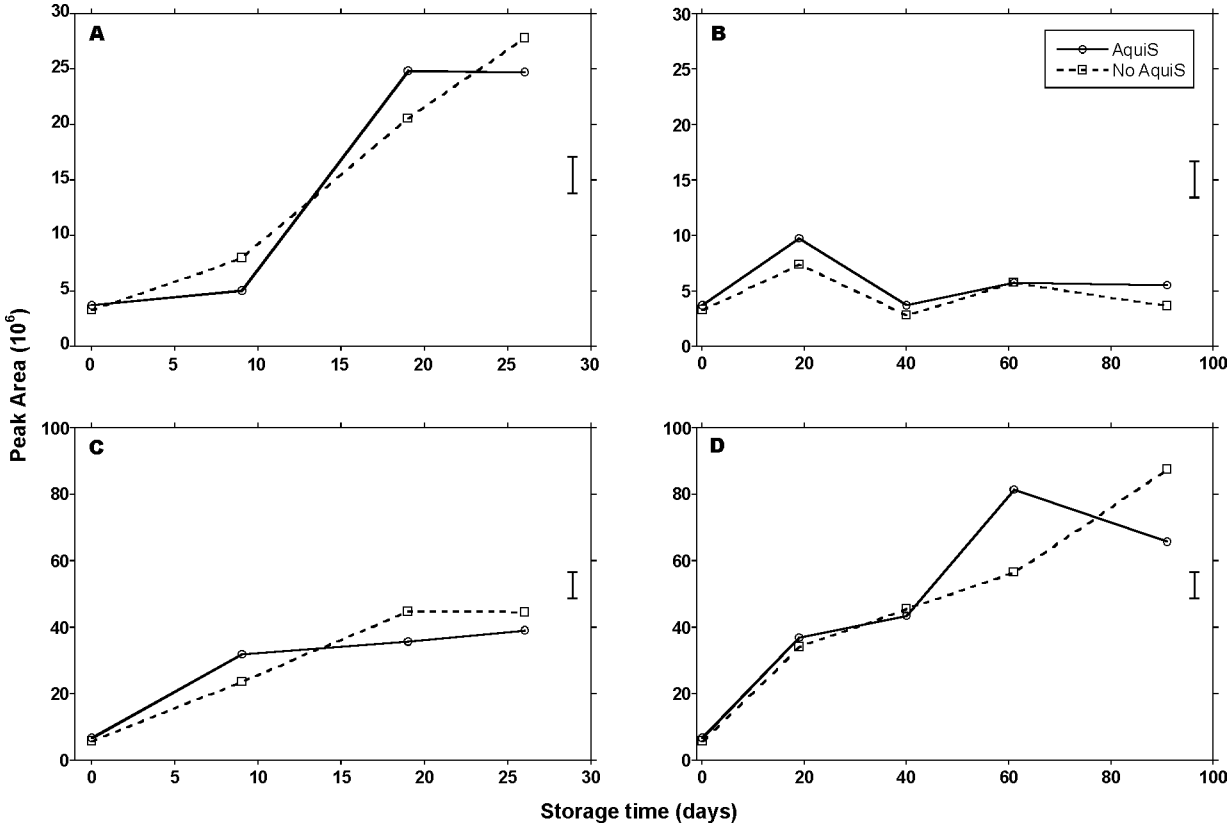

Figure 2. Comparison of levels of acetoin (**A**, air; **B**, modified atmosphere) and acetone (**C**, air; **D**, modified atmosphere) in salmon harvested with or without Aqui-S ($n = 8$).

the fish through the water. The use of an anesthetic during harvesting allows for a positive impact on fish texture (*27*), while not influencing the volatiles (with the exception of *E*- and *Z*-isoeugenol). Isoeugenol is an accepted food additive (*28*), and the increase in the amount present in the fish has not been reported to adversely impact the fish flavor.

Evolution of Salmon Volatiles During Storage. Table 1 summarizes the compounds that could be monitored using the SPME−GC−MS method. By comparing the chromatographic peak responses of compounds at different stages of storage (day 0, 9, 19, 26 for samples stored under AIR; day 0, 19, 40, 61, 91 for samples stored under MA), it was possible to identify compounds whose levels increased, decreased, or remained constant during storage. Interestingly, the behavior during storage was similar within certain classes of compounds (for example, decrease of most short chain saturated aldehydes, and increase of most short chain saturated fatty acids).

Further analysis of the data for compounds that were found to increase or decrease during storage resulted in the identification of compounds that could be classified as follows: (i) compounds that increased throughout storage regardless of treatment (for example, acetone, butanoic acid); (ii) compounds that decreased throughout storage regardless of treatment (for example, 2,3-pentanedione, *Z*-4-heptenal); and (iii) compounds that showed a clear difference in evolution with the different storage conditions (AIR, MA). Since the focus of this research was on monitoring differences between AIR and MA storage of fresh salmon, only compounds showing a difference in evolution during storage under AIR and under MA will be discussed further.

Differences between Air and Modified Atmosphere (MA) Storage. Most of the compounds that showed differences between storage conditions could be divided into the following categories: (i) increasing under AIR and little or no change under MA; (ii) increasing under AIR and lower rate of increase under MA; and (iii) decreasing under AIR and lower rate of decrease under MA.

Compounds that clearly increased under AIR while undergoing little or no change under MA conditions were acetoin, ethyl benzene, propyl benzene, and styrene. The evolution of these compounds during storage of fresh salmon is illustrated in **Figure 3**. The trends for these compounds are almost identical, with a rapid increase in levels for AIR stored salmon and little or no change observed for MA stored salmon. For salmon stored under AIR, styrene started increasing immediately while acetoin, ethyl benzene, and propyl benzene showed a lag before increases occurred, similar to the lag observed before microbial growth became logarithmic (**Figure 1H**). Acetoin is a compound produced from microbial catabolism of glycogen and is one of the most abundantly produced compounds in aerobic microbial fermentation (*29*). Increasing levels of acetoin have been linked to the growth of *Photobacterium phosphorium* (*30*). Ethyl benzene, propyl benzene, and styrene have previously been identified in crab and crayfish (*31*−*36*). The origins of ethyl benzene, propyl benzene, and styrene are unknown; however, it has been suggested that carotenoids are precursors of benzene derivatives found in meat and fish (*35*).

Most short chain fatty acids increased during storage, with the increase being similar regardless of treatment. Exceptions to this were 3-methyl butanoic acid (**Figure 4A**) and acetic acid (**Figure 4B**). The evolution of 3-methyl butanoic acid was similar to that of acetoin, ethyl benzene, propyl benzene, and styrene (showing only a slight increase in fish stored under MA). The level of acetic acid increased at a slower rate in fish stored

Table 1. Overview of Changes in Volatile Compounds During the Storage of Fresh Salmon under Air and Modified Atmosphere

RI	name	decrease ($p < 0.05$)	increase ($p < 0.05$)	no significant trend[a]	identification method[b]
	sulfurous compounds				
1191	2-ethyl thiophene			1.871 (51)	MS, RI[c]
1408	dimethyl trisulfide		all[d]		MS, RI[e]
1723	formyl thiophene			0.993 (23)	MS, RI[c,f]
	alkanes				
1001	decane			1.060 (36)	MS, RI, ref
1094	undecane			176.2 (55)	MS, RI, ref
1196	dodecane			111.8 (33)	MS, RI, ref
1299	tridecane			33.48 (34)	MS, RI, ref
1401	tetradecane			6.840 (31)	MS, RI, ref
1502	pentadecane			108.3 (31)	MS, RI, ref
	aldehydes				
715	propanal	all			MS, RI
1096	hexanal	all			MS, RI, ref
1109	2-methylbut-2-enal	AIR		MA 4.413 (67)	MS, RI
1146	*E*-2-pentenal	all			MS, RI, ref
1202	heptanal	AIR, MA[g]	MA[g]		MS, RI, ref
1238	*E*-2-hexenal	all			MS, RI, ref
1260	*Z*-4-heptenal	all			MS, RI
1307	octanal	all			MS, RI, ref
1412	nonanal	AIR		MA 4.973 (50)	MS, RI
1428	*E,E*-2,4-hexadienal			3.263 (30)	MS, RI, ref
1610	*E,Z*-2,6-nonadienal		AIR	MA 1.127 (47)	MS, RI, ref
1735	4-ethyl benzaldehyde			1.627 (44)	MS, RI, ref
	ketones				
728	acetone		all		MS, ref
1072	2,3-pentanedione	all			MS, RI, ref
1312	acetoin		AIR	MA 5.079 (55)	MS, RI, ref
1342	2,3-octanedione	AIR		MA 5.315 (43)	MS, H
1595	*E,E*-3,5-octadien-2-one	all			MS, RI
1679	acetophenone			2.828 (23)	MS, RI, ref
	alcohols				
1159	1-butanol		all		MS, RI, ref
1174	1-penten-3-ol	all			MS, RI, ref
1264	1-pentanol			25.66 (27)	MS, RI, ref
1328	cyclopentanol	all			MS, RI, ref
1337	*Z*-2-penten-1-ol	all			MS, RI, ref
1367	1-hexanol			8.456 (31)	MS, RI, ref
1464	1-octen-3-ol	all			MS, RI, ref
1471	1-heptanol	AIR		MA 15.97 (31)	MS, RI, ref
1505	2-ethyl-1-hexanol			3.255 (34)	MS, RI, ref
	acids				
1477	acetic acid		all		MS, RI, ref
1650	butanoic acid		all		MS, RI, ref
1688	3-methyl butanoic acid		all		MS, RI, ref
1756	pentanoic acid		all		MS, RI, ref
1866	hexanoic acid		all		MS, RI, ref
1972	heptanoic acid			2.700 (32)	MS, RI, ref
2079	octanoic acid			2.560 (27)	MS, RI, ref
2468	benzoic acid			2.508 (34)	MS, RI, ref
	phenolic compounds				
2035	phenol			1.572 (25)	MS, RI, ref
2288	*Z*-isoeugenol			Aqui-S 3.292 (36) no Aqui-S trace	MS, RI, ref
2383	*E*-isoeugenol			Aqui-S 21.47 (32) no Aqui-S 2.577 (44)	MS, RI, ref
	aromatics				
1139	ethyl benzene		AIR	MA 7.710 (35)	MS, RI, ref
1224	propyl benzene		AIR	MA 4.138 (24)	MS, RI[h]
1277	styrene		AIR	MA 3.371 (48)	MS, RI, ref

[a] Values shown are average peak area in millions (coefficient of variation, %), $n = 16$ (AIR, MA, Aqui-S, no Aqui-S data), $n = 32$ (combined data). [b] Identification confirmed by MS, mass spectra; RI, Kovats retention indices published in Kondajoyan and Berdague (*43*), unless indicated otherwise (see footnotes *c*, *e*, *f*, *h*); H, identified by homologous series; ref, identified by comparison to authentic standards. Unless confirmed by comparison to authentic standards, compounds are considered as tentatively identified. [c] Madruga and Mottram (*44*). [d] "All" indicates that all the treatments showed the same general trend (increase or decrease over time). [e] Le Guen et al. (*45*). [f] Prost et al. (*46*). [g] MA storage: compound decreased from day 0 to day 40, then increased from day 40 to day 91. [h] Vejaphan et al. (*36*).

under MA than in fish stored under AIR. The increase in the levels of 3-methyl butanoic acid and acetic acid results from microbial fermentation, with 3-methyl butanoic acid being a metabolite of leucine and acetic acid a product of glucose fermentation (*37*). The slower rate of increase for these compounds in MA stored salmon could therefore be explained

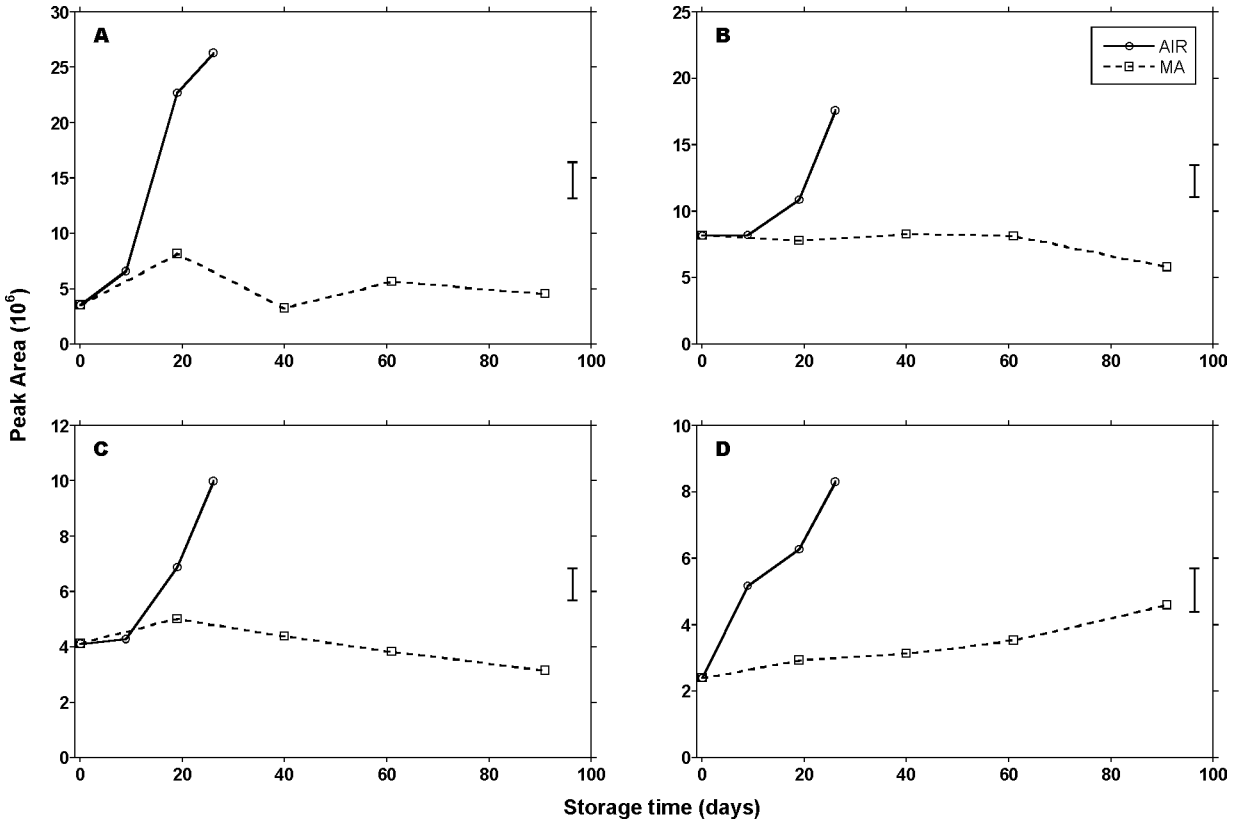

Figure 3. Compounds increasing in air stored salmon and not under modified atmosphere (**A**, acetoin; **B**, ethyl benzene; **C**, propyl benzene; **D**, styrene) ($n = 16$).

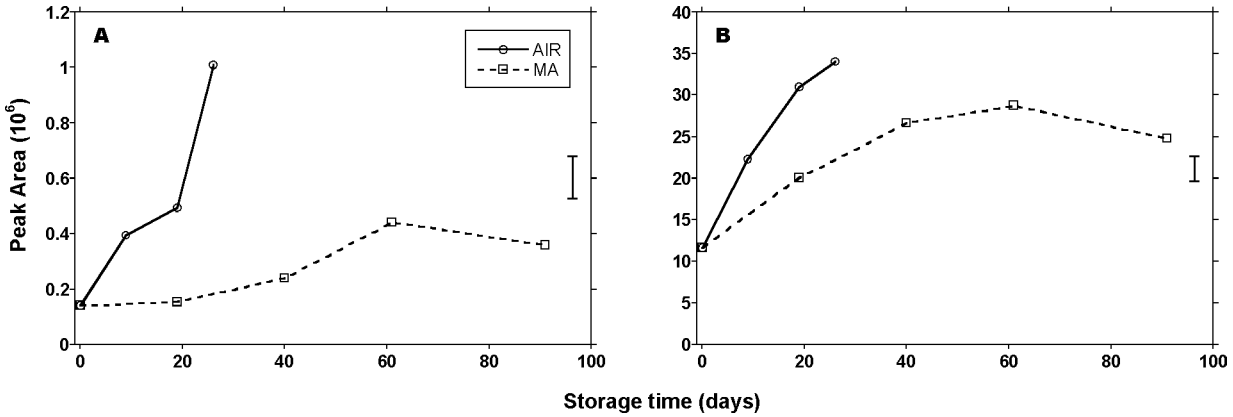

Figure 4. Short chain fatty acids with reduced rate of increase under modified atmosphere (**A** 3-methyl butanoic acid; **B**, acetic acid) ($n = 16$).

by the inhibitory effect of CO_2 on microbial growth. A similar effect of CO_2 on the rate of increase of acetic acid levels in salmon was found by de la Hoz et al. (*2*).

Several alcohols decreased at a lower rate in the MA stored salmon than in the salmon stored under AIR. **Figure 5** shows the evolution of 1-heptanol, cyclopentanol, *Z*-2-penten-1-ol, and 1-penten-3-ol during storage. Short chain saturated aldehydes (hexanal, heptanal, octanal, nonanal) and unsaturated aldehydes (*E*-2-pentenal, *E*-2-hexenal) were found to follow a similar trend to the alcohols (**Figure 6**). According to the literature, several of these alcohols and aldehydes are commonly associated with fresh fish flavor (*38*−*41*). Characteristic fresh fish flavor compounds include volatile 6-, 8-, and 9-carbon aldehydes, ketones, and alcohols, which arise from lipoxygenase action on long chain polyunsaturated fatty acids (*38*, *41*). The clear decrease in the rate of loss of these fresh fish volatiles in MA stored salmon, which results from both microbial action and endogenous enzyme action, could be due to both the inhibitory effect of CO_2 on microbial growth and the absence of oxygen decreasing the rate of enzymatic breakdown for these compounds.

Correlation of Fish Volatile Compound Changes with Sensory Analysis Data. An earlier SPME study (*25*), carried out alongside the previously published research by Fletcher et al. (*4*), found that the changes observed for a few compounds (acetoin, cyclopentanol, 1-penten-3-ol) correlated well with changes in the sensory quality index score of the fresh salmon. The correlation coefficients for correlation between changes in the quality index values and volatile peak areas were close to or equal to 1, with negative values indicating markers of freshness and positive values indicating markers of spoilage. The current research confirmed the trends observed for these compounds, and these compounds are therefore excellent potential markers for the quality of fresh salmon (spoilage

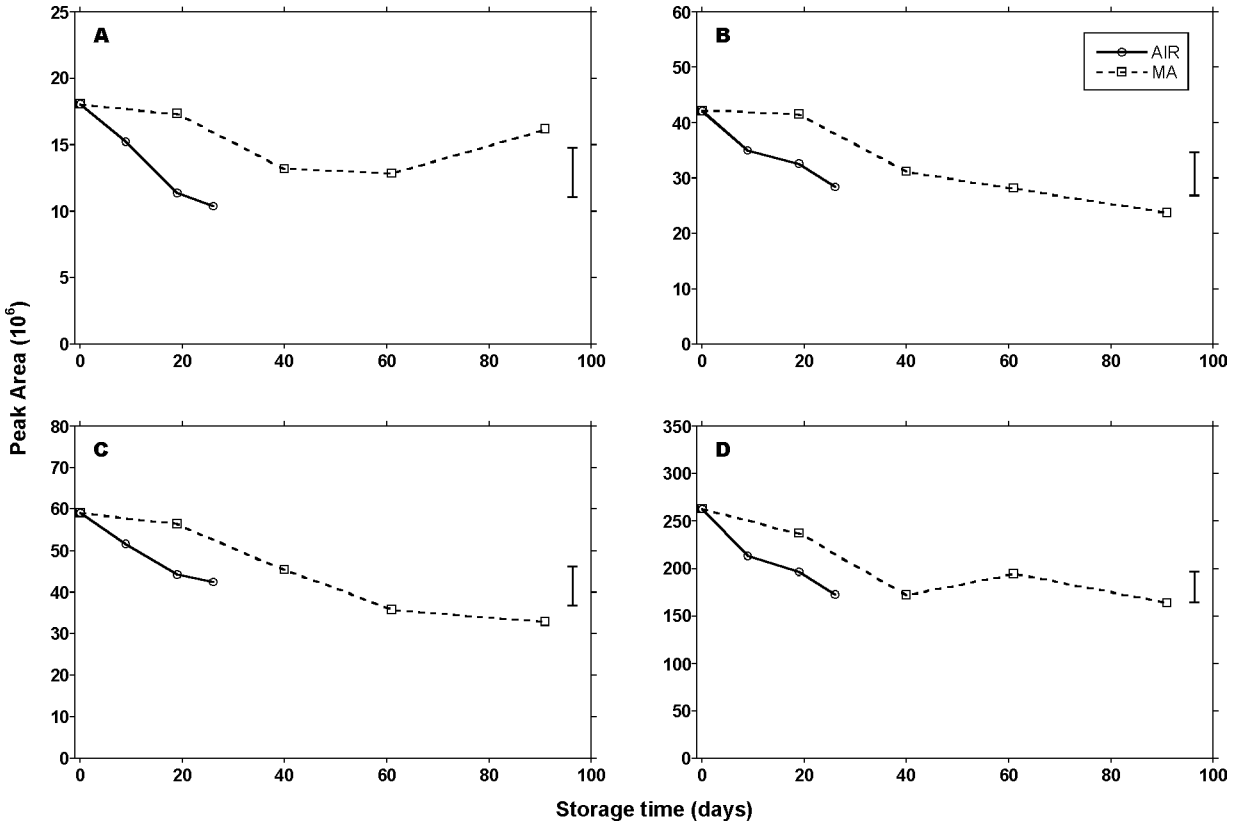

Figure 5. Evolution of alcohols during the storage of fresh salmon under air and under modified atmosphere (**A**, 1-heptanol; **B**, cyclopentanol; **C**, *Z*-2-penten-1-ol; **D**, 1-penten-3-ol) ($n = 16$).

marker: acetoin; freshness markers: cyclopentanol, 1-penten-3-ol). As the changes occurring for ethyl benzene, propyl benzene, styrene, 3-methyl butanoic acid, and acetic acid followed a similar trend to acetoin, it is expected that these compounds are also potential spoilage markers. In addition, it is expected that 1-heptanol, *Z*-2-penten-1-ol, 1-octen-3-ol, as well as the aldehydes (hexanal, heptanal, octanal, nonanal, *E*-2-pentenal, *E*-2-hexenal) could also serve as freshness markers due to the similarity of their evolution profiles to those of cyclopentanol and 1-penten-3-ol during storage.

Potential Development of the SPME−GC Method for Quality Control. The adsorption performance of SPME fibers can vary depending on the history of the fiber (for example, temperature of exposure, number of exposures) and between different SPME fiber batches. The current SPME method was designed to decrease the introduction of variation in the results due to the SPME fiber. As it is not practical to limit the use of an SPME fiber to only 10 analyses, it is necessary to apply a method of standardization to allow for variation in the data due to the SPME fiber characteristics. The use of an internal standard is not practical in this case because these effects on adsorption are different for different compounds. Also, it would be difficult to ensure homogeneous distribution of the internal standard in the fish powder sample. A method of standardization that has been successfully applied for the analysis of dairy samples is the analysis of a so-called reference sample alongside the samples under study (*42*). The ratio of a given volatile compound peak area in the sample over the peak area of the same compound in the reference sample can then be used to accurately and consistently calculate the amount of that volatile in the sample. The same reasoning can be applied to any volatile compound. Under these conditions, the use of a fresh salmon sample as a reference would allow for SPME to be used to monitor salmon quality regardless of SPME fiber batch or age.

The ratios of acetoin (stored fish/fresh fish) were calculated for the data from Dufour et al. (*25*) and for the current data. As shown in **Figure 7**, the ratios for acetoin in AIR stored salmon samples were similar for the two studies. Cutoff ratios (conservatively set at the point beyond which less than 75% of panelists accept the salmon and more than 25% reject the salmon; sensory score of 12) were determined for the different treatments using the sensory analysis results and data from Dufour et al. (*25*). These cutoff ratios and the corresponding days of spoilage beyond sensory acceptability for the current data are shown in **Table 2**. As shown in **Table 2**, a ratio of 4.6 to 5 was determined to be indicative of the salmon no longer being acceptable in terms of sensory analysis. A cutoff ratio of 4.6 could thus be used to indicate sensory acceptability of any given salmon sample. According to the acetoin cutoff ratio, the salmon stored under air in the current study was no longer acceptable on day 15. The MA salmon samples' ratio for acetoin, however, did not exceed 2.5 throughout storage (91 days). Unlike the samples in the earlier study detailed by Fletcher et al. (*4*) and Dufour et al. (*25*), where microbial numbers had reached almost 8 log cfu/g by day 40 in samples stored under MA, the MA stored samples in the current study did not exceed 5 log cfu/g throughout storage (91 days). It therefore appears that, under conditions where microbial spoilage is successfully inhibited, it will be necessary to look at the ratio of another compound (such as one of the freshness markers) to determine the sensory acceptability cutoff point. This would require further research to link sensory acceptability to decreasing levels of fresh salmon volatile compounds in samples where microbial growth is inhibited. As there was no formal sensory evaluation carried out in the current trial, the current data cannot be used to conclusively indicate shelf life extension of MA stored salmon. However, the authors did examine each raw portion of fish, and no off-odor, obvious color change, or slime

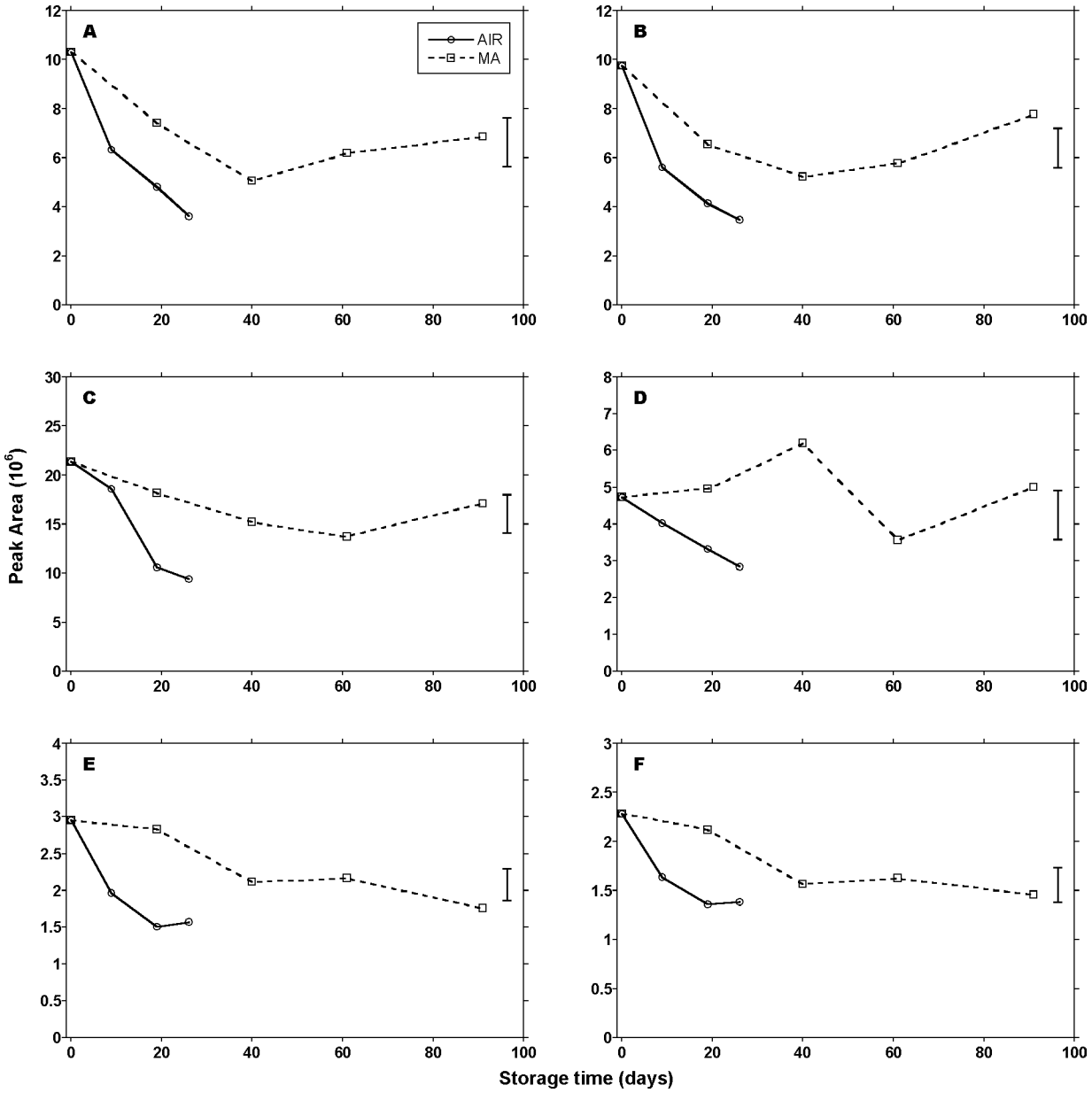

Figure 6. Evolution of aldehydes during the storage of fresh salmon under air and under modified atmosphere (**A**, hexanal; **B**, heptanal, **C**, octanal; **D**, nonanal; **E**, *E*-2-pentenal; **F**, *E*-2-hexenal) ($n = 16$).

was noted in the MA stored samples (in contrast to AIR stored samples). This, combined with low microbial counts, would suggest that the MA samples had not spoiled after 90 days, which may reflect the low initial microbial counts and/or the composition of the microflora present on the fish. Fletcher et al. (*18*) carried out formal sensory evaluation on similarly prepared samples, and although microbial growth was substantially higher than in the current study, the trained panel only described samples stored for 90 days as slightly spoiled. In that case, the microbial flora was dominated by lactic acid bacteria, which do not necessarily spoil seafood but rather have been suggested as potential biopreservative agents in some cases (*47*, *48*).

Final Comments. An SPME−GC method for the analysis of salmon volatile compounds was developed to monitor the quality of the chilled fish during storage under modified atmosphere. Using this method, it was possible to identify several potential markers for salmon freshness (cyclopentanol, 1-penten-3-ol, *Z*-2-penten-1-ol, 1-octen-3-ol, hexanal, octanal, *E*-2-pentenal, *E*-2-hexenal) and for spoilage of salmon (acetoin, ethyl benzene, propyl benzene, styrene, 3-methyl butanoic acid, acetic acid). These compounds showed clear differences between AIR and MA stored samples. With the exception of *E*- and

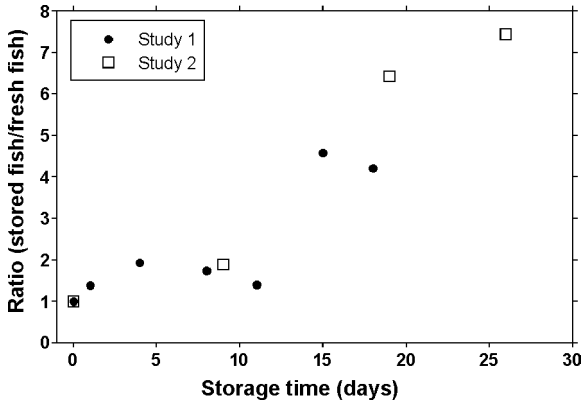

Figure 7. Ratios (stored fish/fresh fish) for acetoin in salmon samples stored under air (study 1, Dufour et al. (*25*); study 2: current data).

Table 2. Cutoff Ratios for Acetoin (Indirect Marker for Sensory Acceptability) in Stored Salmon Samples

	Dufour et al. study (*25*)			current study	
storage treatment[a]	sensory score	ratio	day	ratio	day
AIR9	12	5	1.8	na	
AIR	12	4.6	15	4.6	15
MA	12	5	22	4.6	na

[a] Abbreviations indicate the following: AIR9, stored in air at 9 °C; AIR, stored in air at 0 °C; MA, stored in 40:60 v/v CO_2:N_2 at 0 °C.

Z-isoeugenol, the use of AQUI-S to harvest salmon (rested harvesting) did not impact the levels of the volatiles monitored. A cutoff ratio (stored fish level/fresh fish level) of 4.6 for acetoin was indicative of spoilage beyond sensory acceptability. Further research is needed to: (i) determine cutoff ratios for other marker compounds that can be used to indicate the sensory acceptability cutoff point in samples of salmon where microbial spoilage is low or is successfully inhibited, and (ii) test the consistency of the SPME method with the use of a reference sample to take into account fiber variations and changes.

ABBREVIATIONS USED

ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; cfu, colony forming units; GC, gas chromatography; GC−MS, gas chromatography−mass spectrometry; HX, hypoxanthine; i.d., internal diameter; IMP, inosine 5′-monophosphate; INO, inosine; MA, modified atmosphere; REML, residual maximum likelihood; sPCA, salt plate count agar; SPME, solid-phase microextraction; TMA-N, trimethylamine nitrogen; TVBN, total basic volatile nitrogen.

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