Microbiological, chemical and sensory spoilage analysis of raw Atlantic cod (*Gadus morhua*) stored under modified atmospheres

L. Kuuliala¹,²*, Y. Al Hage¹, A.-G. Ioannidis¹,³, M. Sader⁴, F.-M. Kerckhof⁵, M. Vanderroost¹, N. Boon⁵, B. De Baets⁴, B. De Meulenaer³, P. Ragaert¹ and F. Devlieghere¹

¹Laboratory of Food Microbiology and Food Preservation, Department of Food Safety and Food Quality, Part of Food2Know, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, B-9000 Ghent, Belgium

²Paper Converting and Packaging Technology, Department of Materials Science, Faculty of Engineering Sciences, Tampere University of Technology, P.O. Box 589, FI-33101 Tampere, Finland

³Research Group Food Chemistry and Human Nutrition, Department of Food Safety and Food Quality, Part of Food2Know, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, B-9000 Ghent, Belgium

⁴KERMIT – Research Unit Knowledge-based Systems, Department of Mathematical Modelling, Statistics and Bioinformatics, Part of Food2Know, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, B-9000 Ghent, Belgium

⁵Center for Microbial Ecology and Technology (CMET), Ghent University, Coupure Links 653, B-9000, Ghent, Belgium

*Corresponding author. Laboratory of Food Microbiology and Food Preservation, Department of Food Safety and Food Quality, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, B-9000 Ghent, Belgium; Tel.: +32-(0)9 264 92 03; E-mail address:

Lotta.Kuuliala@UGent.be
Abstract

During fish spoilage, microbial metabolism leads to the production of volatile organic compounds (VOCs), characteristic off-odors and eventual consumer rejection. The aim of the present study was to contribute to the development of intelligent packaging technologies by identifying and quantifying VOCs that indicate spoilage of raw Atlantic cod (*Gadus morhua*) under atmospheres (%v/v CO₂/O₂/N₂) 60/40/0, 60/5/35 and air. Spoilage was examined by microbiological, chemical and sensory analyses over storage time at 4 or 8 °C. Selected-ion flow-tube mass spectrometry (SIFT-MS) was used for quantifying selected VOCs and amplicon sequencing of the 16S rRNA gene was used for the characterization of the cod microbiota. OTUs classified within the *Photobacterium* genus increased in relative abundance over time under all storage conditions, suggesting that *Photobacterium* contributed to spoilage and VOC production. The onset of exponential VOC concentration increase and sensory rejection occurred at high total plate counts (7-7.5 log). Monitoring of early spoilage thus calls for sensitivity for low VOC concentrations.

Keywords

Amplicon sequencing; *Photobacterium*; SIFT-MS; sensor; volatile organic compound
1. Introduction

Raw fish is highly perishable due to the intrinsic properties of the product and inevitable microbial activity. Spoilage of fish is primarily caused by microbial growth and metabolism and is characterized by changes in the sensory properties that lead to unacceptable product quality (Gram & Huss, 1996; Gram & Dalgaard, 2002; Gram et al., 2002). Shelf life of fish is affected by several factors, including storage temperature, fish species, initial microbial contamination and packaging conditions (Sivertsvik, Jeksrud, & Rosnes, 2002). Even though $10^7$ CFU/g has generally been considered as a maximum acceptable microbial load for fish (Stannard, 1997), sensory rejection has typically been found at microbial levels between $10^6$-$10^9$ CFU/g (Dalgaard, Mejlholm, Christiansen, & Huss, 1997; Mikš-Krajnik, Yoon, Ukuku, & Yuk, 2016; Nuin et al., 2008; Parlapani, Mallouchos, Haroutounian, & Boziaris, 2014; Parlapani, Verdos, Haroutounian, & Boziaris, 2015).

Specific spoilage organisms (SSOs) typically constitute a fraction of the initial microbiota and their outgrowth eventually leads to unacceptable changes in the product quality (Gram & Dalgaard, 2002). The microbiota of fresh marine fish generally consists of psychrotrophic Gram-negative rod-shaped bacteria along with Gram-positive microbes (Gram & Huss, 1996). In marine fish stored under refrigerated aerobic conditions, *Pseudomonas* and *Shewanella* spp. have been observed to be dominating (Gram, Trolle, & Huss, 1987; Gram & Huss, 1996; Gram & Dalgaard, 2002; Vogel, Venkateswaran, Satomi, & Gram, 2005), whereas *Photobacterium phosphoreum* has been identified as an SSO of Atlantic cod (*Gadus morhua*) under different modified atmosphere packaging (MAP) conditions (Dalgaard et al., 1997; Dalgaard, 1995; Debevere & Boskou, 1996).
Odor is one of the most important quality determinants for fish freshness (Olafsdottir, Jonsdottir, Lauzon, Luten, & Kristbergsson, 2005). As a result of microbial metabolism, volatile organic compounds (VOCs) are often produced, which leads to the production of characteristic off-odors and off-flavors. Typical compounds associated with fish spoilage include acids, alcohols, aldehydes, amines, ketones and sulfides (Gram & Dalgaard, 2002). The spoilage potential of SSOs is characterized by their qualitative ability to produce off-odors, whereas spoilage activity refers to the quantitative ability to produce spoilage metabolites (Gram & Dalgaard, 2002; Gram et al., 2002). Thus, evolution of spoilage-related VOCs could be used for fish quality evaluation during storage. Different approaches for characterizing the VOC profile have been applied to marine fish species such as cod (Fernández-Segovia, Escriche, Gómez-Sintes, Fuentes, & Serra, 2006; Noseda et al., 2010), salmon (Jónsdóttir, Ólafsdóttir, Chanie, & Haugen, 2008; Jørgensen, Huss, & Dalgaard, 2001; Macê et al., 2013; Mikš-Krajnik et al., 2016), sea bream (Parlapani et al., 2014; Parlapani et al., 2015; Soncin, Chiesa, Panseri, Biondi, & Cantonì, 2009), sea bass (Parlapani, Haroutounian, Nychas, & Boziaris, 2015), hake (Baixas-Nogueras, Bover-Cid, Vidal-Carou, Veciana-Nogués, & Mariné-Font, 2001), mackerel (Alfaro, Hernández, Baliano-Zuazo, & Barranco, 2013) and turbot (Xu et al., 2014).

Intelligent packaging technologies aim at improving the quality and safety of the packaged product and/or informing about its status by detecting, sensing, communicating, recording or applying another intelligent function (Yam, Takhirstov, & Miltz, 2005). Among these technologies, sensors that convert physical or chemical information into an informative signal have been considered to have high potential for future applications (Ghaani, Cozzolino, Castelli, & Farris, 2016; Kerry, O’Grady, & Hogan, 2006; Vanderroost, Ragaert, Devlieghere, & De Meulenaer, 2014). The use of sensor technologies for monitoring VOCs indicating fish spoilage
could enhance the detection of spoilage in individual packages, thus improving quality evaluation and reducing food and packaging material waste throughout the supply chain. Even though different applications for sensor-based quality monitoring of fish have been examined (Bhadra, Narvaez, Thomson, & Bridges, 2015; Chung, Le, Tran, & Nguyen, 2017; Efremenko & Mirsky, 2017; García et al., 2017; Morsy et al., 2016; Pacquit et al., 2006; Pacquit et al., 2007; Perera, Pardo, Barrettino, Hierlermann, & Marco, 2010), there is still a limited number of studies focusing on direct and real-time quantification of the VOC profile produced in the package headspace during storage time, aiming at the development of intelligent packaging technologies.

Efficient quality monitoring of fish spoilage calls for fast, non-destructive and sensitive methods. However, conventional quality analyses of fish packaged under modified atmospheres (MAs) are commonly destructive and time consuming, such as the determination of total volatile basic nitrogen (TVB-N) by steam distillation (Pacquit et al., 2006) or plate counts. Several technologies have been used for rapid and accurate characterization of VOCs, including gas chromatography-mass spectrometry (GC-MS) (Béné, Hayman, Reynard, Luisier, & Villettaz, 2001; G. Duflos et al., 2010; Edirisinghe, Graffham, & Taylor, 2007; Fernández-Segovia et al., 2006; Grimm, Lloyd, Batista, & Zimba, 2000; Jaffrès et al., 2011; Leduc et al., 2012; Mikš-Krajnik et al., 2016; Z. Zhang, Li, Luo, & Chen, 2010) and electronic noses (Natale et al., 2001; Olafsdottir et al., 2005; Zaragozá et al., 2014). On the other hand, selective-ion flow-tube mass spectrometry (SIFT-MS) can be used for non-destructive and sensitive real-time quantification of VOCs from the package headspace. The technology is based on reactions between precursor ions (H$_3$O$^+$, NO$^+$, O$^+$) and target compounds, followed by the quantification of the resulting product ions on the basis of their mass to charge (m/z) ratio. SIFT-MS has previously been validated for fish metabolite research (Noseda et al., 2010) and used for VOC analysis of different food...
products, including seafood (Noseda et al., 2012), meat (Carrapizo et al., 2015; Olivares, Dryahina, Španěl, & Flores, 2012), fruit (Zhang et al., 2013; Zhang, Samapundo, Pothakos, Sürengil, & Devlieghere, 2013; Zhang et al., 2014) and cheese (Castada, Wick, Taylor, & Harper, 2014; Castada, Wick, Harper, & Barringer, 2015; Langford et al., 2012).

Identification and quantification of VOCs related to spoilage is of high importance for the development of food quality monitoring. Establishing a relation between VOC production, microbial growth (both in total amount and in specific microorganisms) and sensorial quality is needed as the basis for the development of intelligent packaging solutions. In the present study, spoilage of Atlantic cod packaged under modified atmospheres was analyzed by following microbial growth, VOC concentrations and sensory quality during refrigerated storage. SIFT-MS was used for the real-time quantification of VOCs from the package headspace and amplicon (NGS) sequencing of the 16S rRNA gene was used for the characterization of the cod microbiota at different stages of storage. The results of the present study contribute to the development of intelligent packaging technologies within the CheckPack project (VLAIO grant number 130036).

2. Materials and methods

2.1. Raw material

For each individual storage experiment, Atlantic cod (minimum body weight ca. 4.5 kg) was caught in the North Atlantic Ocean (FAO zone 27), gutted, filleted and stored under ice. The fish was transported to Belgium by air and delivered to the Laboratory of Food Microbiology and Food Preservation (LFMFP) in polystyrene boxes under ice.

2.2. Packaging and storage
Cod fillet portions (217 ± 5 g) were packaged under different atmospheres with a gas-product ratio 2:1 using a tray sealer MECA 900 (DecaTechnic, Herentals, Belgium), multilayer packaging trays (PP/EVOH/PP, oxygen transmission rate 0.03 cm³/tray*24h at 23 °C and 50 % R.H.) and top film (PA/EVOH/PA/PP, oxygen transmission rate 6.57 cm³/m²*24h*atm at 23 °C, 50 % R.H. and 1 atm). Three different atmospheres and two storage temperatures were applied (Table 1): independent batches of fish were used for each of the five storage experiments. In the present study, the storage experiments are referred to as H4, H8, L4, L8 and Air, where the notation of the MA conditions indicates high (H) or low (L) oxygen content and temperature in Celsius degrees (4 or 8). For the determination of background concentrations possibly originating from the packaging materials and/or heat sealing, sample-free packages (blanks) with similar gas atmospheres were prepared. The packages were stored at (4.0 ± 0.7) or (8.0 ± 0.4) °C until the day of analysis. On a regular basis, three randomly selected packages were analyzed. After sampling, the remaining fish portion was packaged under vacuum using high barrier film bags (oxygen transmission rate < 2.7 cm³/m²*24h*bar at 23 °C and 0 % R.H.) and stored at -32 °C for no longer than 120 days (sensory evaluation) or one year (amplicon sequencing).

2.3. Microbiological analysis

For microbiological analysis, 30 ± 0.1 g of individual fillet was aseptically weighed into a sterile stomacher bag and diluted ten times in physiological saline peptone solution (PPS; 0.85 % m/v NaCl, 0.1 % m/v peptone). The samples were homogenized in Stomacher Lab Blender (LED Techno, Heusden-Zolder, Belgium) for one minute and appropriate decimal dilutions were prepared in PPS. The total psychrotrophic count (TPC) was determined on Marine Agar (MA; Difco Le Pont de Claux, France) by spread plating, lactic acid bacteria (LAB) on Man Rogosa Sharpe Agar (MRS; Oxoid, Hampshire, UK) or modified MRS (mMRS; yeast extract 4.0 g/L,
Lab-Lemco powder 8.0 g/L, peptone 10.0 g/L, sorbitan mono-oleate (Tween 80) 1 ml/L, dipotassium hydrogen phosphate 2 g/L, sodium acetate 5 g/L, triammonium citrate 2 g/L, magnesium sulphate 0.2 g/L, manganese sulphate 0.05 g/L; pH 8.6 at 25 °C; 20 % glucose solution 100 mL/L after autoclaving) by pour plating, hydrogen sulfide (H$_2$S) producers on Iron Agar Lyngby (IAL; Oxoid) supplemented with L-cysteine (Fluka, Steinheim, Germany) by pour plating, Pseudomonads on Pseudomonas Agar (PA; Oxoid) supplemented with Pseudomonas CFC supplement SR 103E (Oxoid) by spread plating and Brochotrix thermosphacta on Streptomycin Sulfate Thallous Acetate Actidione Agar (STAA; Oxoid) supplemented with selective supplement SR 151E (Oxoid) by spread plating. Plates were incubated at 22 °C for 2 (PA and STAA), 3 (MRS and IAL) or 5 days (MA).

2.4. Quantification of spoilage related VOCs by SIFT-MS

A selected-ion flow-tube mass spectrometer (Voice 200, Syft Technologies™, Christchurch, New Zealand) was used for quantifying a predefined set of VOCs in the package headspace. Principles of the instrument have been described elsewhere (Noseda et al., 2010). The compounds (Table 2) were selected on the basis of previous research and literature survey. The package headspace was sampled with a flow rate of 32 ml/min during 60 seconds (preparation 10s, sample 50s) through a septum inserted on the package lid and the VOC concentrations were averaged over eleven data points. Two consecutive gas samples per package were analyzed. During sampling, the headspace was connected to atmospheric air with a needle inlet in order to avoid package collapse and subsequent change in the internal conditions of the package. Respectively, empty packages (blanks, n=9-14) from each headspace-temperature combination were randomly analyzed throughout the storage time and used for determining the limit of
quantification (LOQ) of each compound and for background subtraction. Concentrations of the VOCs were determined with the LabSyft software (Syft Technologies™).

The relative standard deviation (SD%) of each VOC concentration during a SIFT-MS measurement was calculated as

\[ SD\% = \frac{SD_m}{x_m} \times 100\% \]  

(1)

where \(x_m\) is the average and \(SD_m\) the standard deviation of a single SIFT-MS measurement (n=11).

VOCs with concentrations exceeding 25 % average relative standard deviation during the entire storage time within a certain packaging condition were excluded from further analysis.

The Limit of Quantification (LOQ) was calculated with the International Union of Pure and Applied Chemistry (IUPAC) equation (Mocak, Bond, Mitchell, & Scollary, 1997):

\[ \text{LOQ} = x_{bl} + 6 \times SD_{bl} \]  

(2)

where \(x_{bl}\) is the total average and \(SD_{bl}\) the standard deviation of the blanks. Background was subtracted from the measured concentrations that exceeded the LOQ: the reported results are measured concentrations minus \(x_{bl}\).

2.5. Headspace composition (% CO₂/O₂), pH and color measurements

The headspace gas composition (% v/v CO₂/O₂) was analyzed with a gas analyzer (CheckMate® 9900 CO₂/O₂, Dansensor A/S, Ringsted, Denmark). pH was determined as an average of three consecutive measurements from randomly selected spots in individual fillets within 30 minutes after opening the package using a pH electrode (Lab® 427, Mettler Toledo GmbH, Schwerzenbach, Switzerland) connected to a pH meter (SevenEasy, Mettler Toledo GmbH). The product color was determined as an average of ten measurements from randomly selected fillet spots by a
spectrophotometer (CM 2500d, Konica Minolta Sensing Inc., New Jersey, USA) and related SpectraMagic™ NX color data software. Color was measured through a small Petri dish (diameter 230 mm) using the CIE L’ a’ b’ color space with a standard 10° observer and Illuminant D65.

2.6. Sensory evaluation

Sensory evaluation was based on olfactory evaluation and performed in individual booths under red light (UGent Sensolab). A panel of 8-12 persons having experience in sensory evaluation of fish was formed from the laboratory staff at LFMFP. One out of three daily replicates (A-C) was randomly selected and used per testing session. The samples were thawed at 2 °C overnight, cut to 5.0 ± 0.1 g portions and presented to the panelists at 4 °C in odor-free, transparent plastic cups (diameter 67 mm; AVA, Temse, Belgium), closed with lids (AVA) and labelled with three-digit random codes generated with Excel 2013 (Windows).

Ranking tests (ISO, 2006) were used to determine if significant differences occurred between different stages of storage within a certain packaging condition. Four samples were presented to the panelists to be ranked from least fresh (1) to most fresh (4). For conditions H4, H8, and L4, a second ranking test was performed for the critical days identified by the first test. The collected data was subjected to a Friedman test followed by a Least Significant Difference test (Excel 2013 for Windows) in order to determine whether significant differences occurred between samples from different days of storage.

Acceptance tests were used to determine the quality of cod samples from different stages of storage within a certain packaging condition. Four samples were presented to the panelists along with a fresh reference (day 0) from the same lot. A five-point scale (very good, good, satisfactory, marginal, spoiled) was used for the evaluation.
2.7. Amplicon sequencing

16S rRNA gene amplicon sequencing analysis was used for the characterization of the cod microbiota over storage time. Three samples stored at -32 °C were selected to represent early, intermediate and late stages of storage (Table 1). One randomly selected sample out of three daily replicates (A-C) was used for the analysis.

A phenol/chloroform extraction procedure with mechanical disruption using a FastPrep device (Vilchez-Vargas et al., 2013) was used for the extraction of DNA. Bacterial cells were aseptically collected from the frozen sample surface by swabbing. An individual swab was placed in an Eppendorf tube with 200 mg glass beads and 1000 µl of lysis buffer (100 mM Tris; 100 mM EDTA; 100 mM NaCl; 1 wt/vol % polyvinylpyrrolidone; 2 wt/vol % sodium dodecyl sulphate; 50 ml water; pH 8). The tube was transferred to the FastPrep-24 instrument (MP Biomedicals, Santa Ana, California, USA) and disrupted twice at 1400 rpm for 60 s. After centrifuging at maximum speed for 5 min, phenol-chloroform-isoamilic alcohol (500 µl; pH 7) was added to the supernatant and the solution was thoroughly vortexed and centrifuged at maximum speed for 60 s. Chloroform (700 µl) was added to the supernatant, mixed by vortexing and centrifuged at maximum speed for one minute. The resulting upper phase was divided into two Eppendorf tubes (450 µl per tube) where sodium acetate (3M; 45 µl) was added, followed by mixing and addition of isopropyl alcohol (-20 °C; 500 µl). The solution was mixed by inverting, stored for one hour at -20 °C and centrifuged at maximum speed for one minute at 4 °C. The resulting pellet was dried and dissolved into T10E1 (100 µl).

Library preparation and sequencing was carried out at LGC Genomics (Germany) according to the procedure presented by De Vrieze et al. (2016). The PCR mix contained 1 ng of DNA extract and PCRs showing low yields were further amplified for 5 additional cycles if needed.
Sequencing was done on an Illumina MiSeq platform using v3 Chemistry (Illumina, San Diego, California, USA) along with a mock community that was included in triplicate in the sequencing run to assess the sequencing quality. The mock community consisted of the genomic DNA of 12 species from 10 different phyla and was pooled to an equimolar concentration of 16S rRNA gene copies based on Q-PCR with the Illumina primers.

The mothur software package v. 1.38.0 (Schloss et al., 2009) and guidelines developed by P. Schloss (Miseq sop.12th October 2016; Kozich, Westcott, Baxter, Highlander, & Schloss, 2013) were used for processing the amplicon sequencing data. From the total number of forward and reverse reads, contigs with lengths outside of the 2.5 - 97.5 % quantiles or sequences with ambiguous base calls were removed. Remaining unique sequences were aligned to the mother-reconstructed SILVA Seed alignment v. 123 (Pruesse et al., 2007). Unique sequences were pre-clustered within a distance of 1/100 nucleotides and chimeras were screened with UCHIME (Edgar, Haas, Clemente, Quince, & Knight, 2011). Next, sequences were classified using RDP v. 14 (Cole et al., 2009) and Wang’s algorithm.

Non-bacterial or unidentified sequences were removed and the remaining OTUs were clustered using average linkage and 97 % sequence identity. Single-read OTUs were considered as likely errors and discarded from further analyses. The alpha diversity was examined by rarefaction curves and community richness estimators Chao1984 (Chao, 1984), ChaoBunge2002 (Chao & Bunge, 2002) and ACE-1 (Chao & Lee, 1992), diversity estimators Shannon (Shannon & Weaver, 1949), Simpson (Simpson, 1949) and inverse Simpson, and evenness estimator Pielou (Pielou, 1966).

3. Results
3.1. Headspace composition (% CO₂/O₂)

The development of headspace gas concentrations (CO₂/O₂) is presented in Table 3. Under high O₂ concentrations (H4 and H8), simultaneously, an initial increase in oxygen levels and a decrease in carbon dioxide levels were observed, the latter likely due to the dissolution of carbon dioxide into the food product. Under all tested conditions, oxygen content decreased and carbon dioxide content subsequently increased after several days of storage at the time of progressing microbial growth (see 3.3.).

3.2. pH and color

The evolution of pH and color variables L*, a* and b* is presented in Table 3. Throughout storage time, pH was 6.33 ± 0.12, 6.57 ± 0.16, 6.53 ± 0.17, 6.68 ± 0.15 and 6.70 ± 0.05 under the conditions H4, H8, L4, L8 and Air, respectively. In addition to some increase in yellowness (b*) as a function of time under MAP conditions, differences in color values were mostly not detected over time or between different storage conditions.

3.3. Microbiological analysis

Results of the microbiological analysis are presented in Fig. 1. Generally, more rapid growth was observed on all tested media under air when compared to the MAP conditions at the respective storage temperature (4 °C). Initially (day 0), high TPC (Fig. 1A) were typically enumerated on MA. The limit of 7.0 log CFU/g was exceeded after 2 days under air storage at 4 ºC, whereas at both MAP conditions this limit was reached within 2 days at 8 ºC and 4 days at 4 ºC. Under low O₂ concentrations (L4 and L8), stationary phase was reached after 4 days at 8 ºC and 8 days at 4 ºC, which closely coincides with the total depletion of oxygen from the package headspace (Table 3). TPC of cod packaged under MAP was typically 0.5-1 log higher on MA than on IAL (Fig. 1A-B).
LAB enumerated on MRS (Fig. 1C) were able to grow especially well at 8 °C. Oxygen concentration had little effect on LAB growth under MAs until complete depletion from the headspace. Respective enumerations were obtained on modified MRS (results not shown). On the other hand, growth of H$_2$S producers (Fig. 1D) was promoted by low oxygen concentrations. Their growth was highly similar to LAB under low oxygen concentrations (L4 and L8), whereas stationary growth was observed after 4 days under H8 and six days under H4. Under Air, H$_2$S producers reached higher levels than LAB.

*Pseudomonas* spp. growth (Fig. 1E) was favored by storage under air and effectively inhibited by elevated carbon dioxide concentrations (60 %). The initial level of *B. thermosphacta* (Fig 1F) was between 2.5 - 4 log CFU/g and increased by at least 2.5 log CFU/g during storage under all tested conditions.

### 3.4. Quantification of VOCs

The VOC concentrations determined by SIFT-MS exceeding the LOQ and having relative standard deviation below 25 % (Supplementary table 1) are presented in Figures 2-4 as a function of TPC enumerated on MA. In addition to these compounds, acetone exceeded the LOQ under L8 (104 µg/m$^3$ on day 7) and ammonia under L4 (9.0 µg/m$^3$ on day 13). When the LOQ was not exceeded, concentration was marked as 0 in Figs 2-4. The differences between the blank averages and the LOQ (Supplementary table 1) could be attributed to the deviation between blanks. In most cases, concentration of a certain VOC was constant or slightly increasing throughout storage in the blanks. However, ethanol concentration increased in the MAP blanks by a factor of 1000 or more by the end of storage.

### 3.4.1. Alcohols
Levels of alcohols in the package headspace are presented in Fig. 2. Ethanol, 3-methyl-1-butanol, isobutyl alcohol and 2,3-butanediol eventually exceeded the LOQ under most of the tested conditions. Ethanol yielded higher concentrations than the other studied compounds. However, a high initial ethanol concentration and increasing trend as a function of storage time were typically also detected in the blanks, leading to high LOQs that were only exceeded under high O₂ conditions. Concentrations of 3-methyl-1-butanol and 2,3-butanediol started to increase as 7.0 log CFU g⁻¹ TPC was exceeded and reached up to 500 µg m⁻³. Evolution of these compounds was similar under all tested MAP conditions, whereas lower quantities were produced under air. On the other hand, isobutyl alcohol was produced in low quantities and primarily under L4.

3.4.2. Ketones, esters and acids

During refrigerated storage, two ketones (2-pentanone and acetoin), two esters (ethyl acetate and ethyl propanoate) and one acid (acetic acid) were analyzed. Due to high relative standard deviations and/or LOQs, only ethyl acetate, acetic acid and 2-pentanone were quantified (Fig. 3). Increase of ethyl acetate concentration followed a similar trend under every tested condition, whereas other compounds did not exceed the LOQ under all conditions and remained below 200 µg m⁻³ throughout storage. Under air storage, only ethyl acetate exceeded the LOQ, whereas acetic acid was primarily quantified under low O₂ concentrations.

3.4.3. Amine compounds

Of all tested amine compounds, only trimethylamine (TMA) concentrations increased above the LOQ during storage (Fig. 4). At a certain level of microbial growth, higher concentrations of TMA were produced under low O₂ concentrations than under high O₂ or air. Under low O₂
concentrations at 4 °C (L4), some high concentrations were quantified at relatively low microbial levels. This happened during the late days of storage when TPC was decreasing.

3.4.4. Sulfur compounds

The time evolution of the sulfur compounds is presented in Fig. 4. Dimethyl disulfide and hydrogen sulfide had a relative standard deviation over 25 %. Relative standard deviation of methyl mercaptan was below 25 % only under condition L8. Dimethyl sulfide (DMS) was typically quantified at low microbial levels (TPC < 7 log CFU g⁻¹) and was the only sulfuric compound to exceed LOQ under air. Concentrations of DMS did often remain relatively stable throughout storage. Under air or low O₂ MAP, higher concentrations were detected than under high O₂ MAP at a respective level of microbial growth.

3.6. Sensory evaluation

Figures 2-4 present the individual VOC concentrations as a function of sensory rejection (%). A sample was considered rejected if labelled as marginal or spoiled. The onset of VOC concentration increase typically coincided with approximately 25 % rejection, irrespective of the identity of the VOC. At ≥ 50 % rejection, TPC enumerated on MA was generally over 7.5 log.

Friedman and LSD tests were used for analyzing significant differences among the ranking data. The Friedman test indicated no significant differences (α = 0.05) between samples from different days of storage under Air or in the second test of L4, which is why subsequent LSD tests were not carried out. On the basis of LSD tests (Fig. 5), significant differences (α = 0.05) between samples indicated perceivable change in product quality. Under conditions H4, H8, L4 and L8, a change in olfactory quality was observed approximately between days 6-8, 3-5, 4-8 and 3-5, respectively. These changes closely coincide with 50 % rejection (Fig. 2-4).
3.7. 16S rRNA gene sequencing

Rarefaction curves of samples from intermediate to late days of storage commonly showed trends to level off (Supplementary fig. 1), indicating appropriate sampling depth for most of these samples. Even though relatively high species diversity was estimated in samples from early stages of storage (Supplementary table 2), the low read counts were likely insufficient for appropriate sampling of diversity. Alpha diversity analysis indicated that ACE-1 (Chao & Lee, 1992) was the only stable richness estimator for the studied dataset (Supplementary table 3). Under modified atmospheres, the ACE-1 index suggested that community richness increased during the early days of storage and decreased during the late days, respectively. However, within 95 % confidence intervals, this was only observed under condition H8. On the other hand, diversity indices showed that community diversity was highest in the beginning of storage (day 0) under all tested conditions (Supplementary table 4). Diversity was lowest during intermediate storage under MA conditions and in the end of storage under air.

After data processing, 503 OTUs were retained at the 97 % sequence identity threshold and a high variation in the number of reads was observed between samples (Supplementary table 2). The relative distribution of the eight most abundant genera is presented in Fig. 6. Initial microbiota (day 0) were generally diverse under all tested conditions. Even though Acinetobacter, Flavobacterium, Photobacterium, Pseudomonas and Psychrobacter were the most abundant genera on day 0, their proportion of the total microbiota was relatively small. However, under condition L8, Psychrobacter and Flavobacterium were dominating and a relatively high proportion of Photobacterium was detected under H8.

The Photobacterium genus became dominant in relative abundance over storage time under all tested conditions. Initially, Photobacterium formed ca. 30 % of the total microbiota under H8.
and less than 15% of under H4, L4, L8 and Air. On the later days of storage, over 88% was detected under both MAP conditions at 4 °C. Under MA conditions, the relative abundance of \textit{Photobacterium} was highest during intermediate storage and decreased to some extent by the end of storage, thus increasing community diversity (Supplementary table 4). At higher storage temperature (8 °C), \textit{Photobacterium} decreased from 78 to 60% under H8 and 65 to 46% under L8: under these conditions, \textit{Acinetobacter}, \textit{Brochothrix} and \textit{Carnobacterium} were also able to grow. However, a lower number of reads was also obtained from day 7 samples when compared to day 4 samples under these conditions. Under air, 70 and 86% of \textit{Photobacterium} was detected on days 2 and 3 of storage.

4. Discussion

Growth of SSOs is dependent on the packaging and storage conditions. \textit{Photobacterium phosphoreum} has been identified as an SSO of marine fish under elevated CO\textsubscript{2} concentrations (Dalgaard et al., 1997; Dalgaard, Mejlholm, & Huss, 1997; Gram & Dalgaard, 2002; Leroi, 2010). In the present study, the \textit{Photobacterium} genus became indeed dominating under all tested MAP conditions. Since \textit{P. phosphoreum} and \textit{P. iliopiscicarium} are able to grow on MA (Broekaert, Heyndrickx, Herman, Devlieghere, & Vlaemynck, 2011), the results suggest that TPC enumerated on MA reflects the growth of this genus. Furthermore, it was observed that ca. 7 log CFU/g is needed for the onset of exponential VOC increase and ca. 7.5 log CFU/g for 50% rejection. The results are in line with Dalgaard et al. (1997) for cod fillets stored under 60/40/0 and 60/0/40 (CO\textsubscript{2}/O\textsubscript{2}/N\textsubscript{2}) at 0 °C. The results thus suggest that representatives of the \textit{Photobacterium} genus contribute to the increase in VOC concentrations and sensory rejection and that the onset of exponential VOC increase can be observed at relatively high microbial levels.
When stored under air, *Pseudomonas* and *Shewanella* spp. have commonly been considered as SSOs of refrigerated or iced marine fish (Gram & Huss, 1996; Gram & Dalgaard, 2002). Parlapani et al. (2015) observed *Pseudomonas* and H₂S producers to be dominating in sea bass stored both under air and MAP (60/10/30 % CO₂/O₂/N₂); under MAP, LAB and *B. thermosphacta* were observed to be co-dominating. Respectively, both enumeration (Fig. 1) and sequencing (Fig. 6) results of the present study indicate *Pseudomonas* growth under air and inhibition under MAP. Under MA conditions, high carbon dioxide concentrations are known to inhibit pseudomonads (Gram & Huss, 1996).

Enumeration of *Photobacterium* can be affected by the properties of the growth media. Broekaert et al. (2011) identified MA to be more suitable for the enumeration of marine bacteria than IAL. In the present study, the difference typically observed between TPC enumerated on MA and IAL likely reflects the dominance of the *Photobacterium* genus. In the beginning of storage (day 0), highly similar results are obtained on both media, whereas higher counts are generally enumerated on MA on later days of storage (Fig. 1). According to the oligotyping results (Fig. 6), respectively, the *Photobacterium* genus typically forms a small fraction of the initial microbiota and majority at later stages of storage. Since *P. phosphoreum* is sensitive to heat, pour plating temperatures (< 50 °C) have been suggested to lead into underestimation of its growth (Dalgaard et al., 1997). Incubation temperature of 23-25 °C or higher has also been suggested to inhibit *P. phosphoreum* growth (Dalgaard et al., 1997); however, similar enumeration results were obtained in the present study on MA incubated at 22 or 15 °C (results not shown).

Elevated CO₂ concentrations have been reported to favor the growth of CO₂ tolerant LAB (Gram & Dalgaard, 2002; Leroi, 2010). Analogously, the present enumeration results on MRS suggests
that facultative anaerobic LAB were able to grow under both MAP conditions. Even though acetate-containing MRS has been reported to inhibit certain LAB such as carnobacteria (Leroi, 2010), comparative enumeration on MRS and mMRS resulted in highly similar CFU levels (results not shown). According to the sequencing results (Fig. 6), the relative abundance of carnobacteria was higher under MA conditions when compared to storage under air. High CO₂ concentration also had an inhibitive effect on H₂S producers under MAP when compared to air storage, especially under high O₂ conditions. An additional inhibitive effect of O₂ was also observed by López-Caballero et al. (2001), which was suggested to be due to synergistic effect between the gases.

An increase in concentrations of several alcohols was detected in the present study. Respectively, ethanol, 3-methyl-1-butanol and 2,3-butanediol have frequently been identified as potential spoilage indicators of marine fish under air and/or MAP in several studies (Duflos, Coin, Cornu, Antinelli, & Malle, 2006; Mikš-Krajnik et al., 2016; Olafsdottir et al., 2005; Parlapani et al., 2014; Parlapani et al., 2015; Parlapani et al., 2015). Olafsdottir et al. (2005) observed increasing concentrations of ethanol, 2-methyl-1-propanol, 3-methyl-1-butanol and 2,3-butanediol for aerobically stored cod fillets at 0.5 °C. Ethanol and 2-methyl-1-propanol were suggested to have importance in early detection of spoilage despite non-continuous increase. Duflos et al. (2006) found several alcohols including ethanol, 3-methyl-1-butanol and 2,3-butanediol to increase in cod, mackerel and whiting stored under vacuum at 4 °C for ten days. Production of different alcohols has been associated with several microbial species among LAB, Shewanella, Pseudomonas, P. phosphoreum and B. thermosphacta (Casaburi, Piombino, Nychas, Villani, & Ercolini, 2015; Hernández-Macedo et al., 2012; Noseda et al., 2012). In the present study, the
dominance of Photobacterium suggests that the production of alcohols could be largely attributed to this genus.

The H$_2$S concentrations remained low under all tested conditions. Low H$_2$S production by *P. phosphoreum* has been observed in other studies (Dalgaard, Gram, & Huss, 1993). In Danish marine fish, *Shewanella baltica* has been identified as the main H$_2$S producer (Vogel et al., 2005). Even though *S. putrefaciens* has high spoilage potential due to the production of intensive off-odors, high levels (8 log CFU/g) are needed for off-odor production (Dalgaard, 1995). The present results thus support the conclusion that significant VOC production can only be observed at relatively high microbial levels.

TMA is produced by bacteria that utilize trimethylamine oxide (TMAO) for anaerobic respiration and results in ammonia-like or “fishy” odors characteristic for spoiled marine fish (Gram & Dalgaard, 2002). Oxygen has been observed to inhibit the reduction of TMAO into TMA as well as to reduce the growth of TMA-producing *P. phosphoreum* (Boskou & Debevere, 1997; Dalgaard et al., 1997). This is in line with the results of the present study. Since TMA concentration was notably higher at 50% rejection than its human olfactory threshold (OT) 6 µg m$^3$ (Devos, Patte, Rouault, Laffort, & Van Gemert, 1990), TMA was likely to contribute to the rejection of the samples.

Even though VOCs are often produced in low quantities, their effect on the perceived quality of the fish can be significant if they have low OTs. Alcohols have generally high OTs, whereas sulfur and amine compounds often become detectable at very low quantities (Devos et al., 1990). However, OTs are commonly determined for single compounds from a continuous airflow. Furthermore, OT values indicate the lowest quantity of a VOC that can be perceived by the
panelists, instead of indicating whether it is considered acceptable. Acceptance of an odor may
depend on cultural, social and economic aspects, as well as the characteristics of the food
product. Since olfactory evaluation of fish freshness is based on the overall smell, OTs and
acceptability of VOCs are likely dependent on the composition of the whole VOC profile.
Instead of using single compounds for quality and spoilage evaluation of fish, multiple-
compound quality indices have shown promising potential (Jørgensen et al., 2001).

In the present study, the concentration of several VOCs increased as a function of microbial
growth. Under most of the tested conditions, increase in 2,3-butanediol, ethanol, ethyl acetate, 3-
methyl-1-butanol and trimethylamine were observed. All these compounds have been recognized
as fish spoilage metabolites (Duflos et al., 2006; Olafsdóttir et al., 1997). For example, ethanol,
ethyl acetate and/or 3-methyl-1-butanol have also been associated with the spoilage of several
non-seafood products packaged under modified atmospheres (Casaburi et al., 2015; Nieminen,
Dalgaard, & Björkroth, 2016; Zhang et al., 2013; Zhang, Samapundo, Pothakos, Sürengil et al.,
2013): monitoring of such compounds could enhance the applicability of an intelligent packaging
solution into a wider range of food products. Since the Photobacterium genus was highly
abundant under all storage conditions, differences in its metabolism could contribute to the
observed differences in the VOC profiles between the tested storage conditions. Onset of
exponential concentration increase was typically observed between TPC 7 - 7.5 log CFU/g and
25 - 50% rejection. Respectively, late increase of VOC concentrations in relation to microbial
growth has also been detected in other studies (Olafsdottir et al., 2005). Detection of early
spoilage thus requires that low concentrations of relevant VOCs can be detected.

5. Conclusions
Different packaging and storage conditions affect the evolution of fish microbiota and the generated VOCs in the package headspace. In the present study, the SIFT-MS technology allowed the real-time quantification of VOCs directly from the package headspace. This approach eliminated the need of sample preparation procedures, while allowing fast and sensitive analysis of the VOC profile over storage time. The obtained results directly represent the quality deterioration of fish and thus the reality that a sensor needs to be able to respond to during storage.

Packaging and storage conditions affect the evolution of the VOC profile and should be considered in the selection of spoilage indicators. In the present study, increase in 2,3-butanediol, ethanol, ethyl acetate, 3-methyl-1-butanol and trimethylamine concentrations during storage suggests that these compounds could be used in detecting spoilage of raw Atlantic cod. However, since VOC concentrations typically remain at low quantities even at the late stage of storage, detection of early spoilage calls for sensitivity for low concentration ranges.

Acknowledgments

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solid-phase Microextraction—Gas Chromatography—Mass spectrometry for analyzing fish

Hernández-Macedo, M. L., Contreras-Castillo, C. J., Tsai, S. M., Da Cruz, S. H., Sarantopoulos,


with micro-organisms in blown pack spoilage of brazilian vacuum-packed beef. *Letters in

Applied Microbiology, 55*(6), 467-475. doi:10.1111/lam.12004


Sensory characteristics of spoilage and volatile compounds associated with bacteria isolated

from cooked and peeled tropical shrimps using SPME–GC–MS analysis. *International


doi:http://dx.doi.org/10.1016/j.ijfoodmicro.2011.04.008


rapid detection as quality indicators of cold smoked salmon (salmo salar). *Food Chemistry,


produced by spoilage bacteria in vacuum-packed cold-smoked salmon (salmo salar)

analyzed by GC-MS and multivariate regression. *Journal of Agricultural and Food

Chemistry, 49*(5), 2376-2381. doi:10.1021/jf0009908


active and intelligent packaging systems for meat and muscle-based products: A review.

*Meat Science, 74*(1), 113-130. doi:http://dx.doi.org/10.1016/j.meatsci.2006.04.024


quantification of volatile bases produced on Atlantic cod (Gadus morhua). *Journal of Agricultural and Food Chemistry, 58*(9), 5213-5219. doi:10.1021/jf904129j


Figure captions

Fig. 1. Counts of total viable psychrotrophic bacteria (A-B), lactic acid bacteria (C), H$_2$S producers (D), pseudomonads (E) and *Brochothrix thermosphacta* (F) in Atlantic cod fillet portions stored under conditions H4 (60 % CO$_2$ /40 & O$_2$/0 % N$_2$ at 4 °C), H8 (60/40/0 8 °C), L4 (60/5/35 4 °C), L8 (60/5/35 8 °C) and air (4 °C).

Fig. 2. Concentrations (µg m$^{-3}$) of alcohols quantified by SIFT-MS as a function of total viable psychrotrophic counts (TPC) or sensory rejection % in Atlantic cod fillet portions stored under conditions H4 (60 % CO$_2$ /40 & O$_2$/0 % N$_2$ at 4 °C), H8 (60/40/0 8 °C), L4 (60/5/35 4 °C), L8 (60/5/35 8 °C) and air (4 °C).

Fig. 3. Concentrations (µg m$^{-3}$) of ketones, esters and acids quantified by SIFT-MS as a function of total viable psychrotrophic counts (TPC) or sensory rejection % in Atlantic cod fillet portions stored under conditions H4 (60 % CO$_2$ /40 & O$_2$/0 % N$_2$ at 4 °C), H8 (60/40/0 8 °C), L4 (60/5/35 4 °C), L8 (60/5/35 8 °C) and air (4 °C).

Fig. 4. Concentrations (µg m$^{-3}$) of amines and sulfur compounds quantified by SIFT-MS as a function of total viable psychrotrophic counts (TPC) or sensory rejection % in Atlantic cod fillet portions stored under conditions H4 (60 % CO$_2$ /40 & O$_2$/0 % N$_2$ at 4 °C), H8 (60/40/0 8 °C), L4 (60/5/35 4 °C), L8 (60/5/35 8 °C) and air (4 °C).

Fig. 5. Ranks (1=least fresh, 4=most fresh) assigned to cod fillet samples from four different days of storage under conditions H4 (60 % CO$_2$ /40 & O$_2$/0 % N$_2$ at 4 °C), H8 (60/40/0 8 °C), L4 (60/5/35 4 °C) and L8 (60/5/35 8 °C). Storage days with different postscripts (a-c) within a condition are significantly different (p<0.05).
Fig. 6. Composition of microbiota in Atlantic cod fillet portions stored under conditions H4 (60% CO$_2$/40 & O$_2$/0 % N$_2$ at 4 °C), H8 (60/40/0 8 °C), L4 (60/5/35 4 °C), L8 (60/5/35 8 °C) and air (4 °C), determined by amplicon (NGS) sequencing of the 16S rRNA gene.
Fig. 1.
Fig. 2.
Fig. 3.
Fig. 4.
Fig. 6.
Table 1. Packaging and storage conditions used in the study. Samples from days denoted with (*) were studied by amplicon sequencing.

<table>
<thead>
<tr>
<th></th>
<th>H4</th>
<th>H8</th>
<th>L4</th>
<th>L8</th>
<th>Air</th>
</tr>
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<td>60/40/0</td>
<td>60/5/35</td>
<td>60/5/35</td>
<td>air</td>
</tr>
<tr>
<td>(% CO₂/O₂/N₂)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature</td>
<td>4</td>
<td>8</td>
<td>4</td>
<td>8</td>
<td>4</td>
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<tr>
<td>(°C)</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Days of analysis</td>
<td>0°, 4°, 5°, 6°, 7°, 8°, 11°, 13°</td>
<td>0°, 3°, 4°, 5°, 6°, 7°</td>
<td>0°, 4°, 5°, 6°, 7°, 11°, 13°</td>
<td>0°, 3°, 4°, 5°, 6°</td>
<td>0°, 1°, 2°, 3°</td>
</tr>
</tbody>
</table>
Table 2. Product ions of volatile organic compounds (VOCs) quantified with SIFT-MS, respective mass to charge ratios (m/z), branching ratios (b) and reaction rate coefficients (k).

<table>
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<tr>
<th>VOC</th>
<th>Precursor ion</th>
<th>m/z</th>
<th>b (%)</th>
<th>k</th>
<th>Product ion</th>
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<td><strong>Acids</strong></td>
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<tr>
<td>Acetic acid</td>
<td>H₂O⁺</td>
<td>61</td>
<td>100</td>
<td>2.6 E-09</td>
<td>CH₃COOH⁺</td>
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<td>NO⁺.CH₃COOH</td>
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<td>50</td>
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<td>CH₂COOH⁺</td>
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<td><strong>Alcohols</strong></td>
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<td>Ethanol</td>
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<td>100</td>
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<td>C₂H₅O⁺</td>
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<td>H₂O⁺</td>
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<td></td>
<td></td>
<td>C₂H₅O⁺.H₂O</td>
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<td>2,3-butanediol</td>
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<td>100</td>
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<td>C₅H₁₀O⁺⁺</td>
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<td>100</td>
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<td>C₃H₇O⁺⁺</td>
</tr>
<tr>
<td>isobutyl alcohol</td>
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<td>73</td>
<td>95</td>
<td>2.4 E-09</td>
<td>C₃H₇O⁺⁺</td>
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<tr>
<td>2-pentanone</td>
<td>NO⁺</td>
<td>85</td>
<td>100</td>
<td>2.4 E-09</td>
<td>C₅H₁₀O⁺⁺</td>
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<td><strong>Aldehydes</strong></td>
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<td>2-methylpropanal</td>
<td>O₂⁺</td>
<td>72</td>
<td>70</td>
<td>3.0 E-09</td>
<td>C₂H₅O⁺⁺</td>
</tr>
<tr>
<td>3-methylbutanal</td>
<td>NO⁺</td>
<td>85</td>
<td>100</td>
<td>2.4 E-09</td>
<td>C₅H₁₀O⁺⁺</td>
</tr>
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<td>C₂H₅O⁺⁺</td>
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<tr>
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<td>116</td>
<td>100</td>
<td>3.1 E-09</td>
<td>NO⁺.C₅H₁₀O⁺⁺</td>
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<tr>
<td><strong>Sulfur compounds</strong></td>
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<td></td>
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<tr>
<td>Hydrogen sulfide</td>
<td>H₂O⁺</td>
<td>35</td>
<td>100</td>
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<td>H₂S⁺</td>
</tr>
<tr>
<td>O₂⁺</td>
<td>34</td>
<td>100</td>
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<td>H₂S⁺</td>
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</tr>
<tr>
<td>Methyl mercaptan</td>
<td>H₂O⁺</td>
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<td>100</td>
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<td>CH₃SH⁺⁺</td>
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<td>Dimethyl sulfide</td>
<td>H₂O⁺</td>
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<td>(CH₃)₂S.H⁺⁺</td>
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<td>62</td>
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<td>(CH₃)₂S⁺⁺</td>
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<tr>
<td>Dimethyl disulfide</td>
<td>H₂O⁺</td>
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<td>100</td>
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<td>(CH₃)₂S₂⁺⁺</td>
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<td>Dimethyl trisulfide</td>
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<td>C₅H₁₂S₃⁺⁺</td>
</tr>
<tr>
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<td>126</td>
<td>100</td>
<td>1.9 E-09</td>
<td>C₅H₁₂S₃⁺⁺</td>
<td></td>
</tr>
<tr>
<td><strong>Esters</strong></td>
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<td>Ethyl acetate</td>
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<td>90</td>
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<td>NO⁺.CH₃COOC₂H₅</td>
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<td>Ethyl propanoate</td>
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<td>95</td>
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<td>2.5 E-09</td>
<td>NO⁺.C₂H₅COOC₂H₅</td>
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<tr>
<td><strong>Amines</strong></td>
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<td>Ammonia</td>
<td>H₂O⁺</td>
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<td>100</td>
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<td>NH₄⁺</td>
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<td>100</td>
<td>2.4 E-09</td>
<td>NH₄⁺</td>
<td></td>
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<tr>
<td>Dimethylamine</td>
<td>H₂O⁺</td>
<td>46</td>
<td>100</td>
<td>2.1 E-09</td>
<td>(CH₃)₂N.H⁺⁺</td>
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<tr>
<td>Trimethylamine</td>
<td>H₂O⁺</td>
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<td>(CH₃)₃N.H⁺⁺</td>
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<td>59</td>
<td>100</td>
<td>1.6 E-09</td>
<td>(CH₃)₃N⁺⁺</td>
<td></td>
</tr>
<tr>
<td>Series</td>
<td>Time of storage (d)</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>--------</td>
<td>---------------------</td>
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<tr>
<td><strong>O2</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>H4</td>
<td>41.17 ± 0.15</td>
<td>51.9 ± 1.6</td>
<td>51.77 ± 2.12</td>
<td>49.8 ± 1.9</td>
<td>49.33 ± 1.9</td>
</tr>
<tr>
<td>H8</td>
<td>42.47 ± 0.81</td>
<td>49.63 ± 1.27</td>
<td>39.57 ± 18.8</td>
<td>47.57 ± 0.58</td>
<td>46.17 ± 1.53</td>
</tr>
<tr>
<td>L4</td>
<td>5.09 ± 0.12</td>
<td>4.26 ± 0.18</td>
<td>2.8! ± 0.44</td>
<td>2.41 ± 0.79</td>
<td>1.67 ± 0.52</td>
</tr>
<tr>
<td>L8</td>
<td>4.47 ± 0.51</td>
<td>4.36 ± 0.09</td>
<td>2.81 ± 0.87</td>
<td>0.73 ± 0.16</td>
<td>0.07 ± 0.11</td>
</tr>
<tr>
<td>Air</td>
<td>20.73 ± 0.06</td>
<td>20.3 ± 0.1</td>
<td>19.67 ± 0.06</td>
<td>16.53 ± 1.4</td>
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<td>H4</td>
<td>55.83 ± 0.25</td>
<td>44.07 ± 0.65</td>
<td>43.43 ± 1.97</td>
<td>43.47 ± 0.83</td>
<td>46.17 ± 1.57</td>
</tr>
<tr>
<td>H8</td>
<td>55.43 ± 2.12</td>
<td>47 ± 1.04</td>
<td>37.5 ± 13.6</td>
<td>49.33 ± 0.38</td>
<td>50.17 ± 1.86</td>
</tr>
<tr>
<td>L4</td>
<td>56.27 ± 1.02</td>
<td>42.87 ± 0.51</td>
<td>41.1 ± 2.17</td>
<td>44.17 ± 2.27</td>
<td>41.07 ± 0.55</td>
</tr>
<tr>
<td>L8</td>
<td>56.47 ± 0.51</td>
<td>45.13 ± 1</td>
<td>43.77 ± 2.4</td>
<td>47.87 ± 4.11</td>
<td>45.53 ± 1.61</td>
</tr>
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<td>1.37 ± 0.21</td>
<td>1.3 ± 0</td>
<td>4.77 ± 1.31</td>
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<tr>
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<td>6.34 ± 0.16</td>
<td>6.2 ± 0.11</td>
<td>6.36 ± 0.13</td>
<td>6.34 ± 0.15</td>
<td>6.33 ± 0.09</td>
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<tr>
<td>H8</td>
<td>6.42 ± 0.04</td>
<td>6.41 ± 0.13</td>
<td>6.66 ± 0.21</td>
<td>6.6 ± 0.01</td>
<td>6.54 ± 0.1</td>
</tr>
<tr>
<td>L4</td>
<td>6.68 ± 0.24</td>
<td>6.3 ± 0.1</td>
<td>6.4 ± 0.08</td>
<td>6.38 ± 0.04</td>
<td>6.62 ± 0.14</td>
</tr>
<tr>
<td>L8</td>
<td>6.88 ± 0.07</td>
<td>6.56 ± 0.04</td>
<td>6.71 ± 0.02</td>
<td>6.48 ± 0.17</td>
<td>6.71 ± 0.1</td>
</tr>
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<td>6.68 ± 0.08</td>
<td>6.7 ± 0.05</td>
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<td>60.3 ± 1.59</td>
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<td>58.37 ± 1.15</td>
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<td>61.1 ± 1.81</td>
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<td>59.97 ± 1.7</td>
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<td>-3.07 ± 0.44</td>
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<td>-3.35 ± 0.23</td>
</tr>
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<tr>
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<td>-0.14 ± 1.86</td>
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<td>-0.32 ± 1.8</td>
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