Multivariate statistical analysis for the identification of potential seafood spoilage indicators

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Abstract

Volatile organic compounds (VOCs) characterize the spoilage of seafood packaged under modified atmospheres (MAs) and could thus be used for quality monitoring. However, the VOC profile typically contains numerous multicollinear compounds and depends on the product and storage conditions. Identification of potential spoilage indicators thus calls for multivariate statistics. The aim of the present study was to define suitable statistical methods for this purpose (exploratory analysis) and to consequently characterize the spoilage of brown shrimp (*Crangon crangon*) and Atlantic cod (*Gadus morhua*) stored under different conditions (selective analysis). Hierarchical cluster analysis (HCA), principal components analysis (PCA) and partial least squares regression analysis (PLS) were applied as exploratory techniques (brown shrimp, 4 °C, 50%CO_2/50%N_2) and PLS was further selected for spoilage marker identification. Evolution of acetic acid, 2,3-butanediol, isobutyl alcohol, 3-methyl-1-butanol, dimethyl sulfide, ethyl acetate and trimethylamine was frequently in correspondence with changes in the microbiological quality or sensory rejection. Analysis of these VOCs could thus enhance the detection of seafood spoilage and the development of intelligent packaging technologies.

Keywords

Hierarchical cluster analysis; intelligent packaging; principal components analysis; partial least squares regression analysis; selected-ion flow-tube mass spectrometry

1. Introduction

Modified atmosphere packaging (MAP) is commonly used for perishable food products such as seafood in order to inhibit or delay microbial growth and thus to extend the shelf life and quality of the packaged product. During microbiological spoilage of foodstuffs, decomposition of available nutrients by microbial activity can lead to the generation of volatile organic compounds (VOCs) associated with both primary and secondary metabolism (Wang, Li, Yang, Ruan, & Sun, 2016). Growth of specific spoilage organisms (SSOs) and subsequent production of off-odors into the package headspace eventually causes consumer rejection (Gram & Dalgaard, 2002). Consequently, odor is considered as one of the most important seafood quality parameters (Olafsdottir, Jonsdottir, Lauzon, Luten, & Kristbergsson, 2005; Olafsdóttir et al., 1997).

Microbial spoilage of fish may manifest itself as sweet, fruity, ammonia-like, putrid and sulfuric off-odors. VOCs contributing to the odor of fish can be divided into three groups, specifying compounds associated with freshness (C_6-C_9 alcohols and carbonyl compounds), lipid oxidation (aldehydes) and microbiological spoilage (Olafsdóttir et al., 1997). According to Olafsdóttir et al. (1997), microbiological spoilage odor is generally due to compounds such as ammonia, ethanol, ethyl acetate, hydrogen sulfide, 3-methyl-1-butanol, methyl mercaptan and trimethylamine. However, the composition and the development of the VOC profile are affected by several factors, including food product, headspace gas composition, temperature, initial contaminating microbiota and microbial metabolism (Wang et al., 2016).

Brown shrimp (*Crangon crangon*) is highly susceptible to microbiological spoilage. Shrimp contains high amounts of free amino acids and other readily available nutrients for microbial growth (Zeng, Thorarinsdottir, & Olafsdottir, 2005). Unlike other crustaceans, shrimp cannot be

kept alive for extended periods before processing (Adams & Moss, 2008). Currently, the shelf life of preservative-free cooked brown shrimp is maximally 4-6 days under refrigerated conditions (Broekaert, Heyndrickx, Herman, Devlieghere, & Vlaemynck, 2013).

Since microbial activity is the main cause of fish spoilage (Gram & Dalgaard, 2002), identification and quantification of VOCs produced during microbial metabolism under different packaging and storage conditions could enhance efficient quality analysis of the packaged product. Evolution of these spoilage indicators in relation to microbial growth and sensory rejection could be used for the development of intelligent packaging applications. Generally, concentrations of VOCs that indicate spoilage can be expected to increase as a function of storage time and progressing microbial growth. However, VOCs are produced and degraded as a result of several biological and chemical processes. Furthermore, certain odors may be considered as a part of natural odor in one foodstuff and rejected in another product (Gram & Dalgaard, 2002). Thus, the complexity of concentration evolution and acceptancy as well as the wide number of potential spoilage indicators calls for multivariate statistical analysis.

Different statistical methods have been applied to multivariate microbiological and chemical data, including hierarchical cluster analysis (HCA), principal components analysis (PCA) and partial least squares regression analysis (PLS). Previously, PCA has been applied to the comparison of different food products (Blixt & Borch, 2002), microbiota (Hierro et al., 2005; Verginer, Leitner, & Berg, 2010), treatments (Ciesa et al., 2013) or times of storage (Duflos et al., 2010; Fik, Surówka, Maciejaszek, Macura, & Michalczyk, 2012). PLS has been used for the analysis of progressing microbial growth on the basis of VOC concentrations (Jørgensen, Huss, & Dalgaard, 2001; Marín et al., 2007; Storer, Hibbard-Melles, Davis, & Scotter, 2011) and also applied along with HCA or PCA (Argyri, Doulgeraki, Blana, Panagou, & Nychas, 2011; Argyri,

Mallouchos, Panagou, & Nychas, 2015; Blixt & Borch, 2002; Mataragas, Skandamis, Nychas, & Drosinos, 2007; Mikš-Krajnik, Yoon, Ukuku, & Yuk, 2016; Siroli et al., 2014; Vervoort et al., 2012; Wibowo, Grauwet, Gedefa, Hendrickx, & Van Loey, 2015).

The aims of the present study were to 1) determine suitable multivariate statistical methods for characterizing the VOC profile of seafood (exploratory analysis) and 2) consequently identify the most potential spoilage indicators of Atlantic cod (*Gadus morhua*) and brown shrimp stored under different modified atmosphere (MA) conditions (selective analysis). Firstly, HCA, PCA and PLS were applied as exploratory techniques to microbiological, chemical and/or sensory data. Comparison of the three techniques was carried out using a dataset collected during refrigerated storage of seafood (brown shrimp, 4 °C, 50%CO_2/50%N_2) where selected-ion flow-tube mass spectrometry (SIFT-MS) was used for the quantification of VOCs from the package headspace. On the basis of the exploratory analysis, PLS was chosen to be used in selective analysis. Independent PLS analyses were carried out for data collected during spoilage of Atlantic cod (Kuuliala et al. submitted manuscript) and brown shrimp under different packaging and storage conditions.

2. Materials and methods

2.1. Data collection

The datasets used in the study were collected during individual storage experiments of brown shrimp (2x) or Atlantic cod (5x) and used for exploratory (brown shrimp, 4 °C, 50%CO_2/50%N_2) or selective (all storage experiments) statistical analyses.

2.1.1 Brown shrimp

The two individual storage experiments of brown shrimp consisted of sample preparation and packaging, real-time quantification of VOCs with SIFT-MS, microbiological analysis and sensory evaluation.

2.1.1.1 Raw material

Brown shrimp were caught in the North Atlantic Ocean (FAO zone 27) in October and November 2015. The shrimp were sorted according to size and washed before cooking according to normal Belgian fishing practices. No additives or preservatives such as benzoic or sorbic acid were added during processing. After cooking, the shrimp were cooled and stored overnight in plastic bags under ice. The shrimp were brought onshore the following morning and directly transported to the Laboratory of Food Microbiology and Food Preservation (LFMFP, UGent) where the batch was hand peeled. During peeling, shrimp were kept on ice in plastic bags while avoiding direct contact between shrimp and ice. Shrimp portions of 150 ± 2 g were packaged at 2:1 headspace-product ratio with a tray sealer (MECA 900, DecaTechnic, Herentals, Belgium) using multilayer packaging trays (PP/EVOH/PP, oxygen transmission rate 0.03 cm^3/tray*24h at 23 °C and 50 % R.H.) and top film (PA/EVOH/PA/PP, oxygen transmission rate 6.57 cm^3/m^2*24h*atm at 23 °C, 50 % R.H. and 1 atm). Two individual batches of shrimp were independently packaged under modified atmospheres ($CO_2/O_2/N_2$ %) 50/0/50 or 30/0/70 and stored at (4.0 ± 0.7) °C prior to analyses. Analyses were carried out on days 0 (day of packaging), 3, 5, 7, 10 and 12 for three randomly chosen packages (A-C). New replicates A-C were analyzed on each day of storage due to the destructive nature of the microbiological analyses. After sampling, the remaining shrimp was packaged under vacuum using high barrier film bags (oxygen transmission rate < 2.7 cm^3/m^2*24h*bar at 23 °C and 0 % R.H.) and stored at -32 °C for no longer than 70 days.

2.1.1.2 Quantification of spoilage related VOCs by SIFT-MS

The principles of selected-ion flow-tube mass spectrometry have been described in previous studies (Noseda et al., 2010). VOCs (Table 1) were selected on the basis of previous research and literature and quantified from the package headspace by a spectrometer (Voice 200, Syft Technologies^{TM}, Christchurch, New Zealand). Package headspace was sampled through a septum inserted on the package lid with a flow rate of 25.6 ml/min for 60 seconds (preparation 10s, sample 50s) and the concentrations were averaged over eleven data points. A certain package was sampled twice. During sampling, the headspace was connected to atmospheric air with a needle inlet in order to avoid collapse and changes in the internal conditions of the package.

The relative standard deviation ($SD_{\%}$) of each VOC concentration during an individual SIFT-MS scan was calculated as follows:

$$SD_{\%} = SD_m/x_m*100\ \% \quad (1)$$

where x_m is the average and SD_m the standard deviation of a single SIFT-MS scan (n=11). VOCs with concentrations exceeding 25 % average $SD_{\%}$ during the entire storage time within a certain packaging condition were considered not to allow sufficiently accurate quantification and were thus excluded from further analyses.

2.1.1.3 Microbiological analysis

Each shrimp sample of 30 ± 0.1 g was aseptically weighed into a sterile stomacher bag, diluted ten times in physiological saline peptone solution (PPS; 0.85 % NaCl, 0.1 % peptone) and homogenized in Stomacher Lab Blender (LED Techno, Heusden-Zolder, Belgium) for one minute. Appropriate decimal dilutions were prepared in PPS. Total psychrotrophic count (TPC)

was determined on Marine Agar (MA; Difco Le Pont de Claix, France) spread plates after incubation at 22 °C for five days.

2.1.1.4 Sensory evaluation

Sensory evaluation was performed in individual booths under red light (UGent Sensolab). A panel having experience in sensory evaluation of fish was formed from the laboratory staff at LFMFP. For both independent shrimp batches, two testing sessions with eight to ten panelists were organized on consecutive days. During both sessions, four shrimp samples from different days of storage were evaluated. One out of three daily replicates (A-C) was randomly selected and used per testing session. Prior to evaluation, the frozen (-32 °C) samples were cut to 5.0 ± 0.1 g portions and stored overnight at 2 °C. The samples were presented to the assessors 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, along with a fresh reference (day 0) from the same batch. A five-point scale (very good, good, satisfactory, marginal, spoiled) was used in the olfactory evaluation. Marginal or spoiled was considered as rejection.

2.1.2 Atlantic cod

Atlantic cod data collected during storage under modified atmospheres (% $CO_2/O_2/N_2$) 60/40/0 and 60/5/35 at (4.0 ± 0.7) or (8.0 ± 0.4) °C and air at (4.0 ± 0.7) (Kuuliala et al. submitted manuscript) was used in the study. The VOC data was processed correspondingly to brown shrimp (see 2.1.1.3). VOCs with concentrations exceeding 25 % average relative standard deviation during the entire storage time within a certain packaging condition were excluded from further analyses.

2.2 Exploratory analysis

Exploratory analysis techniques were applied to data collected during the storage of brown shrimp under modified atmosphere 50/0/50 (% $CO_2/O_2/N_2$) at 4 °C.

2.2.1 Hierarchical cluster analysis (HCA)

Agglomerative HCA was used for the analysis of the VOC data. The method is based on the identification of groups among objects (samples or variables) on the basis of similarity in their properties. Samples are clustered on the basis of the similarity in their variable profiles and variables on the basis of similarity between their patterns. In agglomerative clustering, each object initially represents an individual cluster. The most similar clusters are progressively joined together to larger clusters until one collective cluster is formed (Rendall et al., 2015). N objects are thus processed by N – 1 clustering steps (Almeida, Barbosa, Pais, & Formosinho, 2007). The process depends on how the similarity of objects is assessed (distance) and how new clusters are formed from subclusters (linkage). Euclidean or Manhattan distance measures are commonly used for continuous variables, whereas common linkage methods include single, complete, average, centroid and Ward (Smoliński, Walczak, & Einax, 2002).

HCA was carried out using Euclidean distance and average linkage. Individual replicate packages A-C of each day were treated as samples and individual VOCs as variables. Both measured concentrations (non-transformed values) as well as logarithmic and/or standardized (z-scores) concentrations (transformed values) of the VOCs were used in the analyses. R 3.3.1 (R Core Team, 2016) was used for producing heat maps (clustering of variables) with function pheatmap() from package **pheatmap** (Kolde, 2015) and dendrograms (clustering of samples) with function pvclust() from package **pvclust** (R. Suzuki & Shimodaira, 2015). Approximately unbiased (AU) p-values included in the dendrograms indicate how the clustering is supported by

192 the data: the greater the p-value, the greater the reliability of the clustering (Shimodaira, 2002; R.
193 Suzuki & Shimodaira, 2006).

194 ***2.2.2 Principal components analysis (PCA)***

195 PCA was used for the characterization of VOCs and their evolution during storage. The method
196 can be used for extracting the most important information from a dataset containing several
197 intercorrelated variables by determining a series of new variables (Abdi & Williams, 2010).
198 These principal components (PCs) are linear combinations of the original variables and
199 uncorrelated with each other. The first PC retains most of the total variance of the data and
200 following PCs retain most of each residual variance, respectively (Chen, Li, Ouyang, & Zhao,
201 2014). PCA can thus be used for simplifying the description of the dataset and for the
202 determination of underlying variables, similarity among samples and correlation among variables
203 (Abdi & Williams, 2010; Mataragas et al., 2007).

204 Logarithmic and standardized VOCs were used in the analysis. R 3.3.1 was used for producing
205 biplots describing both samples (replicate packages) and variables (VOCs) with function
206 prcomp() from package **stats** (R Core Team, 2013). Suitability for data reduction was analyzed
207 with Bartlett's sphericity test using function bart_spher() and sampling adequacy with Kaiser-
208 Meyer-Olkin (KMO) test (Kaiser, 1970) with function KMOS() from package **REdaS**
209 (Hatzinger, Hornik, Nagel, & Maier, 2014; Maier, 2015). KMO test result gives the level of
210 sampling adequacy as marvelous (> 0.90), meritorious (> 0.80), middling (> 0.70), mediocre (>
211 0.60), miserable (> 0.50) or unacceptable (< 0.50) (Kaiser, 1974).

212 ***2.2.3 Partial least squares regression (PLS)***

213 Partial least squares regression analysis (PLS) can be used for modeling one or more response
214 variables (Y) with several predictor variables (X) that can be noisy and highly collinear. On the
215 basis of the original X-variables, new orthogonal variables are defined as linear combinations
216 where the coefficients of the original X-variables are referred to as weights. The new variables
217 are used for modeling the X variables and predicting the Y variables. The part of the data that is
218 not explained by the model is referred to as residuals: high Y residuals indicate insufficient
219 model performance (Wold, Sjöström, & Eriksson, 2001). The influence of an X-variable on the
220 Y-response can be expressed with a Variable Importance in Projection (VIP) coefficient which
221 gives the weighed sum of squares of the PLS weights. The VIP coefficients indicate which X-
222 variables have highest importance in explaining the Y-variance (Farrés, Platikanov, Tsakovski,
223 & Tauler, 2015). Even though high regression coefficients can also be used for determining
224 predictor variables that have high importance on the response, the VIP coefficients summarize
225 the importance of the variable for both Y and X matrices (Wold et al., 2001).

226 PLS was used for the analysis of predictor and response variables with JMP v. 12 using the
227 NIPALS algorithm and leave-one-out cross validation. Logarithmic and standardized VOCs
228 were used as predictor variables and time, TPC or sensory rejection % as the response variable.
229 Logarithmic transformation of predictor variables was used in order to achieve linear relationship
230 with all response variables. The number of factors was chosen so that the root mean predicted
231 residual sum of squares (PRESS) was at its minimum. VIP values and regression coefficients
232 were determined for all VOCs.

233 ***2.3 Selective statistics***

234 Selective statistical analyses were applied to data collected during storage of Atlantic cod and
235 brown shrimp under all tested conditions. On the basis of the exploratory analysis, PLS was

chosen for the determination of most potential spoilage indicators. Logarithmic and standardized VOCs were used as predictor variables and TPC or rejection % as the response variable. When using TPC as the response variable, independent packaging conditions were separately analyzed and samples were excluded from the analysis if stationary or declining TPC had been reached. When using rejection % as the response variable, data from all independent packaging conditions per seafood product was used and VOCs were excluded from the analysis if over 25 % relative standard deviation was observed under any of the tested conditions. Following selection criteria were used for the spoilage indicators: 1) positive correlation with the dependent variable, 2) VIP > 1, and 3) positive regression coefficient. JMP v. 12 was used for all analyses.

3. Results and discussion

The majority of the VOCs had an average relative standard deviation below 25 % and were included in the analyses. Six VOCs were excluded from the analyses of brown shrimp under 50 % CO_2: 3-methyl-1-butanol, acetoin, 2-pentanone, dimethyl amine, dimethyl disulfide and hydrogen sulfide. Under 30 % CO_2, additional excluded VOCs were acetic acid, 2,3-butanediol and isobutyl alcohol. The excluded VOCs correspond well to the compounds that were excluded from the cod data (Table 2). Fluctuation of the concentrations of these VOCs during a SIFT-MS scan did not allow sufficiently accurate quantification and thus excluded them from most potential spoilage indicators.

3.1 Exploratory analysis

3.1.1 Hierarchical cluster analysis (HCA)

Clustering of VOCs produced during the storage of brown shrimp under (% CO_2/O_2/N_2) 50/0/50 at 4 °C is presented as heat maps (Fig. 1) and clustering of samples as dendrograms (Fig. 2).

Both Figures 1-2 indicate the similarity of the objects (variables or samples) within the studied dataset as a tree structure. In the heat maps, VOC concentrations are expressed on a color scale representing measured concentrations (Fig. 1A) or their transformed values (Fig. 1B-D). Generally, similarity of objects in the same cluster decreases as smaller clusters are merged into larger ones since objects that are clustered together sooner are more similar than those clustered at a higher distance (Rendall et al., 2015).

Clustering of VOCs was affected by the applied data transformations. When non-logarithmic and non-standardized data was used (Fig. 1A), VOCs were clustered on the basis of their concentration ranges. This highlighted the high differences observed in initial concentration levels as well as in the production of different VOCs during storage time. Ethanol was the only VOC that exceeded 10^4 ug m^{-3}, which is why it dominated the color scale and the analysis of VOC evolution was thus not possible. Even though logarithmic conversion of non-standardized data (Fig. 1C) allowed better separation of VOCs, the variables were still clustered on the basis of concentration ranges and resulted in subclusters containing VOCs from highest (ethanol, ethyl acetate, ethylene oxide, trimethylamine) to lowest (2,3-butanediol, isobutyl alcohol, ammonia) concentrations. An overall increase in several VOC concentrations and separation between early (0-3) and remaining (5-12) days of storage could be observed. Respective average logarithmic TPC were 6.51 ± 0.53 and 7.62 ± 0.24 CFU g^{-1}. On the other hand, clustering of non-logarithmic and standardized data (Fig. 1B) allowed the comparison of VOC evolution since VOC concentrations were presented on the same scale. Several subclusters of VOCs were formed and three main types of VOC patterns were identified on their basis. Firstly, concentration of ten out of fourteen VOCs generally increased as a function of storage time. Secondly, three VOCs (ethyl acetate, ethylene oxide, trimethylamine) reached highest concentrations on days 5-7 and

281 decreased thereafter. In addition, high initial concentrations of butanone led to the formation of a
282 separate cluster that did not show any clear pattern. Respectively, clustering of logarithmic and
283 standardized data (Fig. 1D) emphasized the evolution of VOCs during storage time.

284 Clustering of samples (Fig. 2) showed that replicate packages of a given day of storage
285 commonly had a short distance and were clustered together at low heights, whereas samples
286 from the earliest and latest days of storage were finally joined at a relatively high distance. The
287 results thus indicate that the VOC profile was usually highly similar between samples from a
288 given day of storage and the most different between samples from the early and late days of
289 storage. AU values were typically high, indicating that the clustering was well supported by the
290 data. Non-logarithmic and non-standardized data (Fig. 2A) separated days 0-7 from days 10-12.
291 Samples from the early days of storage (0-3) were highly similar, which is in good
292 correspondence with the VOC concentration patterns (Fig. 1). Otherwise (Fig. 2B-D),
293 intermediate days (5-7) clustered together with late days (10-12) sooner than with the early days.

294 The choice of distances and linkages affects the clustering results and depends on the dataset and
295 purpose of application. Different alternatives can be compared during exploratory analysis
296 (Rendall et al., 2015; Smoliński et al., 2002). Euclidean distance or correlation coefficient are
297 most commonly used as distance measures together with different linkages. In the present study,
298 preliminary comparison of different linkages resulted in slightly different dendrograms, whereas
299 highly similar results were obtained when comparing different distances (results not shown).
300 Since different transformations were applied to the VOC data in order to examine the similarity
301 both in terms of values and evolution, Euclidean distance and average linkage were chosen.
302 When clustering is based on average linkage, distance of two objects from separate clusters can
303 be either smaller or larger than the average distance of the clusters, which might lead into under-

or overestimating the distance between two objects. Even though using single linkage might avoid this phenomenon, problems caused by outliers and cluster density differences limit the use of this linkage (Almeida et al., 2007).

Concentrations of VOCs that are produced as a result of microbial metabolism can be expected to increase exponentially during the log phase of microbial growth. The three main VOC patterns identified in the present study are analogous to VOC groups observed by Küntzel et al. (2016) during the *in vitro* growth of *Mycobacterium avium* ssp. *paratuberculosis*. VOCs that were increasing throughout storage time were associated with microbial growth, whereas those that reached a peak during storage were suggested to be produced by microbes and decrease after a change in their metabolism (Küntzel et al., 2016). In the present study, logarithmic transformation supported the monitoring of microbiologically related changes in VOC concentrations and separated the samples below and beyond 7 log TPC, whereas non-logarithmic concentrations emphasized the differences in concentration magnitudes and thus separated the late days of storage from the rest. This indicated not only that exponential increase in VOC concentrations occurred after exceeding 7.0 log TPC, but also that the VOC concentrations were still low at this point when compared to the late days of storage. Development of food monitoring systems should thus be sensitive enough in order to detect the onset of exponential concentration increase.

HCA is often applied in the beginning of exploratory analysis in order to characterize the internal structures within a dataset (Smoliński et al., 2002). In the present study, HCA provided an overview of the VOC profile both in terms of concentration range and evolution. Most of the VOCs were increasing as a function of time and microbial growth, suggesting that these VOCs could be considered as potential spoilage indicators. However, since logarithmic transformation

and scaling may emphasize small concentration changes and natural variation in the data, significance of the observed changes should be evaluated prior to statistical analyses.

3.1.2 Principal components analysis (PCA)

Fig. 3 presents the PCA scores and correlation loadings as a two-dimensional biplot where the scores represent samples (independent packages) and correlation loadings indicate the relationships between the individual VOCs. The first two latent variables PC1 and PC2 were linear combinations of the original variables (VOCs) and explained 86.5 % of the total variance within the data. Result of the KMO test (0.62) indicated sufficient sampling adequacy and significance of the Bartlett's sphericity test ($p < 2.22*10^{-16} < 0.05$) suitability for data reduction.

Separation between samples from different days of storage could be observed on the biplot (Fig. 3). The closer the samples are located on the biplot, the higher is the similarity between their VOC profiles (Vervoort et al., 2012). Four main groups of samples could be identified in good correspondence with the clustering results (Fig. 1D and 2D): day 0, day 3, days 5-7 and days 10-12. Butanone was associated with fresh samples, whereas most of the VOCs were characteristic for late stages of storage. On the other hand, correlation loadings could be used for evaluating correlations between VOCs as well as their occurrence in different samples. Closely located VOCs are highly positively correlated, whereas projection in opposite directions indicates negative correlation. Respectively, VOCs that characterize a certain sample group are closely located to the respective scores (Vervoort et al., 2012). In the present study, the main VOC groups identified by PCA (Fig. 3) corresponded to those determined by hierarchical clustering (Fig. 1D). Most of the VOCs were highly positively correlated and characteristic to the late days of storage. Isobutyl alcohol, 2-propanol and acetone were most closely associated with late storage (days 10-12), whereas ethyl acetate, ethylene oxide and trimethylamine were

characteristic to intermediate to late storage due to the decreasing concentrations after day 7. Butanone was negatively correlated with the other VOCs and associated with fresh samples.

The scores of Fig. 3 illustrated an arch-shaped trend. The "horseshoe" is formed when the second axis is distorted in relation to the first axis (Lewis & Menzies, 2015). In the present study, this phenomenon was most likely due to the effect of time. VOC concentrations increased as a function of time, which is why most of the observed variance within the data was caused by progressing time and thus VOC evolution. The first principal component was thus likely related to time, whereas the second principal component had no clear biological interpretation.

3.1.3 Partial least squares regression (PLS)

The PLS plots show the correlation between VOCs and time (Fig. 4A), TPC (Fig. 4B) or sensory rejection % (Fig. 4C) and describe both samples (scores) and VOCs (correlation loadings) along with the response variable. When variables are located between the 75 and 100 % circles, more than 75 % of their variance is explained by the first two latent variables. The importance of a VOC in explaining the variance in the dataset decreases towards the origin of the biplot (Vervoort et al., 2012). VOCs that are projected away from the origin and towards the response variable are highly positively correlated with the response, whereas projection in opposite direction indicates negative correlation (Vervoort et al., 2012; Wibowo et al., 2015). The respective VIP vs. regression coefficient plots (Fig. 4D-F) show the impact of each VOC on the linear models. VOCs with a high VIP coefficient have high impact on the response variable and regression coefficient indicates whether the impact is positive or negative. In the present study, the PLS biplots were analyzed according to the principles presented for PCA (see 3.1.2).

371 Most of the analyzed VOCs were close to the time vector (Fig. 4A), indicating that the VOC
372 concentrations were increasing as a function of time. Respectively as observed in the heatmaps
373 (Fig. 1) and PCA biplot (Fig. 3), butanone was negatively correlated with time and associated
374 with day 0 samples. Furthermore, in case of nine out of fourteen VOCs, 75-100 % of variance
375 was explained by the first two latent variables, indicating that these VOCs had a strong
376 correlation with time. Acetone and methyl mercaptan were the most positively correlated VOCs
377 with time. The VIP plot (Fig. 4D) identified six out of fourteen VOCs having VIP > 1 and a
378 positive regression coefficient: acetone, ammonia, 2,3-butanediol, dimethyl sulfide, ethanol and
379 methyl mercaptan. These VOCs also had positive correlations with time.

380 Even though most of the studied VOCs were positively correlated with TPC (Fig. 4B), their
381 correlations were typically less positive than between VOCs and time (Fig. 4A). Carbon
382 disulfide, ethyl acetate and trimethylamine had strong positive correlation with TPC, whereas
383 most of the other VOCs had slightly positive correlation with TPC. Respectively, four out of
384 fourteen VOCs had VIP > 1 and positive regression coefficients (Fig. 4E): carbon disulfide,
385 dimethyl sulfide, ethyl acetate and trimethylamine. However, since TPC reached stationary phase
386 after day 5, VOCs that showed decreasing concentrations during late storage were highlighted in
387 the respective PLS model. The observed decrease in VOC concentration is likely affected by
388 several reasons independent of microbial growth, such as degradation into other compounds.
389 VOC concentrations cannot thus be directly related to microbial counts after the stationary phase
390 has been reached.

391 Finally, three main groups of VOCs could be identified in the PLS model for rejection % (Fig.
392 4C). These VOC groups closely coincided with those observed in the respective heatmap (Fig.
393 1D). The concentrations of the majority of VOCs had strong positive correlations with panelist

394 rejection. Seven out of fourteen VOCs had VIP > 1 and positive regression coefficients:
395 ammonia, 2,3-butanediol, dimethyl sulfide, ethanol, ethyl acetate, ethylene oxide and
396 trimethylamine. Acetone, isobutyl alcohol and 2-propanol had less positive or weaker
397 correlations and butanone a negative correlation with rejection %.

398 The VIP value 1 has generally been used as a cut-off limit in variable selection: variables
399 exceeding this limit can be considered to be highly influential (Afanador, Tran, & Buydens,
400 2014; Zaragozá et al., 2014). However, since the VIP approach considers every studied variable,
401 VOCs that have high importance in the model are not necessarily limited to those showing
402 constant increase during storage. This can be observed in Fig. 4D-E where the VIP of butanone
403 nearly exceeded 1 despite its negative correlation with time and TPC (Fig. 4A-B) and negative
404 regression coefficients (Fig. 4D-E). Butanone concentration decreased from 170 to 100 ug m^{-3}
405 during storage, indicating that it is not likely relevant for spoilage analysis. Excluding butanone
406 from the analysis could allow other VOCs to exceed the chosen VIP limit. Selection of variables
407 on the basis of VIP thus gives the most influential VOCs, irrespectively of their impact on the
408 value of the response variable.

409 PLS is commonly used when numerous highly correlated predictor variables are present (Wold
410 et al., 2001). A positive correlation between a VOC and the response indicates that increase in
411 VOC concentration is associated with increase in the response. However, correlation does not
412 necessarily indicate a relationship between the variables. In the present study, multicollinearity
413 between VOCs could be expected because increase in VOC concentrations was related to
414 microbial growth and likely to the same producer microbes (Kuuliala et al. submitted
415 manuscript). Some VOCs might thus correlate with the response even though no direct
416 relationship existed between them. Furthermore, since correlation does not consider the possible

417 dependencies between VOCs, direct relationships between the variables and the response might
418 be hidden because of suppression. This phenomenon could be due to e.g. degradation or
419 consumption of a VOC during storage. For example, several VOCs had a relatively strong
420 positive correlation with consumer rejection (Fig. 4C), although their regression coefficients
421 were negative (Fig. 4F). This could suggest that increase in their concentrations may depend on
422 another VOCs and/or that they do not contribute to unpleasant off-odors.

423 In the present study, storage time was extended beyond consumer rejection. After the moment of
424 rejection (day 5), declining TPC and concentrations of certain VOCs were detected. During
425 extended storage, evolution of VOCs produced during microbial metabolism does not necessarily
426 correlate with TPC, which may interfere with the identification of potential spoilage indicators.
427 Analysis of VOC evolution should thus focus on the log phase of microbial growth.

428 ***3.2 Selective statistics***

429 The results of the exploratory analyses indicate that an overview of the evolution and relevance
430 of VOCs can be obtained with all the analytical methods applied in the present study. However,
431 especially HCA was also associated with demanding results interpretation. Systematic and
432 facilitated determination of spoilage indicators calls for cut-off values and correlation between
433 VOCs and a dependent response variable. PLS regression was thus identified as the most
434 systematic approach for selective analysis. Table 2 presents the most potential spoilage
435 indicators of Atlantic cod and brown shrimp identified by PLS and the selection criteria: positive
436 correlation with the response, VIP > 1 and positive regression coefficient. The number of factors
437 resulting in minimal root mean PRESS was in most cases between 2-7. In case of sensory
438 rejection of Atlantic cod, the minimizing number was 1; two factors were selected on the basis of

van der Voet test (van der Voet, 1994) indicating that the residuals of the model with two factors were not significantly larger than with one factor.

When considering TPC as a dependent variable, several VOCs could be identified for cod. Under at least three out of five storage conditions, 2,3-butanediol, dimethyl sulfide, ethyl acetate, 3-methyl-1-butanol, isobutyl alcohol and trimethylamine fulfilled the selection criteria. These VOCs could thus indicate spoilage under different storage conditions and could be related to the metabolism of representatives from the *Photobacterium* genus (Kuuliala et al. submitted manuscript). Dimethyl sulfide was associated with low oxygen MAP or air, whereas acetic acid was associated with MAP at lower storage temperature (4 °C). When rejection % was used as a dependent variable, the selected VOCs were well in correspondence with the TPC model.

Under the two atmospheres tested for brown shrimp, different VOC profiles were identified. Under 50 % CO_2, most of the VOCs corresponded to the compounds identified for cod under low oxygen concentrations. In addition, carbon disulfide and methyl mercaptan fulfilled the selection criteria. Under 30 % CO_2, only dimethyl sulfide, ethyl acetate and trimethylamine were identified. Respectively, few compounds were identified when rejection % was used as a dependent variable. The results are in good correspondence with VOCs detected in previous studies concerning crustaceans. Noseda et al. (2012) observed a significant increase in acetic acid, ammonia, dimethyl sulfide, dimethyl amine, ethanol, ethyl acetate and trimethylamine in brown shrimp stored under 50 % CO_2 and 50 % N_2. Production of hydrogen sulfide, carbon disulfide and methyl mercaptan was inhibited in the presence of carbon dioxide. Broekaert et al. (2013) observed the production of several respective VOCs in aerobically stored brown shrimp inoculated with *Pseudoalteromonas*. Respectively, increasing concentrations of several compounds including alcohols, aldehydes, ketones and trimethylamine have been observed with

462 other crustaceans (Fall et al., 2012; Laursen, Leisner, & Dalgaard, 2006; Olafsdottir et al., 2005).
463 The wet dog odor of Nordic shrimp has been attributed to the co-culture of *Carnobacterium*
464 *maltaromaticum* and *Brochothrix thermosphacta* (Mejlholm, Bøknæs, & Dalgaard, 2005),
465 particularly to the interaction of their metabolic products (Malcolm Love, 1979).

466 The potential spoilage indicators observed in the present study are produced during microbial
467 metabolism (Casaburi, Piombino, Nychas, Villani, & Ercolini, 2015; Olafsdóttir et al., 1997). 3-
468 methyl-1-butanol has frequently been observed during seafood spoilage (Duflos et al., 2010;
469 Mikš-Krajnik et al., 2016; Parlapani, Mallouchos, Haroutounian, & Boziaris, 2014) and has been
470 associated with cheesy or fruity off-odors (Montel, Masson, & Talon, 1998). In addition, both 3-
471 methyl-1-butanol and 2,3-butanediol have been associated with fermented odor under vacuum
472 (Casaburi et al., 2015). Ethyl esters such as ethyl acetate have been associated with fruity off-
473 odors in meat (Ercolini et al., 2010). Production of dimethyl sulfide results in sulfurous and
474 cabbage-like odors (Ercolini et al., 2010), whereas trimethylamine contributes to the
475 characteristic smell of spoiled marine fish (Gram & Dalgaard, 2002).

476 The results of the present study indicate that several VOCs are produced during refrigerated
477 storage of seafood. Even though some VOCs were identified as potential spoilage indicators
478 under various conditions, single compounds have limited potential in quality analysis because
479 their evolution is dependent on the storage conditions and subject to natural variation. The results
480 are thus in line with previous studies suggesting that the use of multiple compound indices could
481 enhance seafood quality analysis (Jørgensen et al., 2001; Leroi, Joffraud, Chevalier, & Cardinal,
482 2001; Ólafsdóttir, Högnadóttir, Martinsdóttir, & Jónsdóttir, 2000).

483 **4. Conclusions**

The identification of volatile organic compounds (VOCs) related to spoilage allows the analysis of seafood spoilage by following the concentrations of these compounds over storage time. Multivariate statistics provides analytical methods for the characterization and selection of relevant spoilage indicators. In the present study, acetic acid, 2,3-butanediol, isobutyl alcohol, 3-methyl-1-butanol, dimethyl sulfide, ethyl acetate and trimethylamine were most frequently identified as potential spoilage indicators of Atlantic cod and/or brown shrimp under different atmospheres. Due to the complex nature of microbiological spoilage and VOC evolution as well as the wide range of available packaging and storage conditions, seafood quality analysis could thus benefit from the analysis of multiple VOCs instead of single compounds over storage time.

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691

Figure captions

Fig. 1. Hierarchical cluster analysis (HCA) of volatile organic compounds (VOCs) produced during storage of brown shrimp under modified atmosphere (% $CO_2/O_2/N_2$) 50/0/50 at 4 °C. Euclidean distance and average linkage were used for building the heat maps. The columns represent individual VOCs (Table 1) and rows represent shrimp samples labelled with day of storage and replicate A-C. VOCs were analyzed as (A) non-logarithmic and non-standardized, (B) non-logarithmic and standardized, (C) logarithmic and non-standardized and (D) logarithmic and standardized data.

Fig. 2. Hierarchical cluster analysis (HCA) of brown shrimp samples stored under modified atmosphere (% $CO_2/O_2/N_2$) 50/0/50 at 4 °C. Euclidean distance and average linkage were used for building the dendrograms. Approximate unbiased (AU) and bootstrap probability (BP) values are given above the corresponding clusters. The shrimp samples are labelled with day of storage and replicate A-C.

Fig. 3. Principal components analysis (PCA) biplot of brown shrimp stored under modified atmosphere (% $CO_2/O_2/N_2$) 50/0/50 at 4 °C. The shrimp samples (scores) are labelled with day of storage and replicate A-C. The correlation loadings represent individual VOCs.

Fig. 4. Partial least squares (PLS) biplots (A-C) and VIP vs. regression coefficient plots (D-F) of brown shrimp stored under modified atmosphere (% $CO_2/O_2/N_2$) 50/0/50 at 4 °C. VOCs are treated as predictor variables and time (A and D), TPC (B and E) or rejection % (C-F) as response variables. The biplots present samples as scores (day 0: +; day 3: ◊; day 5: Δ; day 7:*; day 10: x; day 12: □) and VOCs as correlation loadings.

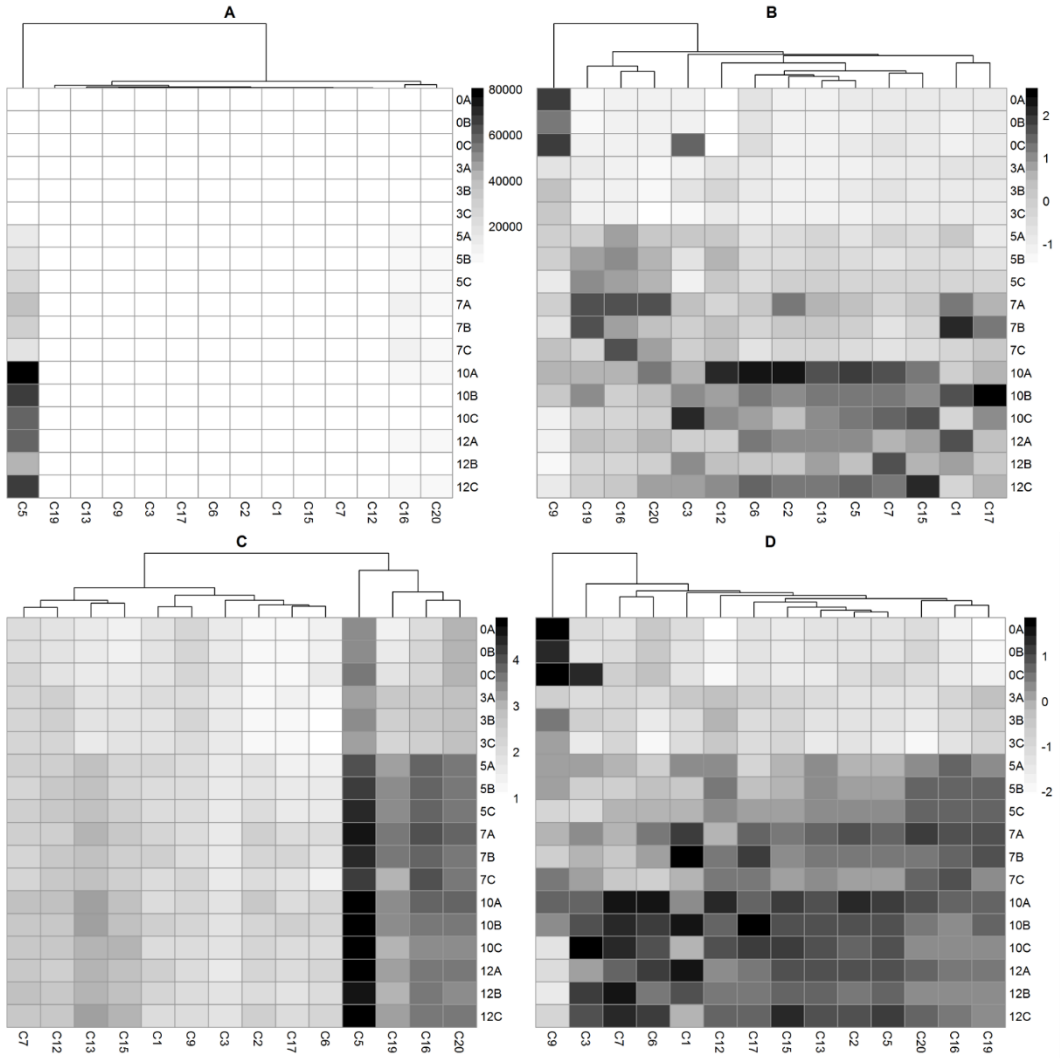
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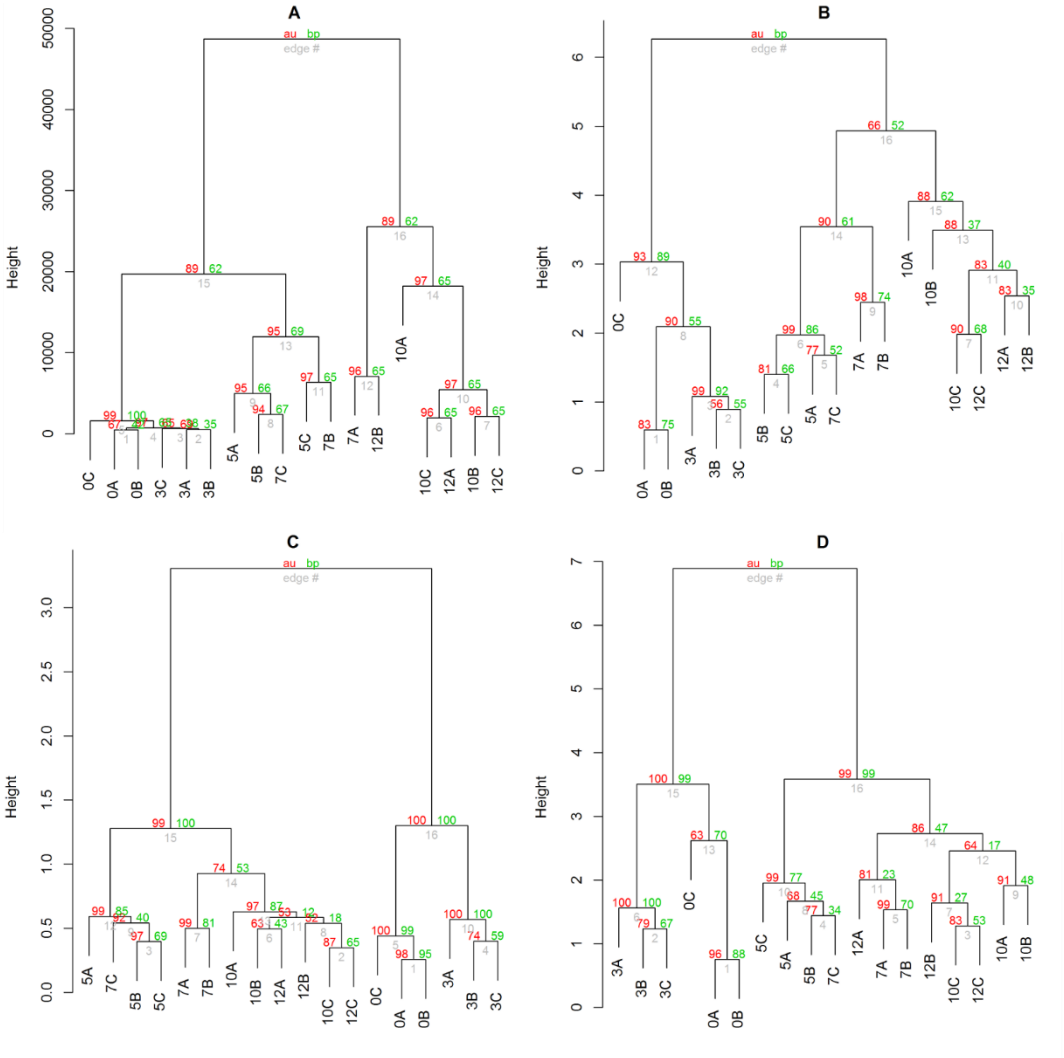
Fig. 1.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10C
12A
10B
10A
12C

Fig. 2.

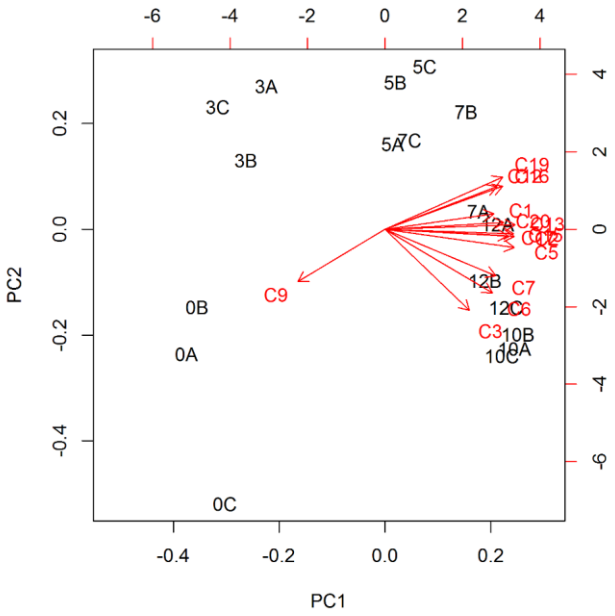
-6
-4
-2
0
2
4
5C
5B
3A
3C
7B
7C
5A
3B
C19
7A
C1
12A
C5
C9
12B
C7
0B
C3
10B
10A
0A
10C
0C
0.2
0.0
-0.2
-0.4
PC2
PC1
-0.4
-0.2
0.0
0.2
4
2
0
-2
-4
-6

Fig. 3.

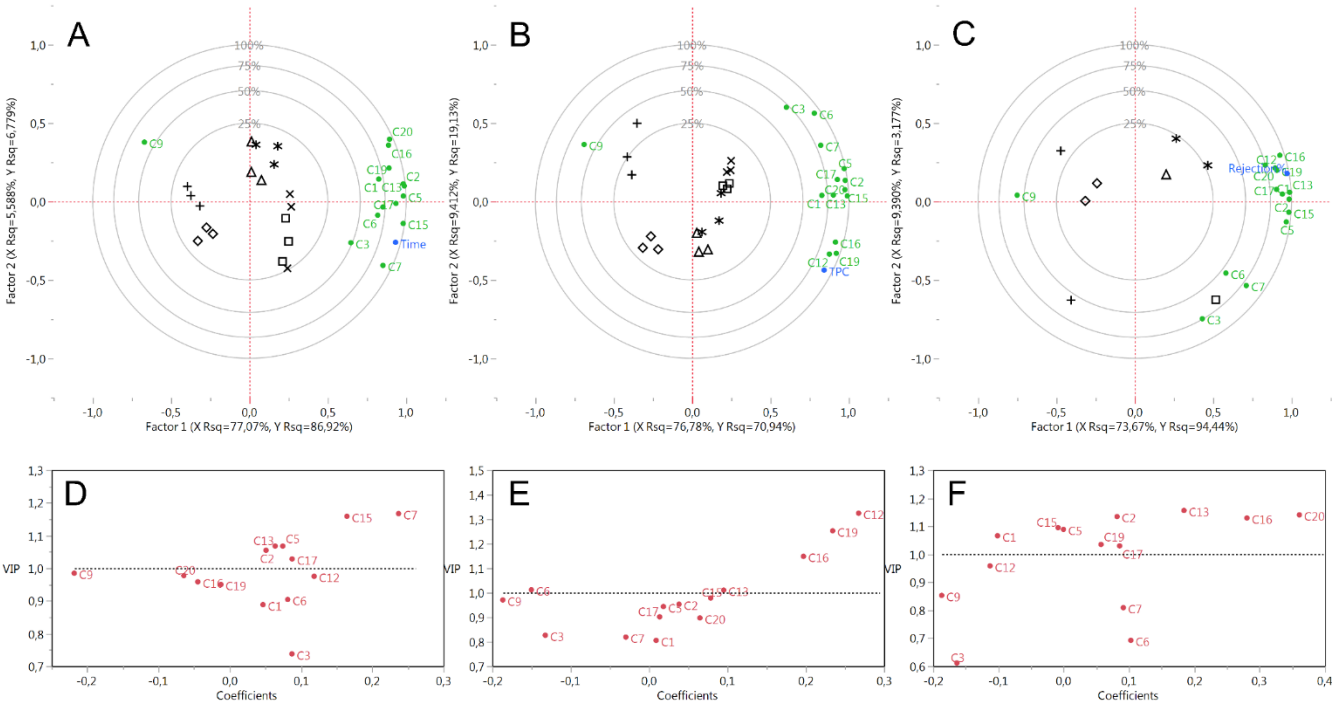
A
B
C
D
E
F
Factor 1 (X Rsq=77,07%, Y Rsq=86,92%)
Factor 2 (X Rsq=5,588%, Y Rsq=6,779%)
Factor 1 (X Rsq=76,78%, Y Rsq=70,94%)
Factor 2 (X Rsq=9,412%, Y Rsq=19,13%)
Factor 1 (X Rsq=73,67%, Y Rsq=94,44%)
Factor 2 (X Rsq=9,390%, Y Rsq=3,177%)
Time
TPC
VIP
Coefficients

Fig. 4.

Table 1. *Product ions of volatile organic compounds (VOCs) quantified with SIFT-MS from the headspace of brown shrimp samples, respective mass to charge ratios (m/z), branching ratios (b) and reaction rate coefficients (k).*

VOC	Code	Precursor ion	m/z	b (%)	k	Product ion
Acids						
Acetic acid	C1	NO^+	90	100	9.0 E -10	$NO^+.CH_3COOH$
		NO^+	108		9.0 E -10	$NO^+.CH_3COOH.H_2O$
Alcohols						
2,3-butanediol	C2	H_3O^+	91	100	3.0 E -09	$C_4H_{10}O_2^+.H^+$
		NO^+	89	100	2.3 E -09	$C_4H_9O_2^+$
2-propanol	C3	H_3O^+	43	80	2.7 E -09	$C_3H_7^+$
3-methyl-1-butanol	C4	H_3O^+	71	100	2.8 E -09	$C_5H_{11}^+$
		NO^+	87	85	2.3 E -09	$C_5H_{11}O^+$
Ethanol	C5	H_3O^+	47	100	2.7 E -09	$C_2H_7O^+$
		H_3O^+	65			$C_2H_7O^+.H_2O$
		H_3O^+	83			$C_2H_7O^+.(H_2O)_2$
Isobutyl alcohol	C6	H_3O^+	57	100	2.7 E -09	$C_4H_9^+$
		NO^+	73	95	2.4 E -09	$C_4H_9O^+$
		O_2^+	33	50	2.5 E -09	CH_5O^+
Ketones						
Acetone	C7	H_3O^+	59	100	3.9 E -09	C_3H_7O+
		NO^+	88	100	1.2 E -09	$NO^+.C_3H_6O$
Acetoin	C8	O_2^+	88	20	2.5 E -09	$C_4H_8O_2^+$
Butanone	C9	NO^+	102	100	2.8 E -09	$NO^+.C_4H_8O$
2-pentanone	C10	H_3O^+	87	100	3.9 E -09	$C_5H_{11}O^+$
		H_3O^+	105		3.9 E -09	$C_5H_{11}O^+.H_2O$
		NO^+	116	100	3.1 E -09	$NO^+.C_5H_{10}O^+$
Sulfur compounds						
Hydrogen sulfide	C11	H_3O^+	35	100	1.6 E -09	H_3S^+
		H_3O^+	53		1.6 E -09	$H_3S^+.H_2O$
		O_2^+	34	100	1.4 E -09	H_2S^+
Carbon disulfide	C12	O_2^+	76	100	7.0 E -10	CS_2^+
Dimethyl sulfide	C13	NO^+	62	100	2.2 E -09	$(CH_3)_2S^+$
Dimethyl disulfide	C14	H_3O^+	95	100	2.6 E -09	$(CH_3)_2S_2.H^+$
		NO^+	94	100	2.4 E -09	$(CH_3)_2S_2^+$
Methyl mercaptan	C15	H_3O^+	49	100	1.8 E -09	$CH_4S.H^+$
		H_3O^+	67		1.8 E -09	$CH_4S.H^+.H_2O$
Esters						
Ethyl acetate	C16	NO^+	118	90	2.1 E -09	$NO^+.CH_3COOC_2H_5$
		O_2^+	31	20	2.4 E -09	CH_3O^+
Amines						
Ammonia	C17	H_3O^+	18	100	2.6 E -09	NH_4^+
		H_3O^+	36		2.6 E -09	$NH_4^+.H_2O$
		O_2^+	17	100	2.4 E -09	NH_3^+
Dimethylamine	C18	H_3O^+	46	100	2.1 E -09	$(CH_3)_2N.H^+$
Trimethylamine	C19	H_3O^+	58	10	2.0 E -09	$C_3H_8N^+$
		H_3O^+	60	90	2.0 E -09	$(CH_3)_3N.H^+$
Others						
Ethylene oxide	C20	NO^+	74	100	1.0 E -10	$C_2H_4O.NO^+$

Table 2. *Most potential spoilage indicators of Atlantic cod (C) and brown shrimp (S) stored under different atmospheres (% $CO_2/O_2/N_2$), determined by PLS regression analysis. TPC or rejection % were used as the dependent variable and VOCs as independent variables.*

	TPC							**Rejection %**	
	C 4 °C 60/40/0	C 8 °C 60/40/0	C 4 °C 60/5/35	C 8 °C 60/5/35	C 4 °C Air	S 4 °C 50/0/50	S 4 °C 30/0/70	C	S
2,3-butanediol	x	x	x	x	x	x	0	x	0
2-methylpropanal						-	-		-
2-pentanone	0	x	0	0	0	0	0	0	0
2-propanol	-	-	-	-	-			-	
3-methyl-1-butanol	x	x	x	x	x	0	0	x	0
3-methylbutanal						-	-		-
Acetic acid	x		x		0	x	0	0	0
Acetoin	0	0	0	0	0	0	0	0	0
Acetone	x				x				
Ammonia						x			
Butanone	-	-	-	-	-			-	
Carbon disulfide	-	-	-	-	-	x		-	
Dimethyl amine	0	0	0	0	0	0	0	0	0
Dimethyl disulfide	0	0	0	0	0	0	0	0	0
Dimethyl sulfide			x	x	x	x	x		x
Dimethyl trisulfide						-	-		-
Ethanol		x							x
Ethyl acetate	x	x	x	x	x	x	x	x	x
Ethyl propanoate	0	0	0	0	0	-	-	0	-
Ethylene oxide	-	-	-	-	-			-	
Hydrogen sulfide	0	0	0	0	0	0	0	0	0
Isobutyl alcohol	x	x		x			0	x	0
Methyl mercaptan	0	0	0		0	x		0	
Trimethyl amine	x	x	x	x	x	x	x	x	x

x: selection criteria (VIP > 1, regression coefficient > 0, positive correlation with dependent variable) were met
-:VOC was not included in the SIFT-MS analysis
0: relative standard deviation > 25 %