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%CO₂/50%N₂) 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 sulfurous off-odors. VOCs contributing to the odor of fish can be divided into three groups, specifying compounds associated with freshness (C6-C9 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,
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 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 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). Two individual batches of shrimp were independently packaged under modified atmospheres (CO₂/O₂/N₂ %) 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 cm³/m²*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™, 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:

$$SD\% = \frac{SD_m}{x_m} \times 100\%$$ (1)

where $x_m$ is the average and $SD_m$ the standard deviation of a single SIFT-MS 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 (% CO₂/O₂/N₂) 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 (% CO₂/O₂/N₂) 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 heatmap() 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
the data: the greater the p-value, the greater the reliability of the clustering (Shimodaira, 2002; R. Suzuki & Shimodaira, 2006).

2.2.2 Principal components analysis (PCA)

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

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

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

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

2.3 Selective statistics

Selective statistical analyses were applied to data collected during storage of Atlantic cod and 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
% CO₂: 3-methyl-1-butanol, acetoin, 2-pentanone, dimethyl amine, dimethyl disulfide and
hydrogen sulfide. Under 30 % CO₂, 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 (% CO₂/O₂/N₂) 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
decreased thereafter. In addition, high initial concentrations of butanone led to the formation of a separate cluster that did not show any clear pattern. Respectively, clustering of logarithmic and standardized data (Fig. 1D) emphasized the evolution of VOCs during storage time.

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

The choice of distances and linkages affects the clustering results and depends on the dataset and purpose of application. Different alternatives can be compared during exploratory analysis (Rendall et al., 2015; Smoliński et al., 2002). Euclidean distance or correlation coefficient are most commonly used as distance measures together with different linkages. In the present study, preliminary comparison of different linkages resulted in slightly different dendrograms, whereas highly similar results were obtained when comparing different distances (results not shown). Since different transformations were applied to the VOC data in order to examine the similarity both in terms of values and evolution, Euclidean distance and average linkage were chosen. When clustering is based on average linkage, distance of two objects from separate clusters can 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 ($p < 2.22 \times 10^{-16} < 0.05$) 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).
Most of the analyzed VOCs were close to the time vector (Fig. 4A), indicating that the VOC concentrations were increasing as a function of time. Respectively as observed in the heatmaps (Fig. 1) and PCA biplot (Fig. 3), butanone was negatively correlated with time and associated with day 0 samples. Furthermore, in case of nine out of fourteen VOCs, 75-100 % of variance was explained by the first two latent variables, indicating that these VOCs had a strong correlation with time. Acetone and methyl mercaptan were the most positively correlated VOCs with time. The VIP plot (Fig. 4D) identified six out of fourteen VOCs having VIP > 1 and a positive regression coefficient: acetone, ammonia, 2,3-butanediol, dimethyl sulfide, ethanol and methyl mercaptan. These VOCs also had positive correlations with time.

Even though most of the studied VOCs were positively correlated with TPC (Fig. 4B), their correlations were typically less positive than between VOCs and time (Fig. 4A). Carbon disulfide, ethyl acetate and trimethylamine had strong positive correlation with TPC, whereas most of the other VOCs had slightly positive correlation with TPC. Respectively, four out of fourteen VOCs had VIP > 1 and positive regression coefficients (Fig. 4E): carbon disulfide, dimethyl sulfide, ethyl acetate and trimethylamine. However, since TPC reached stationary phase after day 5, VOCs that showed decreasing concentrations during late storage were highlighted in the respective PLS model. The observed decrease in VOC concentration is likely affected by several reasons independent of microbial growth, such as degradation into other compounds.

VOC concentrations cannot thus be directly related to microbial counts after the stationary phase has been reached.

Finally, three main groups of VOCs could be identified in the PLS model for rejection % (Fig. 4C). These VOC groups closely coincided with those observed in the respective heatmap (Fig. 1D). The concentrations of the majority of VOCs had strong positive correlations with panelist
rejection. Seven out of fourteen VOCs had VIP > 1 and positive regression coefficients: ammonia, 2,3-butanediol, dimethyl sulfide, ethanol, ethyl acetate, ethylene oxide and trimethylamine. Acetone, isobutyl alcohol and 2-propanol had less positive or weaker correlations and butanone a negative correlation with rejection %.

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

PLS is commonly used when numerous highly correlated predictor variables are present (Wold et al., 2001). A positive correlation between a VOC and the response indicates that increase in VOC concentration is associated with increase in the response. However, correlation does not necessarily indicate a relationship between the variables. In the present study, multicollinearity between VOCs could be expected because increase in VOC concentrations was related to microbial growth and likely to the same producer microbes (Kuuliala et al. submitted manuscript). Some VOCs might thus correlate with the response even though no direct relationship existed between them. Furthermore, since correlation does not consider the possible
dependencies between VOCs, direct relationships between the variables and the response might be hidden because of suppression. This phenomenon could be due to e.g. degradation or consumption of a VOC during storage. For example, several VOCs had a relatively strong positive correlation with consumer rejection (Fig. 4C), although their regression coefficients were negative (Fig. 4F). This could suggest that increase in their concentrations may depend on another VOCs and/or that they do not contribute to unpleasant off-odors.

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

3.2 Selective statistics

The results of the exploratory analyses indicate that an overview of the evolution and relevance of VOCs can be obtained with all the analytical methods applied in the present study. However, especially HCA was also associated with demanding results interpretation. Systematic and facilitated determination of spoilage indicators calls for cut-off values and correlation between VOCs and a dependent response variable. PLS regression was thus identified as the most systematic approach for selective analysis. Table 2 presents the most potential spoilage indicators of Atlantic cod and brown shrimp identified by PLS and the selection criteria: positive correlation with the response, VIP > 1 and positive regression coefficient. The number of factors resulting in minimal root mean PRESS was in most cases between 2-7. In case of sensory 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 % CO₂, 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 % CO₂, 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 % CO₂ and 50 % N₂. 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
other crustaceans (Fall et al., 2012; Laursen, Leisner, & Dalgaard, 2006; Olafsdottir et al., 2005).

The wet dog odor of Nordic shrimp has been attributed to the co-culture of *Carnobacterium maltaromaticum* and *Brochothrix thermosphacta* (Mejlholm, Bøknæs, & Dalgaard, 2005), particularly to the interaction of their metabolic products (Malcolm Love, 1979).

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

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

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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References


crangon) during storage on ice and at 7.5 °C. *Food Microbiology, 36*(2), 123-134.
doi:http://dx.doi.org/10.1016/j.fm.2013.04.009

and the volatilome associated to meat spoilage. *Food Microbiology, 45, Part A*, 83-102.
doi:http://dx.doi.org/10.1016/j.fm.2014.02.002

doi:http://dx.doi.org/10.1016/j.snb.2014.08.025

Ciesa, F., Dalla Via, J., Wisthaler, A., Zanella, A., Guerra, W., Mikoviny, T., . . . Oberhuber, M.
(2013). Discrimination of four different postharvest treatments of ‘Red delicious’ apples
based on their volatile organic compound (VOC) emissions during shelf-life measured by
proton transfer reaction mass spectrometry (PTR-MS). *Postharvest Biology and Technology,
86*, 329-336. doi:http://dx.doi.org/10.1016/j.postharvbio.2013.06.036

characterisation of whiting (merlangius merlangus) using an SPME/GC/MS method and a
statistical multivariate approach. *Journal of the Science of Food and Agriculture, 90*(15),
2568-2575. doi:10.1002/jsfa.4122

Ercolini, D., Casaburi, A., Nasi, A., Ferrocino, I., Di Monaco, R., Ferranti, P., . . . Villani, F.
(2010). Different molecular types of pseudomonas fragi have the same overall behaviour as
doi:http://dx.doi.org/10.1016/j.ijfoodmicro.2010.06.012

Sensory and physicochemical evolution of tropical cooked peeled shrimp inoculated by
brochothrix thermosphacta and lactococcus piscium CNCM I-4031 during storage at 8 °C.

*International Journal of Food Microbiology, 152*(3), 82-90.

doi:http://dx.doi.org/10.1016/j.ijfoodmicro.2011.07.015

importance in projection (VIP) and of the selectivity ratio (SR) methods for variable

doi:10.1002/cem.2736

Fik, M., Surówka, K., Maciejaszek, I., Macura, M., & Michalczyk, M. (2012). Quality and shelf
life of calcium-enriched wholemeal bread stored in a modified atmosphere. *Journal of
Cereal Science, 56*(2), 418-424. doi:http://dx.doi.org/10.1016/j.jcs.2012.06.006

Opinion in Biotechnology, 13*(3), 262-266. doi:http://dx.doi.org/10.1016/S0958-1669(02)00309-9


compound generation in dry fermented sausages by the surface inoculation of selected

doi:10.1007/s00217-004-1083-2

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


doi: http://dx.doi.org/10.1016/j.vibspec.2015.10.002


doi: 10.1002/jsfa.2740300414

doi: http://dx.doi.org/10.1016/j.ijfoodmicro.2006.11.003


doi: http://dx.doi.org/10.1016/j.fm.2015.10.001


**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.
Fig. 2.
Fig. 3.
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).

<table>
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<th>VOC</th>
<th>Code</th>
<th>Precursor ion</th>
<th>m/z</th>
<th>b (%)</th>
<th>k</th>
<th>Product ion</th>
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<td>C₇H₁₁⁺</td>
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<td>H₃S⁺</td>
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<td>NH₄⁺</td>
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<td>C₂H₂O.NO⁺</td>
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</table>
Table 2. Most potential spoilage indicators of Atlantic cod (C) and brown shrimp (S) stored under different atmospheres (% CO₂/O₂/N₂), determined by PLS regression analysis. TPC or rejection % were used as the dependent variable and VOCs as independent variables.

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<th>Rejection %</th>
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<td>C 4 °C</td>
<td>C 8 °C</td>
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<tr>
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<td>60/40/0</td>
</tr>
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<td>2,3-butanediol</td>
<td>x</td>
<td>x</td>
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<td>2-methylpropanal</td>
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<td>-</td>
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<td>2-pentanone</td>
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<td>x</td>
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<td>2-propanol</td>
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<td>-</td>
</tr>
<tr>
<td>3-methyl-1-butanol</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>3-methylbutanal</td>
<td>-</td>
<td>-</td>
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<td>x</td>
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<td>Isobutyl alcohol</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Methyl mercaptan</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Trimethyl amine</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>

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%