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# Volatile Organic Compounds as Quality Markers in Cooked and Sliced Meat Products

**PhD thesis by**
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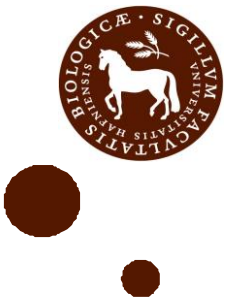


**Volatile Organic Compounds as Quality Markers in Cooked and Sliced Meat Products**
PhD thesis 2011 © Esben Skibsted Holm

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# Abstract

Quality changes in modified atmosphere packed cooked meat products during storage are mainly ascribed to recontamination of the product during the slicing and handling after the heat processing. In this PhD-project these quality changes have been investigated using primarily measurements of the sensory profile, the aroma composition and the microbial composition. The purpose of this was to identify volatile organic compounds (VOC's) which could be used as chemical markers for the sensory quality of cooked and sliced meat products. The potential use of these VOC's as a tool for early detection of sensory spoilage in the meat industry was furthermore investigated. The experimental work of this PhD-project has resulted in 4 papers which investigate different aspects of 'VOC's as quality markers in cooked and sliced meat products'.

PAPER I is an initial study investigating the effect of different experimental conditions on the aroma composition of sliced saveloy. The use of commercial slicing, an increased initial storage temperature and temperature fluctuation during the fourth and final week of the shelf-life period was found to affect the level of 2- and 3 methylbutanal, which was used as an indicator of microbial activity.

In PAPER II the quality changes of saveloy samples sliced at 3 different locations were studied during the fourth and final week of the shelf-life period. The changes in the sensory descriptors sour&old odor and meaty odor were modeled using partial least squares regression (PLS) based on measurements of the aroma composition with gas chromatography-mass spectrometry (GC-MS). In the resulting PLS-models the formation of the microbially produced VOC's acetoin, diacetyl, 2- and 3-methylbutanal and 2- and 3- methylbutanol were found to be closely related to changes in both of the sensory descriptors. These compounds were therefore concluded to be chemical markers for the sensory quality of sliced saveloy. The developed PLS-models, which were based on the 10 VOC's with the strongest relation to the sensory descriptors, moreover provided relatively good predictions of the sensory quality of the saveloy samples.

PAPER III investigated the quality changes in sliced saveloy samples inoculated with monocultures of 4 potential spoilage bacteria or a 1:1:1:1 mixture of these. Inoculation with *B. thermosphacta*, *C. maltaromaticum* or *C. luteola* resulted a significant decrease in the sensory quality of the saveloy samples whereas inoculation with *Leuc. carnosum* only resulted in limited sensory changes of the product. PLS-models describing six sensory descriptors based on measurements of the aroma composition with GC-MS showed that acetoin, diacetyl, 2- and 3-methylbutanal, 2- and 3-methylbutanol and 2-methylpropanol were closely related changes in the sensory profile. This result corresponded well with the observations made in PAPER II. The highlighted VOC's were moreover found to be produced in varying amounts by *B. thermosphacta*, *C. maltaromaticum* and *C. luteola*. PAPER III also studied the changes in the aroma composition shortly after slicing and packing. However, no significant changes in the suggested chemical markers were found between day 1 and week 1 after slicing. This result therefore indicates that the suggested chemical markers have a limited potential for early detection of sensory spoilage in cooked and sliced meat products.

PAPER IV investigated the use proton transfer reaction-mass spectrometry (PTR-MS) as an alternative to 'traditional' measurements of the aroma composition with GC-MS. PTR-MS provides rapid on-line measurement of VOC's, and it is therefore interesting in relation a practical application of the suggested chemical markers. However, PTR-MS does not include a separation of the VOC's in the sample headspace prior to measurement, which complicates the interpretation of the resulting mass spectra. Nevertheless, based in the results presented in PAPER IV, PTR-MS was concluded to be a suitable method for measurement of quality changes in cooked and sliced meat products.

Overall, the results of this PhD-project showed that the suggested chemical markers were able to predict the present sensory quality of cooked and sliced meat product. Moreover, based on the observations made in this PhD-project the possibility of using VOC's for early detection of spoilage of cooked and sliced meat products seems limited. However, further research is needed to make a final conclusion on this matter.

# Resumé

Kvalitetsændringer under lagring af modificeret atmosfære pakkede kogte kødprodukter skyldes hovedsageligt rekontaminering af produktet under håndtering og slicening efter varmebehandlingen. I dette PhD-projekt er disse kvalitetsændringer blevet undersøgt ved måling af den sensoriske profil, aromasammensætningen og den mikrobiologiske sammensætning. Formålet med dette var at identificere aromaforbindelser med potentiale som kemiske markører for sensorisk kvalitet af kogte og slicede kødprodukter. Muligheden for at bruge disse kemiske markører til måling af den sensoriske holdbarhed af kogte og slicede kødprodukter kort tid efter pakning blev endvidere undersøgt. Det eksperimentelle arbejde udført i forbindelse med dette PhD-projekt har resulteret i 4 artikler der behandler forskellige aspekter af brugen af aromaforbindelser som kvalitets markører i kogte og slicede kødprodukter.

ARTIKEL I er en indledende undersøgelse af forskellige eksperimentelle faktorers betydning for aromasammensætningen af slicet kødpølse. Brugen af kommerciel slicening, forhøjet lagringstemperatur i de første 3 uger af forsøget samt temperatursvingninger i den fjerde og sidste uge af forsøget resulterede i koncentrationsændringer af 2- og 3- methylbutanal, der blev brugt som indikator for mikrobiel aktivitet.

I ARTIKEL II blev kvalitetsændringerne i kødpølse slicet på 3 forskellige lokaliteter undersøgt i den fjerde og sidste uge af deres holdbarhedsperiode. Ændringerne i de sensoriske parametre sur&gammel lugt og kød lugt blev modelleret ved brug 'partial least suqares regression' (PLS) ud fra målinger af aromasammensætningen. PLS-modellerne viste, at dannelsen af de mikrobiologisk dannede aromaforbindelser acetoin, diacetyl, 2- og 3-methylbutanal og 2- og 3-methylbutanol var tæt relateret til ændringer i begge sensoriske parametre. Det blev derfor konkluderet at disse aromaforbindelser kan bruges som kemiske markører for den sensoriske kvalitet af kogte og slicede kødprodukter. De førnævnte PLS-modeller var baseret på de 10 aromaforbindelser, der havde størst betydning for de sensoriske parametre. Disse modeller gav endvidere forholdsvist gode prædiktioner af den sensoriske kvalitet af kødpølse prøverne.

ARTIKEL III undersøgte kvalitetsændringerne i slicede kødpølse prøver podet med monokulturer af 4 potentielle fordærvelses bakterier eller en 1:1:1:1 blanding af disse. Podning med *B. thermosphacta*, *C. maltaromaticum* eller *C. luteola* resulterede i et signifikant fald i sensorisk kvalitet af kødpølse prøverne mens podning med *Leuc. carnosum* kun gav et beskedent fald i den sensoriske kvalitet. PLS-modeller, der beskriver 6 sensoriske parametre ud fra aromasammensætningen viste, at acetoin, diacetyl, 2- og 3-methylbutanal, 2- og 3-methylbutanol samt 2-methylpropanol var tæt relateret til ændringer i den sensoriske profil. Dette resultat hænger godt sammen med resultaterne fra ARTIKEL II. De fremhævede aromaforbindelser blev endvidere produceret af *B. thermosphacta*, *C. maltaromaticum* og *C. luteola* i forskelligt niveau.

ARTIKEL III undersøgte også ændringer i aromasammensætningen kort efter slicening og pakning. Der blev dog ikke fundet signifikante ændringer i de kemiske markører mellem dag 1 og uge 3. Baseret på dette resultat vurderes muligheden for at bruge aromaforbindelser som tidlige

markører for den sensoriske holdbarhed af kogte of slicede kødprodukter som værende begrænset.

I ARTIKEL IV blev proton transfer reaktion-massespektrometri (PTR-MS) undersøgt som et alternativ til traditionelle aromamålinger med gaskromatografi-massespektrometri (GC-MS). PTR-MS muliggør hurtige on-line målinger af flygtige forbindelser, og er derfor en interessant metode forhold til en praktisk anvendelse af de kemiske markører foreslået i ARTIKEL II og ARTIKEL III. PTR-MS måling inkluderer ikke en separation af de enkelte aromaforbindelser, hvilket er med til at komplicere fortolkningen af resultaterne. Resultaterne præsenteret ARTIKEL IV viste dog at PTR-MS er en velegnet metode til måling af kvalitetsændringer i kogte og slicede kødprodukter.

Overordnet set lykkedes det i dette PhD-projekt at identificere aromaforbindelser, der kan prædiktere den nuværende sensoriske kvalitet af kogte og slicede kødprodukter. Muligheden for at bruge aromaforbindelser som tidlige markører for sensorisk holdbarhed af disse kødprodukter viste sig, baseret på resultaterne fra dette projekt, at være begrænset. Dette bør dog undersøges yderligere før det kan konkluderes endeligt.

# Preface

This thesis was written as the conclusion of a PhD-project done at the Quality & Technology (Q&T) research group at the Department of Food Science, Faculty of Life Sciences, University of Copenhagen in collaboration with Danish Meat Research Institute (DMRI)-Teknologisk. The work presented in this thesis was done primarily at DMRI-Teknologisk but experimental work was also done at Q&T and at the Department of Biosystems Engineering, Faculty of Agricultural Sciences, Aarhus University. The project was funded by LMC-FOOD, DMRI-Teknologisk and the Faculty of life Sciences at University of Copenhagen whom I would like to thank for making this project possible.

I am very grateful to my supervisors Associate Professor Mikael Agerlin Petersen (Q&T) and Senior Consultant Annette Schäfer (DMRI-Teknologisk) for their guidance and encouragement throughout the project, and moreover for introducing me to the world of GC-MS.

I am also grateful to all the co-authors of my publications for their important contribution to this PhD-project. Anette Granly Koch (DMRI-Teknologisk) has contributed with microbial expertise and inputs to the design of my experiments. Thomas Skov (Q&T) has shared his skills in chemometrics, Matlab and GC-MS data-processing. Anders Peter Adamsen and Anders Feilberg (Department of Biosystems Engineering, Faculty of Agricultural Sciences, Aarhus University) have introduced me to PTR-MS and moreover invited me to work with this technology in their laboratory. Virginia Resconi (Department of Animal Production and Food Science, University of Zaragoza) has shared her experience in working with GC-MS and has moreover been a valued friend.

I would moreover like to thank Jakob Søltoft-Jensen (DMRI-Teknologisk) for including me in various projects and providing valuable inputs to my Project, and Tomas Jacobsen (DMRI-Teknologisk) for patiently sharing his knowledge of microbiology.

Furthermore, I would like to thank the staff at DMRI-Teknologisk and Q&T for providing an excellent working environment throughout the project. The laboratory technicians at DMRI-Teknologisk and Q&T deserve special credit for introducing me to various analyses, and for their general contribution to the experimental work of this PhD-project.

Finally I would like to thank my family, friends for their support and interest in my project. Last but not least I am very grateful to my lovely wife Stine for her general support and for tolerating my absence during the final months of the project.

Esben Skibsted Holm
November 2011

# List of publications

**PAPER I**

E.S. Holm, V. Resconi, A. Schäfer

Effect of slicing and storage on the aroma composition of saveloy

Conference Proceeding from: The 55th International Congress of Meat Science and Technology (ICoMST), Copenhagen 2009. PE8.21, p. 1372-1375

**PAPER II**

E.S Holm, A. Schäfer, T. Skov, A.G. Koch, M.A. Petersen

Identification of chemical markers for the sensory shelf-life of saveloy

Meat Science 90 (2012) 314-322.

**PAPER III**

E.S. Holm, A. Schäfer, A.G. Koch, M.A. Petersen

Investigation and modeling of spoilage in saveloy samples inoculated with four potential spoilage bacteria

Submitted to Journal of Agricultural and Food Chemistry

**PAPER IV**

E.S. Holm, A.P. Adamsen, A. Feilberg, A. Schäfer, M.A. Petersen

Quality changes during storage of cooked and sliced meat products measured with HS-GC-MS and PTR-MS

In preparation for submission

# List of abbreviations

| | |
|---|---|
| ATP | Adenosine 5´-TriPhosphate |
| CI | Chemical Ionization |
| CFU | Colony Forming Units |
| CSS | Consumer simulated storage |
| DMRI | Danish Meat Research Institute |
| EI | Electron Ionization |
| FAA | Free Amino Acids |
| FFA | Free Fatty Acids |
| GC | Gas Chromatrography |
| GC-MS | Gas Chromatography-Mass Spectrometry |
| HETP | Height Equivalent to a Theoretical Plate |
| MA | Modified Atmosphere |
| MS | Mass Spectrometry |
| PC | Principal Component |
| PCA | Principal Component Analysis |
| PLS | Partial Least Squares regression |
| RMSECV | Root Mean Square Error of Cross Validation |
| PTR-MS | Proton Transfer Reaction-Mass spectrometry |
| SSO | Specific Spoilage Organism |
| TIC | Total Ion Current |
| TOF | Time of Flight |
| VOC | Volatile Organic Compound |

# Contents

# 1. Introduction

**Theoretical background**. Shelf-life and product stability are key concepts for the manufacturers of cooked and sliced meat products. A long shelf-life period enables the manufacturers to reach more consumers and hereby increase sales. However, if the shelf-life period is too long consumers could be exposed to spoiled products. The goal of the meat industry should therefore be to manufacture cooked and sliced meat products which have a long shelf-life and maintain a high quality throughout the shelf-life period *(1)*. In order to deliver this, the meat industry must be able to control all factors affecting the shelf-life of their products. However, controlling the shelf-life of cooked and sliced meat products is a complex matter. The development of shelf-life models, which account for the quality changes of specific cooked and sliced meat products during storage, would therefore be of great value to the meat industry.

The overall subject of this PhD-project is quality changes and shelf-life of cooked and sliced meat products. The shelf-life can be defined as the period of time in which a product can be stored without becoming unacceptable from a sensory point of view and without becoming a health risk *(1)*. However, in this thesis it is assumed that the growth of pathogen bacteria and other potential threats to human health are under control. The focus is therefore to model and describe the quality changes in cooked and sliced meat products, which eventually lead to spoilage from a sensory perspective. These quality changes are caused by both chemical and microbial alterations in the product during storage. However, the shelf-life of cooked and sliced meat products is typically limited by microbially induced quality changes during storage *(2-5)*.

The objective of this PhD-project is to identify chemical markers for the sensory quality of cooked and sliced meat products. Early detection of quality changes would enable the manufacturers of cooked and sliced meat products to take the necessary precautions in order to avoid shelf-life problems. The possibility of developing a predictive model for early detection of quality changes in cooked and sliced meat products based on the identified chemical markers will therefore also be investigated.

Odor is an important parameter for evaluation of the eating quality of cooked and sliced meat products. The aroma profile is therefore an obvious place to search for chemical markers for sensory quality. The odor of the product is among the first quality attributes that is perceived by the consumers, and based on the odor impression the product is either accepted or rejected for consumption. The aroma composition of cooked and sliced meat products is moreover affected by both chemical and microbial quality changes during storage. Oxidation of lipids can for example lead to formation of rancid off-odors whereas microbial metabolism can lead to formation of sweet, sour and putrid off-odors. These changes in the aroma composition can be measured by gas chromatography-mass spectrometry (GC-MS) and will moreover eventually be perceived as spoilage by the consumers or in a sensory evaluation.

**Outline of experimental work.** The experimental work of this thesis consists of a series of storage experiments where measurements of the aroma composition of cooked and sliced meat products

by dynamic headspace extraction coupled with GC-MS has been the common denominator. The objective of these storage experiments was to study the quality changes in cooked and sliced meat products using mainly measurements of the aroma composition, the microbial composition and the sensory profile. Chemometric tools, such as principal component analysis (PCA) and partial least squares regression (PLS), were used to clarify these relations and furthermore to model the sensory changes in cooked and sliced meat product based on volatile organic compounds (VOC's).

In PAPER I an initial investigation of factors affecting the aroma composition of sliced saveloy was performed by GC-MS measurements during the storage period. The investigated factors included the initial storage temperature, the use of temperature fluctuations during the final week of storage and industrial slicing vs. experimental slicing.
The changes in the aroma composition, the microbial composition and the sensory profile were investigated in saveloy from 3 different slicing locations during the fourth and final week of the shelf-life period in PAPER II. The objective of this was to model the sensory changes in the product based on GC-MS measurements of the aroma composition using PLS. This should moreover lead to identification of VOC's which could be used as chemical markers for the sensory quality of cooked and sliced meat products.
Based on the results of PAPER II it was evident that microbial reactions were the main reason for spoilage in cooked and sliced meat products. The impact of 4 potential spoilage bacteria (*B. thermosphacta*, *C. luteola*, *Leuc. carnosum* and *C. maltaromaticum*) on the shelf-life of cooked and sliced saveloy was therefore investigated in sliced saveloy during a 4 week storage period in PAPER III. This study also included PLS-modeling of the sensory evaluations based on measurements of the aroma composition with GC-MS. However, the early changes on the aroma composition after slicing and packing were also an important issue in this paper.
GC-MS is considered unsuited for measurements in a production environment. In PAPER IV proton transfer reaction-mass spectrometry (PTR-MS) was therefore tested as an alternative method for at-line measurement of changes in the aroma composition during storage in industrially produced saveloy and 4 other types of cooked and sliced meat products.

**Outline of thesis**. The following chapters in this thesis introduce the theoretical background to some of the relevant areas within the scope of this PhD project.
In chapter 2 the basic theory behind measurement of VOC's is outlined. This includes an introduction to extraction of VOC's by headspace sampling and to separation of the VOC's by gas-chromatography. Some theoretical aspects of mass spectrometry (MS) will moreover be outlined in relation to both GC-MS and PTR-MS.
In chapter 3 a definition of the term 'cooked and sliced meat products' is provided. This is done in order to identify the products for which the results of this PhD-project could be relevant. Moreover, the most important steps in the production of saveloy are described with focus on their impact on product quality and shelf-life.

Chapter 4 describes the microbial changes in cooked and sliced meat products during storage. Factors which have an impact on microbial growth and the rate and the extent of spoilage will be described along with basic concepts of microbial growth and metabolism. Furthermore, the characteristics of some of the most frequently isolated spoilage bacteria in cooked and sliced meat products will be described.

Chapter 5 contains a review of other studies dealing with the modeling of spoilage in cooked and sliced meat products. This includes studies modeling shelf-life based on microbial growth and studies which relate chemical spoilage markers to quality changes in cooked and sliced meat products.

Chapter 6 summarizes the results of the experimental work of this PhD-project and moreover includes some new aspects of these results. The strengths and weaknesses of the results are discussed in relation to their potential application in the meat industry.

The final conclusion of the PhD-project is given in chapter 7.

# 2. Measurement of volatile organic compounds

Measurement of the aroma composition with GC-MS has been the recurrent analysis through this project. The basic aspects of the extraction and measurement of VOC's with GC-MS will therefore be described in this chapter. PTR-MS is an interesting method for on-line or at-line measurements of VOC's, and PTR-MS is therefore a highly relevant method in relation to a potential industrial application of the results achieved in this project. A description of the principles of PTR-MS, which was tested in PAPER IV**,** is therefore also included.

## 2.1 Headspace extraction

The first step in the analysis of the aroma composition of a given food sample is the extraction of the VOC's from the sample. Headspace extraction, which can be either static or dynamic, is a common way to achieve this.

**Static headspace extraction**. In a typical static headspace extraction procedure the sample is placed in a closed glass container on a water bath at a constant temperature. If the temperature is sufficiently high this facilitates the release of VOC's from the sample into the headspace. The release of a specific VOC from the sample depends on its volatility and on how well it adheres to the sample matrix. This can be expressed by the partition coefficient ($K_{VOC}$) shown in Eq. 1. Here $VOC_{headspace}$ and $VOC_{matrix}$ are the concentrations of the specific VOC found in the headspace and in the sample matrix at equilibrium *(6)*.

Eq. 1
$$K_{VOC} = \frac{VOC_{Headspace}}{VOC_{matrix}}$$

In a static headspace extraction procedure the sample is kept on the water bath at constant temperature ideally until equilibrium is reached for all VOC's in the sample. A portion of the headspace is then withdrawn and injected on to the GC-column *(6, 7)*. Only a limited amount of headspace can be injected onto a GC-column. Static headspace is therefore only a suitable method if the partition coefficient of the VOC's of interest is sufficiently high. However, in many food samples a concentration step should be included in order to obtain an amount of VOC exceeding the detection limit in the subsequent GC-MS analysis *(6)*. Therefore dynamic headspace extraction is often the preferred headspace extraction technique.

**Dynamic headspace extraction**. In a dynamic headspace procedure the closed class container is equipped with a purge top as shown Figure 1. Through this purge top the sample is continuously purged with an inert gas which is typically nitrogen. The purge gas sweeps the VOC's from the sample to a trap which retains the VOC's from the sample. Due to the continuous purging equilibrium between the VOC's in the headspace and those retained in the sample matrix is not reached. Therefore VOC's are continuously released from the sample purged on to the trap. This

means that the VOC's released from the sample matrix are concentrated on the trap. The degree of concentration depends on the volume of purging gas used, plus the volatility and rate of the release of the specific VOC's from the sample *(6, 7)*. Typically around 5 volumes of headspace are purged through the system during the sampling *(8)*.
The basic dynamic headspace setup does not give the exact concentration of VOC's in the sample but measures the VOC's extracted at the specific purge and temperature conditions. However, if these conditions are carefully replicated dynamic headspace sampling is a good method for comparison of the aroma composition between samples. For exact quantification of the VOC's in the sample other methods should be considered. Multiple dynamic headspace sampling could be a possibly headspace method for aroma quantification. In this method the sample is continuously purged until depleted from VOC's while the trap is changed in regular intervals.

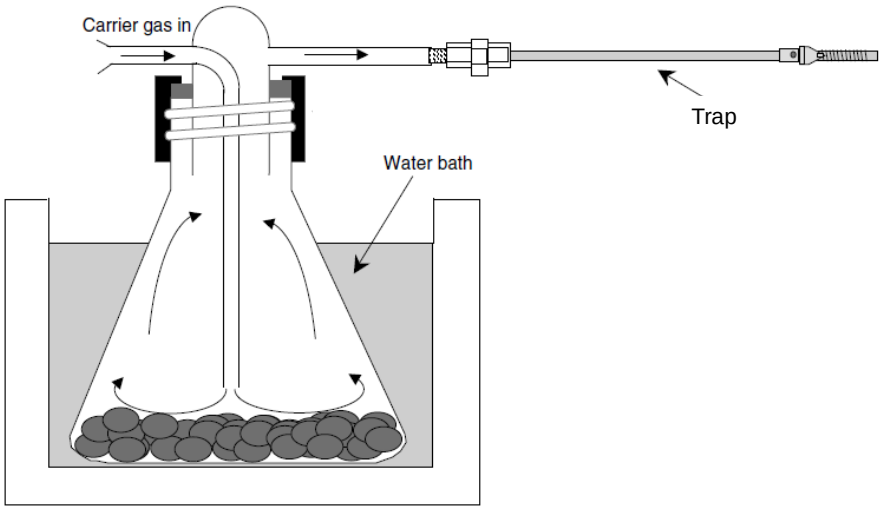


**Figure 1. An overview of aroma extraction using the dynamic head apace method adapted from Elmore, 2009 *(8)*.**

**Sorbent material**. The trap is basically a tube packed with a combination of sorbent materials designed to absorb VOC's and let the purge gas pass through. The sorbent material has a high surface area which means that VOC's passing through the trap are likely to collide and be adsorbed on to it *(6)*. In this PhD-project silcosteel coated traps were packed with a combination of Tenax TA (60-80 Mesh) and carbograph 1TD. Tenax is a polymer of 2,6-diphenylphenol and a general purpose sorbent material which adsorbs a wide range of volatiles fairly well. However, Tenax has relativity low affinity for polar compounds and very volatile hydrocarbons. It can therefore be an advantage to backup Tenax with a more retentive sorbent material *(6, 7)*. Carbograph 1TD retains very volatile molecules and is suitable for both ketones, alcohols, aldehydes and sulphur compounds *(9)*. It is therefore considered an appropriate backup material for Tenax TA in the traps.

**Breakthrough volume**. The adsorption of the VOC's to the sorbent material is not irreversible and the VOC's therefore travel through the trap as the sample is purged during the dynamic headspace sampling. The amount of a specific VOC adsorbed to the trapping material therefore is limited by the breakthrough volume. The breakthrough volume is the volume of purge gas needed for a specific VOC to reach the end of the trap. When the breakthrough volume is reached the amount of the specific VOC on the trap no longer increases with the purge volume. This makes it difficult to compare the amount of the specific VOC's between samples. The breakthrough volume depends the strength of the interaction between the specific VOC and the sorbent material, the amount of sorbent material, the volatility of the VOC and the temperature of the trap *(6)*. Therefore parameters such as temperature, purge gas flow and purge time must be adjusted to avoid extensive breakthrough of important VOC's and moreover to achieve the best possible sampling result.

**Thermal desorption**. Direct desorption from the trap onto the gas chromatographic (GC)-column would result broad peaks in the following GC-analyses. This is because the primary trap is too large for the VOC's adsorbed on the trap to be desorbed quickly. The trap is therefore introduced to the GC-column using a two-stage automated thermal desorption system. In the first stage the primary trap is purged with helium at high temperature (240 °C) which desorbs the VOC's from the sorbent material and onto a cold-trap (-30 °C) packed with Tenax TA. The low temperature slows down the transport of the VOC's through the cold-trap, which helps focusing the VOC's. In the second desorption step the cold trap is heated, and VOC's are desorbed onto the GC-column *(10)*. Because of the previous focusing of the VOC's in the cold trap this desorption proceeds quickly.

**Headspace extraction in this project**. In the dynamic headspace extractions made in this PhD-project 25 g of the sample was placed in a 500 mL glass container, conditioned for 10 min at 30 °C and then purged with a nitrogen flow of 65 mL/min for 15 min. This means that almost 2 volumes of headspace were used for the purging of the sample. However, VOC's were also released from the sample during the conditioning. The volume of purge gas chosen for the sampling is a trade-off between increased sensitivity and the risk of breakthrough of important VOC's. The use of a moderate volume of purge gas for the experimental work of this PhD-project indicates that avoiding extensive breakthrough is regarded important. The breakthrough volume is calculated per gram of sorbent material. The traps were packed with 73 mg Tenax TA (60-80 Mesh) and 100 mg carbograph 1TD. This equals a purge volume of 13.2 L/g Tenax TA and 9.75 L/g carbograph 1TD. The sensitivity of the described headspace sampling method was estimated to be between 5-20 ng VOC per 25 g of sample. This estimate was made based on GC-MS measurement of authentic compound standards of some of the most relevant VOC's found in the headspace of saveloy.

## 2.2 Gas chromatography

**Essential gas chromatography**. The purpose of GC is to separate the VOC's in the extracted sample headspace before they reach the mass spectrometer. The separation takes place in a capillary column coated with a stationary phase. The desorbed sample headspace is loaded on the capillary column where it is transported by an inert carrier gas. The carrier gas, also called the mobile phase, is normally helium or hydrogen. As the VOC's in the sample pass through the column they interact with the stationary phase. The strength of this interaction determines how long time a specific VOC is retained by the column. Differences in the strength of the interaction between specific VOC's and the stationary phase will thus separate the VOC's in the sample in time as they pass through the column *(11, 12)*. The type of stationary phase used should be determined based on the expected composition of VOC's in the sample. Generally polar stationary phases should be chosen for polar compounds *(12)*. The HP-INNOWax column (30 m x 0.25mm with 0.25µm film thickness, Agilent technologies), which has a highly polar stationary phase made from Polyethylene glycol, has been the preferred column in this PhD-project.
The GC-column is placed inside an oven which controls the temperature of the column during the chromatographic analysis. Increasing the column temperature will cause the VOC's to elute faster which evidently will shorten the total time of analysis. The column temperature is used actively to control the chromatographic analysis by running a temperature program. The column temperature is kept relatively low in the beginning of the analysis and gradually increased during the analysis *(11, 12)*. By keeping the temperature relatively low in the beginning of the analysis fast eluting VOC's will be retained in the column long enough to ensure an adequate separation. Furthermore, the high temperatures in the end of the analysis will 'speed up' the elution of the VOC's, which interact strongly with the stationary phase, in order to avoid excessive broadening of the peaks.

**Separation efficiency**. Ideal chromatography results in efficient separation of the VOC's. This implies chromatograms with intense and narrow peaks separated at base line *(12)*. However, as the VOC's travel through the column they are broadened as well as separated. For complex samples it is not always possible to achieve perfect separation of the VOC's but the separation efficiency can be optimized. The Van Deemter equation (Eq. 2) relates the height equivalent to a theoretical plate (HETP) with the linear velocity of the mobile phase (u). HETP is a measure of separation efficiency, or the extent of peak broadening, in the chromatographic analysis. The optimal separation efficiency is achieved at the minimal value of HETP.

Eq. 2 $$HETP = A + \frac{B}{u} + Cu$$

In Eq. 2 the parameters A, B and C are positive constants determined from the properties of the stationary phase and the mobile phase. The A-term describes eddy diffusion and depends on the variation in flow path inside the column. In capillary columns the A-term is very small and can be

neglected *(13)*. The B-term accounts for longitudinal diffusion of the analytes in the column. Diffusion in a gas phase is very fast and the B-term can therefore be important in GC-analyses *(14)*. As seen in the Van Deemter equation longitudinal diffusion (B) has a high impact on the separation efficiency at low velocities of the mobile phase. At higher velocities of the mobile phase the impact of longitudinal diffusion on the separation efficiency is decreased but the impact of the C-term increases. The C-term accounts for the resistance to mass transfer, which relates to the interaction of the VOC's and the stationary phase. If the velocity is increased the peaks are broadened because the resistance to mass transfer of the VOC's is constant. The C-term depends on the column material and on the choice of mobile phase *(11-13)*. As seen in Figure 2 the linear velocity of the mobile phase can be adjusted to reach a lowest possible HEPT according to the Van Deemter equation. However, in practical GC-analysis the linear flow rate can be set above optimum in order to decrease analysis time.

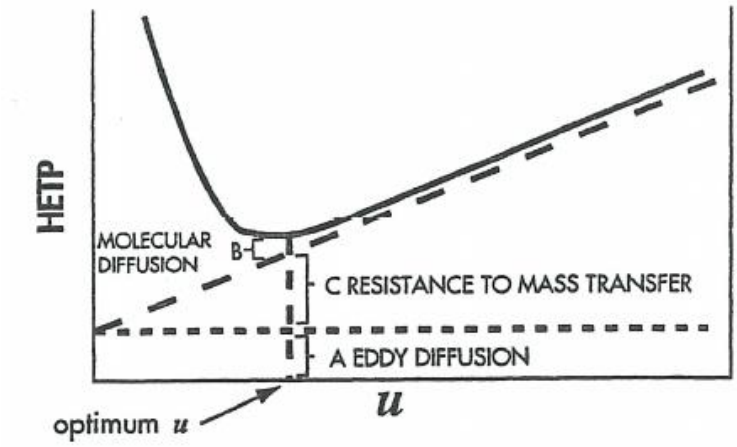


**Figure 2. The relation between the linear flow rate and the separation efficiency (HEPT) as described in the Van Deemter equation. Adapted from Reineccius, 1998 *(12)*.**

## 2.3 Mass spectrometry

The mass spectrometer provides ongoing analysis of the VOC's eluting from the GC. The purpose of MS is to detect, quantify and identify the VOC's in the sample. This is achieved through the three main steps in MS which are ionization, mass analysis and detection.

**The ionization method**. There are several ionization methods which can be applied in MS, however electron ionization (EI) is the most common for GC-MS instruments. Chemical ionization (CI), which is soft ionization method, will be described in relation to the PTR-MS instrument. The electrons, used for ionization of the VOC's, are generated by exposing a filament of rhenium or tungsten metal to an energy of 70 eV. This causes the filament to heat and emit electrons which travel through the ion chamber as they are attracted to a positive electrode *(15)*. EI mass spectra are almost always obtained at 70 eV, as this ensures high ionization efficiency and a desirable amount of fragmentation of the VOC's. From a practical viewpoint it furthermore ensures a common ground for comparison of mass spectra from different GC-MS instruments *(16)*. As the

VOC's from the sample enters the ion chamber and move into the proximity of the electron beam, energy transfers from the electrons to the VOC's. The excessive energy in the molecule causes the VOC to eject an electron and hereby generating a positively charged ion with the molecular mass of the VOC's. This ion is called the molecular ion or parent ion *(15-17)*. The loss of an electron causes the molecular ions of a specific VOC to destabilize and split into different mass fragments. The pattern of this fragmentation is very specific for the compound in question and can therefore be used as a 'fingerprint' of the VOC's in the following identification. The ionization takes place at very low pressure in order to keep the generated ions from reacting with components of atmospheric air. The principles of EI are illustrated in Figure 3.

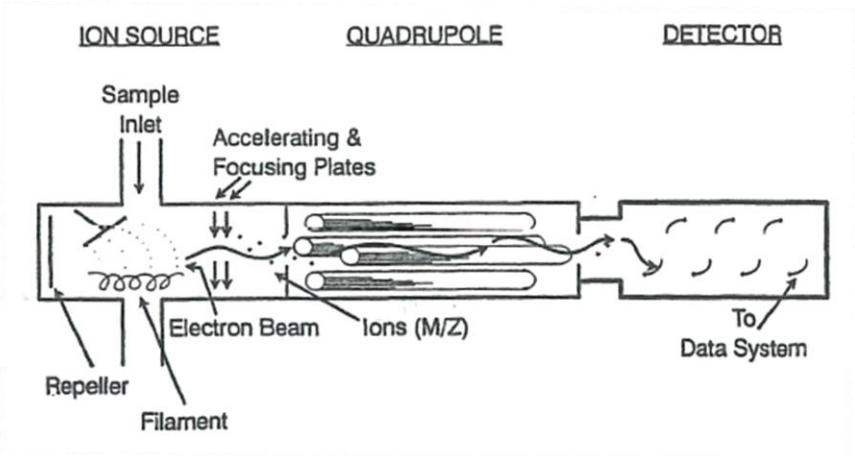


**Figure 3. A schematic overview of the mass spectrometer. Adapted from Smith & Thakur, 1998 *(15)*.**

**The mass analyzer**. As for the ionization methods there are several types of mass analyzers available for MS-applications. However, the quadrupole mass analyzer, which was also used for the GC-MS work presented in this PhD-project, is the most common *(17)*. However, ion trap mass analyzers and 'time of flight' (TOF) mass analyzers are also frequently used. The purpose of the mass analyzer is to filter the fragments generated by the ionization according to their mass to charge (m/z) ratio. In some cases the molecules eject more than one electron during the ionization which means that the fragments will have a higher charge. However, the charge of the ionized fragments is commonly +1 when using EI. This means that the mass analyzer in most cases filters the fragments according to their mass *(15, 17)*. As illustrated in Figure 3 the quadrupole mass analyzer consists of four rods that are used to generate an electric potential which allows mass fragments with a specific m/z to travel through the quadrupole and enter the detector. The rods are pair wise applied an either positive or negative electric potential of equal strength which oscillates between the two pairs of rods. As the positive ion fragment enters the quadrupole it is attracted to the rods with negative potential. However, as this rod changes potential the fragment changes direction, and moves towards the rods which now have a negative potential. For ion fragments, which have mass to charge ratios matching the oscillating electric potential, this movement is stabilized and the fragments are transmitted through the quadrupole. The remaining ion fragments are deflected of the rods. However, the electric potential of the quadrupole is

continuously changed allowing other ion fragments to be transmitted *(15, 17)*. When the complete mass range has been scanned a mass spectrum has been acquired. Quadrupole mass analyzers are commonly able to scan a mass range from between m/z 1 and m/z 10 to between m/z 650 and m/z 1250 *(17)* with a resolution of around 0.3 m/z. The time it takes to acquire a mass spectrum depends on the mass range scanned and the time the quadrupole dwells on each m/z. The quadrupole records mass spectra at a typical frequency of 1-10 spectra per second during the entire GC-run.

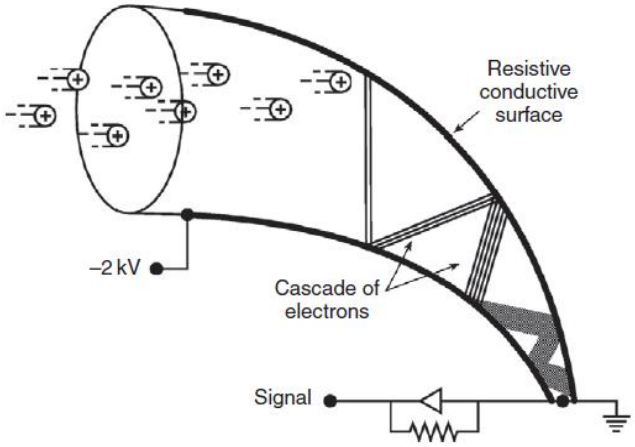


**Figure 4. Schematic overview of an electron multiplier detector. Adapted from Sparkman et al., 2011 *(17)*.**

**The detector**. The detector used in the GC-MS instrument is most often an electron multiplier, which amplifies the signal of the incoming ions. When the ions transmitted through the quadrupole hit the detector they release a number electrons corresponding to their kinetic energy. The higher kinetic energy of ion fragments the more electrons they release. As illustrated in Figure 4 the released electrons multiply as they hit the detector surface again. This results in a cascade of electrons which are detected as an electric signal proportional to the amount of ions that reach the end of the detector *(15)*.

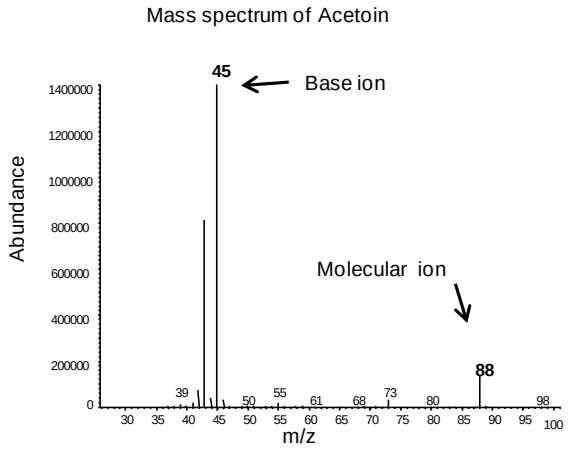


**Figure 5. The mass spectrum of acetoin obtained from a sample of sliced saveloy. The base ion (m/z 45) and the molecular ion (m/z 88) are highlighted.**

**GC-MS data**. The basic output of the GC-MS analysis is the mass spectrum. As illustrated in Figure 5 the mass spectrum has the m/z-ratio on the x-axis and the relative abundance of the ion fragments on the y-axis. The mass spectrum of a given VOC corresponds to the fragments formed during the EI. The masses of the fragments formed and their abundance relative to each other forms a fingerprint characteristic to the VOC in question. As mentioned this fingerprint can be used for identification purposes. The molecular ion and the base ion are two important fragments for identification of a given compound. As illustrated in mass spectrum of acetoin in Figure 5 the base ion is the most abundant ion fragment in the chromatogram whereas the molecular ion has a mass which equals the molecular weight of the compound. In the case of acetoin the base ion and molecular ion have m/z 46 and m/z 88 respectively. Trained mass spectrometrists are able to recognize VOC's by looking at their mass spectrum. However, the NIST mass spectral database *(18)* provides mass spectra of all common chemical compounds, which can be accessed and compared to a specific mass spectrum directly via GC-MS computer software.

The quadrupole scans the selected mass range and continuous to record mass spectra throughout the GC-MS run. The abundance of all ion fragments in a given mass spectrum can be summarized to produce the total ion current (TIC). If the TIC is plotted for each mass spectrum in the order by which they are obtained during the analysis they form the TIC-chromatogram. The TIC-chromatogram therefore contains a three dimensional data structure where each data point has a corresponding mass spectrum. Figure 6 shows the TIC-chromatogram of a sample obtained from the headspace of sliced saveloy where acetoin was found as the peak with a retention time of approximately 10.0 min. The mass spectrum of acetoin, indicated in the figure, was obtained by averaging all the mass spectra contributing to the peak and then subtracting the mass spectrum of the base line (background noise). This data structure furthermore enables detection of co-eluting peaks by analysis of the mass spectra across each chromatographic peak. Co-eluting peaks can moreover be quantified separately based on single target ions if it is possible to find ions which do not overlap between the mass spectra of the co-eluting compounds.

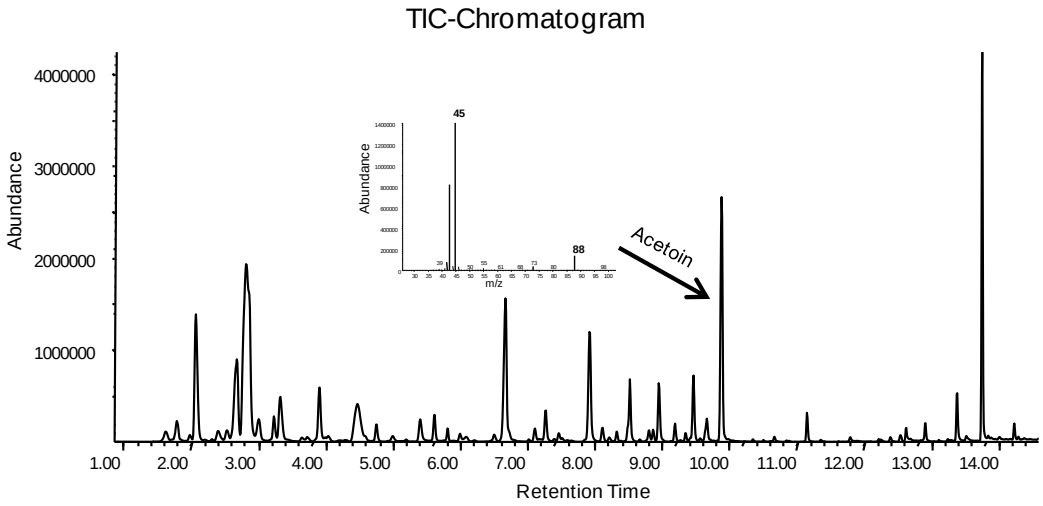


**Figure 6. Shows part of a TIC-chromatogram from the GC-MS measurement of the headspace of a sliced saveloy sample. The retention time is given on the x-axis whereas the summarized abundance of the recorded mass fragments is given on the Y-axis. The averaged mass spectrum of the acetoin peak is moreover shown.**

## 2.4 Proton transfer reaction-mass spectrometry

PTR-MS is an alternative method for measurement of VOC's which provide fast, continuous and direct measurements of a given sample headspace. The result of a PTR-MS measurement can moreover be obtained almost instantaneously. PTR-MS is furthermore a highly sensitive method which allows detection of VOC's in pptv levels in air *(19)*. In PTR-MS measurement the time-consuming GC-analysis is eliminated. The sample preparation and extraction of the VOC's from the sample can also be significantly reduced or even eliminated. The PTR-MS instrument is furthermore robust and relatively easy to operate, which makes it even more suited for on-line measurements in a processing environment.

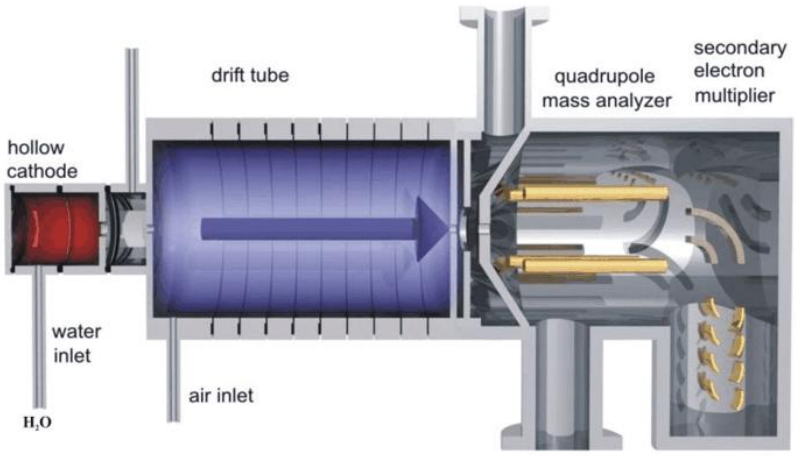


**Figure 7: A schematic overview of the PTR-MS instrument adapted from Ionicon Analytik *(20)*. The proton transfer reaction takes place in the drift tube where the VOC's from the sample are mixed the protonated water.**

**The proton transfer reaction**. In the PTR-MS instrument, illustrated in Figure 7, the VOC's from the sample headspace are ionized chemically by the use protonated water ($H_3O^+$) produced from vaporized water in the hollow cathode. The protonated water is let into the drift tube where it is mixed with the sample headspace containing the VOC's from the sample. In the drift tube the VOC's collide with the protonated water which facilitates the 'proton transfer reaction' shown in Eq. 3 *(19, 21)*.

$$\text{Eq.3} \qquad H_3O^+ + VOC \rightarrow VOC\text{-}H^+ + H_2O$$

The $H_3O^+$-ions are present in much higher density than the VOC's. This increases the chance of a collision between the $H_3O^+$-ion and the VOC's as the molecules travel through the drift tube. The $H_3O^+$-ion can only transfer a proton to molecules with a higher proton affinity than water. The constituents of atmospheric air all have lower proton affinity than water and therefore they do not interfere with the PTR-MS measurements. However, practically all VOC's have higher proton affinity than the water and can therefore be ionized by proton transfer reaction *(19, 21)*.

**The degree of fragmentation**. The product of the proton transfer reaction is the protonated molecular ion (M+1-ion) of the specific VOC. Compared to the molecular ion resulting from EI the M+1-ion is relatively stable. The degree of fragmentation resulting from CI is therefore much lower than what is observed when applying EI. This means the M+1-ion is often the main contributor to the mass spectrum of a specific VOC. However, as previously mentioned the VOC's are not separated before reaching the mass analyzer of the PTR-MS instrument. The PTR-MS instrument therefore records mass spectra containing the all the ionized VOC's present in the sample headspace at any given time. It is therefore a necessity to use the softer CI in the PTR-MS instrument in order to keep the data interpretation relatively simple. However, particularly for alcohols, aldehydes and esters the degree of fragmentation can be a challenge for the interpretation of the PTR-MS data *(22, 23)*. The degree of fragmentation can partly be controlled by changing the reaction conditions in the drift tube. This can be done by manipulating the E/N-ratio, where E is the strength of the electric field in the drift tube and N is the buffer gas density. A high E/N-ratio means increased collision energy and increased fragmentation of the M+1 ions. The degree of fragmentation is lowered by decreasing the E/N-ratio. However, by lowering the E/N-ratio the formation of water clusters ($H_3O^+ \cdot (H_2O)n$, $n \geq 1$) is increased, which can cause problems for the progress of proton transfer reaction *(22)*.

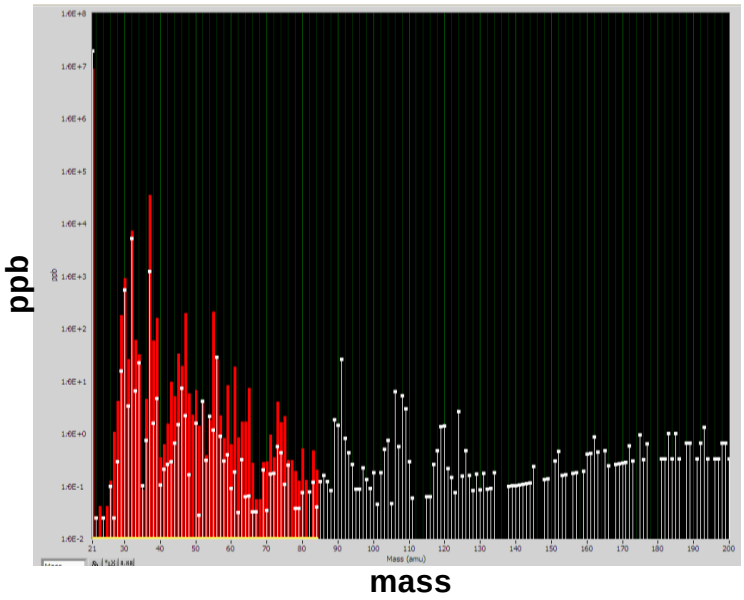


**Figure 8. Illustration of the data acquisition by the PTR-MS instrument. The red bars correspond to the ongoing measurement whereas the white bars represent the previous measurement.**

**PTR-MS advantages and disadvantages**. The PTR-MS continuously draws in sample headspace, scans the selected mass range and records the resulting mass spectra. These mass spectra are moreover obtained almost instantaneously. It is therefore also possible to monitor changes in the concentration of specific VOC's as they occur. The data acquisition by the PTR-MS instrument is illustrated in Figure 8. Here the mass spectrum currently being recorded is showed in red bars whereas the previous mass spectrum is shown in white bars. The high sensitivity of the PTR-MS furthermore enables measurement of VOC's without a previous concentration step *(21)*. PTR-MS is moreover a robust instrument which is relatively easy transported as it does not depend on any gas supply. These abilities make PTR-MS a potential method for on-line measurement of VOC's in many situations. PTR-TOF-MS has for example been used for on-line monitoring of the production of VOC's, including diacetyl and acetoin, during the fermentation of milk *(24)*. In another study by Mayr et al. *(25)* PTR-MS was furthermore used to monitor the production of VOC's during spoilage of vacuum packed fresh meat.

As mentioned, the elimination of the separation step in the PTR-MS technology raises some challenges regarding handling of the data. Even though the fragmentation is limited when using CI it provides some difficulties in the assigning of 'PTR-MS masses' to specific VOC's. The possibility that several VOC's can contribute to the same mass is considered relatively high in a complex samples matrix such as sliced saveloy. This could potentially interfere with measurements of the changes in the concentration of specific VOC's with time. Despite of these potential problems the many advantages of PTR-MS makes it a very interesting method for scientific as well as industrial on-line/at-line measurement of VOC's.

# 3. Cooked and sliced meat products

This chapter gives an introduction to the types of meat products that are included in the scope of this project. The vast majority of the experimental work performed as part of this PhD-project was done on samples of sliced saveloy. However, the results of the project are relevant for cooked and sliced meat products in general. The chapter includes a description of the diversity and production of cooked and sliced meat products along with a description of the aroma composition of the fresh products.

## 3.1 Definition and diversity of cooked and sliced meat products

In this thesis the term 'cooked and sliced meat products' refers to a broad range of industrially processed meat products. The 'cooking process' generally refers to steam cooking or cooking/boiling in a water bath *(26)*. Besides cooking and slicing the processing often involves curing, seasoning, smoking and in some cases pre-roasting. Cooked and sliced meat products include both whole-muscle products, products made from re-formed muscle and meat emulsion products. Pork ham and chicken breast could be examples of whole-muscle products. However, they are also commonly made from re-formed muscle. Examples of products made from a meat emulsion could be different types of meatloaf or different sausages, including saveloy. Terms like cold cuts, luncheon meats and cured meats covers a product range similar to that of 'cooked and sliced meat products'. However, 'cooked and sliced meat products' refers specifically to the two processes which these products have in common from a manufacturers perspective. It is due to the cooking and subsequent slicing that the quality changes observed during storage of these products are similar, and it is these quality changes which are investigated in the present PhD-project.

The diversity of cooked and sliced meat products available on the market is huge and covers products of varying animal origin, flavor composition and product quality. The quality of meat products can be measured in many ways. However, the meat content and type of meat used for production is considered an important quality measure. Re-formed meat products are a good example of the difference in product qualities encountered in practice. High quality re-formed meat products are made from large pieces of lean meat whereas economy quality products are made from meat trimmings, skin emulsion and mechanically deboned meat. Product extension by the addition of increased levels of brine can also be used in order to reduce production costs *(26)*.

In Denmark cooked and sliced meat products are commonly consumed on sliced rye bread or in sandwiches and are found in lunch boxes across the country. The Danish tradition of 'smørrebrød' involves carefully decorated open rye bread sandwiches with plenty of sliced meat, fish or cheese. Due to this tradition the cooked and sliced meat products are furthermore considered an important part of Danish food culture *(27)*. The quality changes of these products during storage are therefore relevant for many Danish consumers.

## 3.2 Production of cooked and sliced meat products

In this section the production of cooked and sliced meat products will be described using saveloy as an example. Whole muscle products and re-formed meat products commonly include a brine injection or tumbling process instead of the cutting and mixing. However, these processes will not be described here.

**Ingredients**. The list of ingredient for production of the saveloy, used for the experimental work of PAPER I-III, is shown in Table 1. Saveloy is a gently seasoned sausage made from a homogeneous mass of pork meat, pork fat, water, potato starch, soy-isolate, spices and additives. The saveloy is seasoned with a mixture of nutmeg, coriander and black pepper. However, the mixture of spices used in cooked and sliced meat products varies greatly depending on product type and tradition. Besides the spices several classes of additives are commonly used in cooked and sliced meat products including: preservatives, stabilizers, antioxidants, flavor enhancers, fillers and thickeners *(28)*. The additives included in the saveloy recipe are among those most commonly found in meat products. Salts have several functions in meat products besides enhancing the flavor. They generally have antimicrobial effect as they lower the water activity. Salts furthermore increase the ionic strength and hereby increase the solubility and the water binding ability of the proteins *(29)*. Nitrite salts are added for their antimicrobial effect but they are also responsible for the stable pink color of most processed meat products. Ascorbate has antioxidative effect but is commonly added to meat products because it accelerates the formation of the pink color caused by nitrite and furthermore stabilizes this color *(28, 30)*. Phosphates, preferably in combination with salts, increase the solubility of the proteins and increase their water holding capacity. This has a positive effect on the stability of the sausage mass and the production yield *(30)*. Starch and soy-isolate both contribute to the desired texture of cooked meat products and furthermore have high water holding capacity. However, they also added as fillers due to their low cost compared to meat protein.

**Table 1: The list of ingredients in used in the production of saveloy for the experimental work of this PhD-project.**

| Ingredient | Content in % | Content in Kg |
|---|---|---|
| Shank and belly meat | 40.0 | 6.0 |
| Pork trimmings | 20.0 | 3.0 |
| Pork fat | 5.0 | 0.75 |
| Water | 26.4 | 3.96 |
| Vacuum salt | 0.7 | 0.10 |
| Nitrite salt | 1.0 | 0.15 |
| Soy-isolate | 2.0 | 0.30 |
| Potato starch | 4.0 | 0.60 |
| Sodium ascorbate | 0.03 | 0.0045 |
| Phosphate | 0.3 | 0.045 |
| Saveloy seasoning mix | 0.6 | 0.090 |
| **Total** | **121.0** | **4780** |

**Cutting and mixing**. The mixture of ingredients for saveloy production is thoroughly cut and mixed in a bowl cutter as shown in Figure 9. The cutting process is optimized to obtain the desired particle size and achieve a sufficient mixing of the ingredients. Together with phosphates and salt this will ensure solubilization of the proteins and generate a strong emulsification of the fat. The solubilized protein immobilizes the added water and emulsifies the fat particles. The protein creates a protective layer around the fat particles in the emulsion, which is preserved and stabilized during the cooking process. In an 'undercut' sausage mass there is a risk that the protein fraction is inadequately solubilized which prevents the protein layer from being formed. In an 'overcut' sausage mass the particle size is too small and their surface area too large for the solubilized protein to create an adequate layer around the fat particles. The cutting process is therefore important in order to ensure optimal emulsion properties of the sausage mass and a high quality of the final product *(30)*.

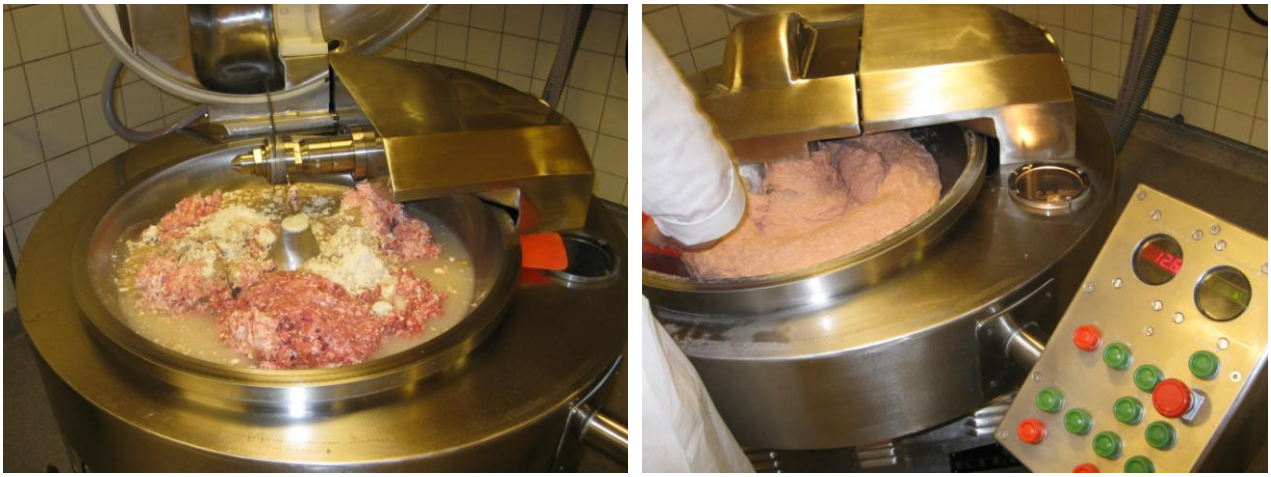

**Figure 9: The ingredients for saveloy production in the bowl cutter before and after cutting and mixing. Photos by V. Resconi.**

**Filling and cooking**. The finished saveloy mass was filled in sterile plastic casings with a diameter of 63 mm and then steam cooked at 80 °C for 50 min, reaching a core temperature of 75 °C. The product was then sprinkled with cold water for 10 min and subsequently cooled to 2 °C. The cooking process induces major changes in the product including denaturation of the proteins and inactivation of enzymes. However, from a shelf-life perspective the main purpose of this process is to destroy the microorganisms in the product *(31)*. A successful cooking process should eliminate the majority of the spoilage bacteria found in the sausage mass. However, sporulating bacteria can survive the cooking process and can be found in the freshly cooked product. These bacteria spores could derive from the spices where they are commonly found *(32)*. However, under the normal processing conditions sporulating bacteria do not play an important role in spoilage of cooked and sliced meat products due to the preservation of the product and the competition from the remaining spoilage flora. The cooking process furthermore generates the desired sensory properties of the product *(31)*. The cooking process also contributes greatly to the formation of the desired flavor, odor, texture and color of freshly cooked meat products.

**Slicing, packing and re-contamination**. Recontamination of the product after the cooking process is a major cause of spoilage in cooked and sliced meat products *(2)*. During slicing and packing the product is subjected to the in-house microbial flora. This flora consists of bacteria present in the air of the processing facility, on the skin of the workers and on the processing equipment *(5, 33-35)*. In cases of insufficient cleaning, the microbial flora can establish biofilm on the processing equipment from where it can contaminate the product *(36)*. However, accumulation of organic material on the slicing equipment during production also poses a risk of contamination, because this serves as a good substrate for various microorganisms *(35)*. In the slicing process the same knife is in direct contact with the surface of each slice of meat product, and this process is therefore of particularly interest in relation to re-contamination. The handling of the product during the removal of the casings prior to slicing also poses a risk of microbial contamination. Thorough cleaning of the processing equipment and personal hygiene are therefore of great importance for a successful slicing and packing of cooked meat products. Prior to slicing, the product is commonly crust frozen which hardens the surface and makes it possible to cut the product in thin slices. Cooked and sliced meat products are normally packed in modified atmosphere (MA) packing or in vacuum. This inhibits the fast growing aerobic spoilage flora and selects for facultative anaerobic and anaerobic bacteria. The saveloy used in the experimental work of this PhD-project was packed in MA-packing containing of 70% $N_2$ and 30% $CO_2$. The presence of $CO_2$ in the packing gas reduces the growth rate of most bacteria, and therefore increases the shelf-life of the product. Furthermore, the microbial metabolites produced in meat products stored in MA- or vacuum- packing are generally less offensive compared to the metabolites produced in the presence of oxygen *(2)*. The formation of microbial metabolites during storage will be described in more detail in chapter 4. The expiry date of Danish cooked and sliced meat products stored in MA-packing at 5 °C is normally set to 3-5 weeks from the day of slicing and packing. The manufacturers moreover generally recommend that the product is stored for a maximum of 2-4 days after package opening.

## 3.3 The aroma composition of cooked and sliced meat products

The composition of VOC's of a given product is closely related to consumer acceptability and eating quality in general *(2, 37)*. The VOC's formed during the cooking process combined with VOC's from the spices added to the product make up the desirable odor of the fresh product. However, during storage the odor of the fresh product changes, which will contribute to the loss of eating quality experienced by the consumers.

The cooking process takes place at temperatures around 80 °C and at high relative humidity. Due to these processing conditions the Maillard reaction only plays a minor role in the formation of VOC's in steam cooked meat products *(38)*. Thermally induced oxidation of lipids during cooking is therefore considered the most important source of VOC's in freshly cooked and sliced meat products. The reaction pathways, which lead to formation of VOC's from unsaturated fatty acids,

are generally the same for thermal oxidation and the oxidation processes leading to rancidity during storage. However, slight differences between the two pathways lead to the different aroma profiles of freshly cooked meat and rancid meat *(39)*. Moreover, the thermal oxidation of lipids also includes the saturated fatty acids which further contribute to the aroma profile of freshly cooked meat products *(40)*. The primary products of lipid oxidation are hydroperoxides which are further degraded to yield VOC's *(41)*. Aldehydes, ketones, alcohols are the most important groups of VOC's deriving from lipid oxidation. In general saturated and unsaturated aldehydes, with 6-10 carbon atoms, have been reported as the major lipid derived volatile compounds of cooked meats. Hexanal is moreover frequently mentioned as a good indicator of lipid oxidation in meat products *(42, 43)*. This corresponds well with the observations made in the experimental work of this project. Table 2 gives a list of the VOC's which were found in highest amounts the in fresh saveloy samples from PAPER III (the uninoculated control samples measured on day 1). From this table it is seen that several lipid oxidation products including aldehydes, ketones and alcohols were isolated from fresh saveloy. 2-pentylfuran, which can be formed by oxidation of linoleic acid, was also found in the fresh saveloy samples *(44)*. Besides the lipid oxidation products the fresh saveloy samples also contained dimethyl disulphide and dimethyl trisulfide. These compounds can be formed from degradation of sulfur containing amino acids in reaction pathways involving the Maillard reaction and Strecker degradation *(45)*. Terpenes were also an important part of the aroma of fresh saveloy. These compounds derive from the spices added to the product and generally have pleasant odors *(45)*. Based on the peak area, these terpenes were the most abundant compound class in the fresh saveloy samples. However, during storage the peak areas of the terpenes generally decreased. In the small sensory assessment of industrially produced saveloy samples in PAPER IV a decrease in spicy odor was observed by several of the assessors during storage. This corresponded well with the decrease in the peak area of several terpenes in the GC-MS measurements.

**Table 2. A list of VOC's which potentially contributes to the fresh odor of the uninoculated saveloy samples in Paper III. The odor description is obtained from Acree & Arn 2004 *(46)*, whereas the aroma threshold values are obtained from van Gemert 2003 *(47)*.**

| Compound | Peak area day 1 /100 | Odor | Threshold (recognition) in air (mg/m$^3$) | Origin |
|---|---|---|---|---|
| **Aldehydes:** | | | | |
| Ethanal | 1813 | Pungent, ether | 0.027-10 | Lipid oxidation |
| Pentanal | 417 | Almond, malt pungent | 0.013-0.22 | Lipid oxidation |
| Hexanal | 1168 | Grass, tallow, fat | 0.02-0.16 | Lipid oxidation |
| Heptanal | 30 | Fat, citrus, rancid | 0.04-0.15 | Lipid oxidation |
| Octanal | 12 | Fat, soap, lemon, green | 0.005-0.01 | Lipid oxidation |
| Nonanal | 99 | Fat, citrus, green | 0.02-0.06 | Lipid oxidation |
| 2 and 3-methylbutanal[a] | 158 | Malt, cocoa, almond | 0.002-0.004 | Maillard+Strecker/microbial |
| **Ketones:** | | | | |
| Acetone | 9414 | - | 1.66 | Lipid oxidation |
| 2-Butanone | 971 | Ether | 16-29 | Lipid oxidation |
| 2-Heptanone | 313 | Soap | 0.045-1.3[b] | Lipid oxidation |
| **Alcohols:** | | | | |
| Ethanol/ isopropyl alcohol | 269648 | | 11-20 | Lipid oxidation/microbial |
| 1-Pentanol | 225 | Balsamic | 1-30 | Lipid oxidation |
| 1-Hexanol | 77 | Resin, flower, green | 0.1-0.38 | Lipid oxidation |
| 2-Heptanol | 24 | Mushroom | 0.1 | Lipid oxidation |
| 1-Octen-3-ol | 79 | Mushroom | 0.012-0.11 | Lipid oxidation |
| 2- and 3-methylbutanol[a] | 26 | Whisky, malt, burnt, wine | 0.26 | Maillard+Strecker/microbial |
| **Furans:** | | | | |
| 2-pentylfuran | 277 | Green bean, butter | 0.27[b] | Lipid oxidation |
| **Sulfides:** | | | | |
| Dimethyl disulfide[a] | 28 | Cabbage, sulfur, gasoline | 0.011-0.017 | Maillard+Strecker/microbial |
| Dimethyl trisulfide[a] | 31 | Cabbage, sulfur fish | 0.0075-0.014 | Maillard+Strecker/microbial |
| **Terpenes:** | | | | |
| α-pinene | 1007 | Pine, turpentine | 25.0-29.0 | Spice mixture |
| α -thujene | 342 | Wood, green, herb | - | Spice mixture |
| Camphene | 47 | Camphor | 26-30 | Spice mixture |
| β-pinene | 849 | Pine, resin, turpentine | 35-38 | Spice mixture |
| Sabinene | 642 | Pepper, turpentine, wood | - | Spice mixture |
| 3-carene | 1020 | Lemon, resin | 4[b] | Spice mixture |
| α-phellandrene | 393 | Dill | - | Spice mixture |
| β-Myrcene | 131 | Balsamic, must, spice | - | Spice mixture |
| α-terpinene | 307 | Lemon | - | Spice mixture |
| Limonene | 519 | Lemon, orange | - | Spice mixture |
| Eucalyptol | 414 | Mint, sweet | - | Spice mixture |
| γ-terpinene | 474 | Gasoline, turpentine | - | Spice mixture |
| p-cymene | 745 | Solvent, gasoline, citrus | - | Spice mixture |
| δ-terpinene | 102 | Pine, plastic | - | Spice mixture |
| α-p-dimethylstyrene | 33 | Citrus, pine | - | Spice mixture |
| Camphor | 57 | Camphor | 3.35 | Spice mixture |
| Linalool | 524 | Flavor, lavender | 0.0033 | Spice mixture |
| Caryophyllene | 129 | Wood, spice | 11-13 | Spice mixture |
| 1-terpinen-4-ol | 481 | Turpentine, nutmeg, must | - | Spice mixture |

**[a] These compounds can be formed in microbial metabolism as well as in Maillard reactions followed by Strecker degradation *(45, 48)*. [b] For these compounds the threshold value of detection is given instead of the recognition threshold value.**

# 4. Microbial changes in cooked and sliced meat products

Both chemical and microbial changes affect the quality of a given meat product during storage. However, several studies have shown that it is primarily the microbial changes which determine the shelf-life of cooked and sliced meat products *(2, 3, 49-51)*. This chapter describes the microbial changes that occur in the cooked and sliced meat products during storage with focus on the relevant spoilage bacteria of meat products and their production of VOC's.

## 4.1 Microbial growth and metabolism

**Stages of Microbial growth.** During slicing and packing cooked meat products are recontaminated with the microbial flora of the workers, the processing equipment and the surrounding air. The initial microbial count of cooked and sliced meat products is typically between $10^2$-$10^4$ colony forming units (CFU)/g depending on the hygienic conditions at the slicing facility *(2, 51-53)*. During the shelf-life period of the cooked and sliced meat products, which in Denmark is typically 3-5 weeks, the total microbial count will increase to $10^7$-$10^9$ CFU/g depending on the product composition and storage conditions. Figure 10 illustrates the stages of microbial growth using growth of *E. coli* at 37 °C in nutrient broth as an example.

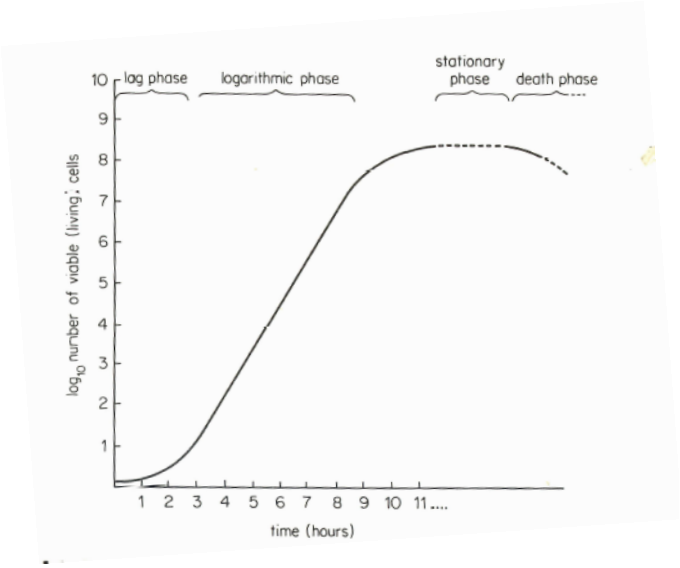


**Figure 10. An overview of the growth phases of microorganisms during spoilage exemplified by *E. coli* in a nutrient broth at 37 °C. In cooked and sliced meat products stored at refrigerated temperatures the time needed to reach maximum microbial counts would be measured in weeks rather than in hours. *E. coli* would moreover not cause spoilage in foods at refrigerated temperatures. Figure adapted from Singleton 1992 *(54)*.**

After bacteria are inoculated in the broth, or after changing environment from the processing equipment to the surface of a meat product, they need time to adapt to the new surroundings. This period of time is called the lag phase. During the lag phase growth and cell division is very slow, or even paused, because the cells need to synthesize the enzymes needed for exploitation of the nutrients in the new growth media. The length of the lag phase depends on the conditions from which the cells came. Cells coming from sparse conditions or cells previously depending on

different nutrients need more time to adapt, which will increase the lag phase *(54)*. However, cells from a culture in exponential growth normally need less time to adapt than cells from the stationary- or death phase *(55)*. After adapting to the new environment the cells starts to divide at increasing rates and the microbial population enters the logarithmic or exponential growth phase. In this phase the population grows at the maximum rate under the given environmental conditions. The growth rate during the exponential phase moreover varies between different bacteria. The population doubling time occurs at a constant rate as the exponential phase while the nutrients are used and the microbial waste products, including VOC's, accumulate. As the primary nutrients starts to deplete the microbial growth rate decreases and the population enters the stationary growth phase *(54, 55)*. In the stationary phase there is no longer a net increase in the population. The onset of the stationary phase can also be caused by microbial waste products which can have an inhibitory effect on growth. Production of organic acids could for example lead to a pH drop which could inhibit further growth of certain bacteria. The stationary phase leads to the death phase in which the population starts to decrease *(54, 55)*. The above description of the growth phases is a simplification of what is observed in cooked and sliced meat products. Here the spoilage flora consists of several species of bacteria that co-exists and compete for the nutrients. The ability of the individual species of bacteria to adapt to the given microenvironment will moreover enable them to grow faster and dominate the spoilage flora of the product.

**Basic microbial metabolism**. In order to grow, bacteria need energy and building blocks for production of biological important compounds. The chemical reactions used to obtain this are referred to as metabolism. Metabolism can be divided into two main categories. Catabolism is the degradation of organic compounds in order to release energy whereas anabolism is the building of biological significant compounds. Bacteria can obtain energy by catabolism of for example sugars, amino acids and fatty acids. However, most of the potential spoilage bacteria in meat products have sugars, commonly glucose, as their preferred substrate for growth *(37)*. The catabolic reactions can generally be split in respiration and fermentation. The aim of both types of reaction pathways is to generate adenosine 5´-triphosphate (ATP) which is the energy currency used by the living cell *(56)*. In fermentation processes the substrate is degraded without the presence of an external oxidizing agent and furthermore typically under anaerobic conditions. Glycolysis is commonly the initial reaction pathway in fermentation of sugars. In the glycolysis one molecule of glucose is converted to two molecules of pyruvic acid producing two ATP as illustrated in Figure 11. The subsequent degradation of pyruvic acid can follow several pathways and yield various end-products depending on the specific bacteria *(56, 57)*. In the respiratory pathways energy is released by oxidative degradation of the substrate, typically by the use of oxygen from the surrounding atmosphere as external electron acceptor. The use of oxygen means that the respiration processes typically yield more energy, in the form of ATP, compared to the fermentation of the same compounds. In a complete respiration glucose can be converted completely to water and carbon dioxide. However, in bacteria the respiration is often incomplete and yield other end products *(58)*.

The VOC's that eventually lead to spoilage of cooked and sliced meat products are end-products or intermediates of the different fermentation and respiration pathways used by the spoilage bacteria to yield energy. The diversity of the VOC's produced depends on the enzyme systems available in the bacteria responsible for spoilage. These enzyme systems enable bacteria to use different metabolic pathways and to switch between the different nutrients available on the product surface. As will be shown later the main VOC's associated with spoilage of meat products derive from metabolism of glucose and free amino acids.

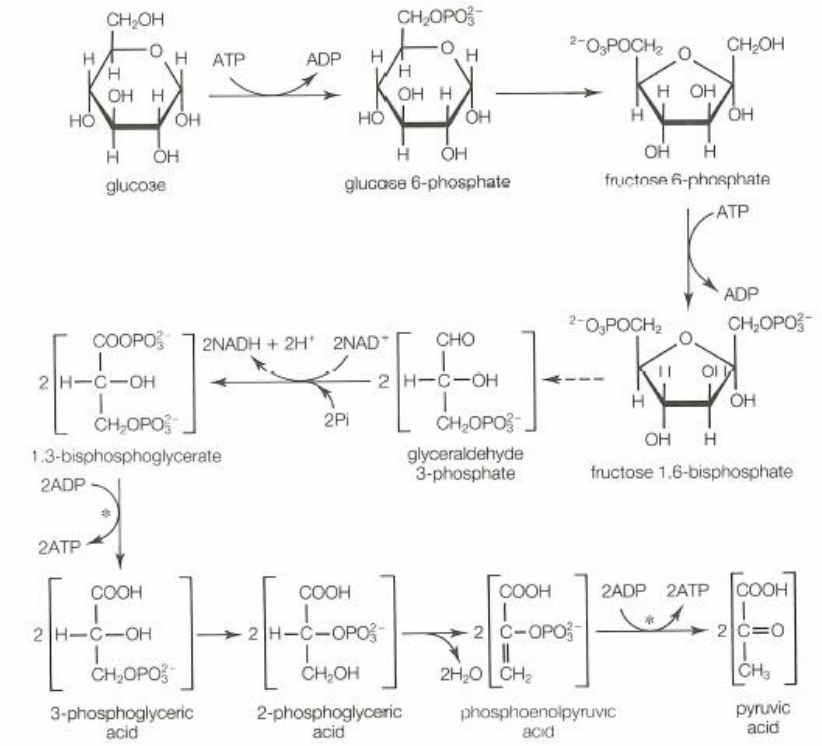


**Figure 11. Schematic overview of the glycolysis or which is the initial pathway of sugar fermentation used by LAB and other meat spoilage organisms. 1 glucose molecule is converted to 2 molecules of pyruvic acid yielding 2 ATP. Pyruvic acid is further metabolized to give various aroma compounds. The figure is adapted from Singleton 1992 *(56)*.**

## 4.2 Factors affecting microbial growth

The growth rate of bacteria depends on their tolerance towards several intrinsic and extrinsic factors *(49, 59)*. The intrinsic factors include the pH, the water activity, the level of preservatives and the availability of nutrients in the product surface. The extrinsic factors include storage temperature, packing atmosphere and the permeability of the packing material. It is the combination of these factors which select the recontaminating flora of a given meat product. The manufacturers of meat products moreover used their knowledge of these factors actively to limit microbial growth or to inhibit growth of specific bacteria *(2, 49, 59)*. The importance of some of these factors is discussed below.

**Temperature**. The temperature is the most important factor in relation to spoilage of cooked and sliced meat products *(49, 59)*. The rate of the metabolic reactions and the general growth rate of bacteria increase with increasing temperature. However, if the temperature increases above the level of optimal growth it starts to have negative impact on the bacterial cells. At high temperature the enzymes and proteins can denature irreversibly. Bacteria can be divided into 3 main categories according to their temperature preference. These are the psychrophiles (low temperature of optimal growth, 12-15 °C), the mesophiles (medium temperature of optimal growth, 30-40 °C) and the thermophiles (high temperature of optimal growth 55-75 °C) *(55, 60)*. The storage conditions of cooked and sliced meat products selects for psychrotrophic bacteria, and these bacteria generally pose the greatest risk of spoilage in cooked and sliced meat products *(2, 49)*. Psychrotrophic bacteria are characterized by their ability to grow at refrigerated temperatures (< 7 °C) though their optimum temperature almost corresponds to that of mesophilic bacteria *(61)*.
The temperature is used actively to control the microbiology of a given meat product. Storage at refrigerated temperatures slows down microbial growth and therefore increases the shelf-life of cooked and sliced meat products. Maintaining a constant and low temperature is therefore a key factor in controlling microbial growth during storage and distribution *(49, 62)*. Nevertheless, temperature fluctuations during transportation, during storage in the supermarkets and during storage in the refrigerators of the consumers is a major cause of premature spoilage in cooked and sliced meat products *(49, 63, 64)*.

**Atmosphere**. Control of the oxygen level in the surrounding atmosphere is another method used to control microbial growth during storage. In the meat industry this can be achieved by packing in MA or vacuum. Bacteria can also be classified according to their preferred atmosphere. Aerobic bacteria grow well in the oxygen level found in atmospheric air whereas anaerobic bacteria lack the respiratory system and cannot use oxygen as electron acceptor. The anaerobic bacteria can be divided into the aerotolerant anaerobes, which are able to grow in the presence of oxygen, and the obligate anaerobes which do not tolerate oxygen *(55)*. Facultative anaerobic bacteria, such as *B. thermosphacta*, prefer growth in the presence of oxygen but are able to grow under anaerobic conditions. Finally, microaerophilic bacteria require oxygen for their metabolism but are sensitive towards high oxygen levels. The packing atmosphere of cooked and sliced meat products is chosen based on knowledge of the growth rates of the potential spoilage bacteria in different compositions of packing gas. In meat and meat products packing in MA or vacuum decrease the rate of spoilage compared to storage in atmospheric air. This is illustrated in Figure 12 using an example adapted from Dainty & Mackey, 1992 *(65)*. Figure 12 shows the growth of different spoilage bacteria in chill-stored raw meat during storage in either atmospheric air or vacuum. From this figure it is seen that fast growing *Pseudomonas* spp. dominate the product when stored in atmospheric air. Moreover, the facultative aerobic *B. thermosphacta* also grow to high number under these conditions. However, in the vacuum packaging *Pseudomonas* spp. and *B. thermosphacta* are unable to compete with LAB's, which are able to grow well under anaerobic

conditions. A comparison of the time scale of two graphs in the figure furthermore show that the growth rate of the LAB's under anaerobic conditions is significantly lower than the growth rate of *Pseudomonas* spp. under aerobic conditions. This illustrates that packing in vacuum increases the shelf-life of raw meat considerably.

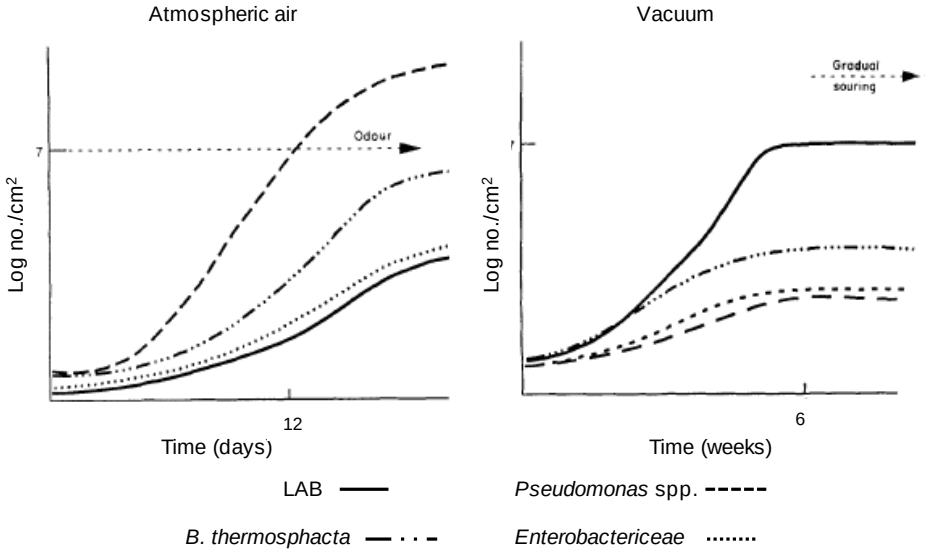


**Figure 12. Growth of different spoilage bacteria in chill stored red meat stored I atmospheric air and vacuum. Modified after Dainty and Mackey 1992 *(65)*.**

It should furthermore be mentioned that $CO_2$ has a general inhibitory effect on microbial growth, and therefore it is commonly used for packing of cooked and sliced meat products. This inhibitory effect is partly caused by the formation of carbonic acid when $CO_2$ is dissolved in water *(60)*. The shelf-life extending effect of packing in pure $CO_2$ was compared to vacuum packing in samples of fresh pork cuts in a study by Gill & Harrison 1989 *(66)*. The results of this study showed that packing in $CO_2$ compared to vacuum packing prolonged the time of storage without apparent spoilage from 2 weeks to 5.5 weeks at 3°C. This extension of the shelf-period was ascribed to inhibitory effect of $CO_2$ on *B. thermosphacta.*

**pH-value**. Most bacteria have their pH-optimum at values between 6 and 8. At pH-values outside this range the important metabolic processes are damaged as the enzyme activities decrease. The pH-value of a given meat product can therefore also be used to inhibit or slow down microbial growth and spoilage *(60)*. The initial pH value of the saveloy used in PAPER I-III was measured to 6.3 *(67)* which is within the limits of optimal growth of most microorganisms. However, during storage spoilage bacteria, for example LAB, can lower the pH-value of a given meat product via their production of organic acids from sugars *(2)*. This production of organic acids often favors the growth of LAB's on the expense of other spoilage bacteria such as B. *thermosphacta (62)*. Typical pH drops observed in cooked and sliced meat products during storage are within the range from 6.5-6.0 to 5.5-5.0 *(2, 4, 51, 68)*.

**Water activity**. The water activity expresses the availability of water which is an important factor for microbial growth. The water activity of a given sample is defined as the vapor pressure of water over the sample relative to the vapor pressure over distilled water. The water activity moreover depends on the concentration of solutes in the water phase. Differences in the water activity between the surroundings and the cytoplasm of the bacterial cells will lead to a transport of water across the cell membrane *(55)*. The adding of salt and other humectants to cooked and sliced meat products has a preserving effect as it decreases the water activity of the surroundings compared to the cytoplasm. This could lead to a transport of water from the bacterial cell to the surroundings. However, the bacteria can manage this by increasing the concentration of solutes in the cytoplasm, which lowers the water activity inside the cell *(60)*. This lowering of the internal water activity will prevent water from leaving the cell. Nevertheless, the growth rates of most spoilage organisms in meat products will suffer from decreasing water activity. Generally gram-negative bacteria, such as *Pseudomonas* ssp. and *Enterobacteriaceae*, are more sensitive to low water activities than gram-positive bacteria, such as LAB and B. *thermosphacta (69)*. The water activity of cooked and cured meat products is typically in the range 0.96-0.99 *(2)*.

## 4.3 Specific spoilage organisms of cooked and sliced meat products

A wide selection of bacteria can be found as part of the in-house microbial flora, and the composition of this flora furthermore varies between different meat processing facilities *(34, 51)*. However, only a fraction of the initial flora is responsible for the eventual spoilage of cooked and sliced meat products. This fraction is called the 'specific spoilage organisms' (SSO's). The natural selection of the SSO's of a given meat product depends on factors such as packing atmosphere, water activity, pH and storage temperature as described above. The use of specific antimicrobial agents also contributes to the selection the SSO's of a given meat product *(49, 70, 71)*. Growth to high numbers has furthermore been stated as a prerequisite for spoilage but a high microbial count does not always equal spoilage. Nevertheless, a total microbial count of $10^7$ CFU/g is often used as a loose indicator of the onset of spoilage *(65, 69, 72, 73)*. However, due to the variation in the spoilage potential of different species of bacteria it is difficult to use the population size as spoilage indicator *(2)*. The spoilage potential of a given bacteria can furthermore change depending on the specific growth conditions in the microenvironment. Korkeala *et al.*, 1989 *(74)* moreover reported a delay in the sensory spoilage of vacuum packed ring sausages after the spoilage flora, which was completely dominated by *lactobacilli*, reached a population size of $10^7$ CFU/g *(74)*. This delay varied between 7 and 30 days at storage temperatures between 2 °C and 12 °C.

A comparison of the literature concerning the spoilage of cooked and sliced meat products shows that the predominant spoilage bacteria of these products, depending on the storage conditions, are *B. thermosphacta*, *Pseudomonas* spp. and wide selection of LAB *(2, 49)*. The role of these bacteria in the spoilage of meat product will be described in the following. A general overview of

the factors affecting the growth of these spoilage bacteria is provided in Table 3. However, the temperature-range, pH-range and salt tolerance provided in the table should be seen as approximate values as they often vary between specific species and strains of bacteria. The microbial production of VOC's is a major focus area of this PhD-project. Therefore Table 4 provides an overview of the production of selected metabolites by *B. thermosphacta*, *Pseudomonas* spp., *Leuc. carnosum* and *C. maltaromaticum* under aerobic and anaerobic conditions. The metabolites included in Table 4 do not represent the complete range of metabolites produced by the selected bacteria. However, the metabolites shown in the table were selected based on their expected role in the formation of spoilage, or based on their significance in the metabolism of the bacteria included in the table.

**Table 3. A general overview of the preferred growth conditions of selected bacteria with spoilage potential in cooked and sliced meat products. The data provided in the table is only approximate values as the variation within the different strains and genera of bacteria is rather large. The table includes bacteria used for inoculation in saveloy in PAPER III plus the general information of LAB and *Pseudomonas* spp.**

| Bacteria | Gram +/- | Aerobe/anaerobe | Salt tolerance (Approx.) | pH-range (Approx.) | Temp.-range (Approx.) | Utilized nutrients | Ref. |
|---|---|---|---|---|---|---|---|
| ***B. thermosphacta*** | + | Facultative anaerobic | max. 6.5-8% | 5-9 | ~0-35°C | G, A | *(75),(76)* |
| **Lactic acid bacteria** | + | Facultative anaerobic, | Generally salt tolerant. max. ~6.5-10% | Generally tolerates low pH | Several psychrotrophic species | G, A Species dependent | *(73)* |
| ***Leuconostoc spp.*** **fx. *carnosum*, *mesenteroides* (LAB)** | + | Facultative anaerobic | 6.5% | Growth may occur at pH 4.8 | ~1-37°C > | G, A | *(73)* |
| ***Carnobacterium* spp.** **fx. *maltaromaticum, divergens* (LAB)** | + | Facultative anaerobic | 5-10% | Sensitive to acid pH. Slow growth at pH< 6 | ~0-40°C | G, A | *(75)* |
| ***Pseudomonas spp.*** | - | Aerobic | Salt sensitive* | Sensitive to acid pH. No growth at pH 4.5 | ~4°C-42°C (opt) 28°C | G, A | *(2),(71), (77)* |
| ***C. luteola*** | - | Facultative anaerobic | <6.5% | Sensitive to acid pH | ≤42°C | G, A | *(77)* |

*No specific values regarding the salt tolerance of *Pseudomonas* spp. were found. However, according to Borch *et al.*, 1996 *(2) Pseudomonas* spp. are 'salt sensitive'.

### 4.3.1 Lactic acid bacteria (LAB)

The packing of cooked and sliced meat products in vacuum or MA generally selects for growth of LAB *(5, 51, 78)*. The LAB which are relevant in cooked and sliced meat products include *Carnobacterium* spp., *Lactobacillus* spp., *Leuconostoc* spp. and *Wiessella* spp. of which different strains can have varying spoilage potential *(4, 69, 78-81)*. Therefore the degree of spoilage caused

by LAB depends on the composition of LAB present in the initial flora, and their ability to adapt to the specific microenvironment of given meat product. Various types of LAB have been shown to be responsible for the spoilage of different meat products whereas other types of LAB have been shown to cause very limited spoilage or none at all. The latter type of LAB has been suggested as bio-protective cultures in cooked and sliced meat products *(82, 83)*. Strains of *Leuc. carnosum* and *C. maltaromaticum* were used for inoculation of saveloy samples in PAPER III, and the characteristics of these two bacteria are therefore described below after a general introduction to spoilage by LAB.

**General signs of spoilage by LAB.** Besides the formation of off-odors, spoilage by LAB can become apparent in several other ways. The general souring of meat products stored under anaerobic condition due to the formation of lactic acid and other organic acids is a common sign of spoilage by LAB *(2, 5)*. The formation of ropy or milky slime is also quite often observed as a sign of spoilage by LAB in vacuum packed meat products. Korkeala *et al*., 1988 *(84)* reported the formation of ropy slime in sausages caused by *Lactobacillus* and *Leuconostoc* species whereas Samelis *et al.*, 2000 *(51)* reported the formation of a clear exudate on sliced and vacuum packed ham and turkey breast stored a 4°C for 30 days. This was attributed to *Leuc. mesenteroides*. Fermentation of sugars by some LAB yields $CO_2$ which can result in 'blown packs' and a loss of consumer acceptability. The formation of blown packs due to excessive $CO_2$ production has been attributed to different LAB including *Leuconostoc spp*. and *Lactobacillus spp*. *(2, 5, 51)*. An example of a 'blown pack' caused by $CO_2$ formation by *Leuc. carnosum* is given in Figure 13.

**Figure 13. An example of a slightly 'blown pack' caused by *Leuc. carnosum* in PAPER III. The package shown in the figure was kept beyond the extent of the experiment.**

In the presence of oxygen some LAB are furthermore able to produce $H_2O_2$ which may result in green spots on the surface of cooked and sliced meat products due to oxidation of nitrosohaemochrome. $H_2O_2$-producing LAB species include *Lactobacillus spp*. and *Leuconostoc spp*. *(2)*. In a study made on vacuum packed morcilla de Burgos (Spanish blood sausage/black pudding) inoculated with *Leuc*. *mesenteroides* and/or *W*. *viridescens* by Diez *et al.*, 2009 *(72)* the formation of gas, green spots, slime and off-odors were all detected by a sensory panel during a 75 days

storage period. This illustrates that the described indicators of spoilage by LAB can develop simultaneously during storage. Furthermore, it may be a combination of these indicators which lead to rejection of the product by the consumers or a sensory panel.

***Leuconostoc* spp.** *Leuc. carnosum* and *Leuc. mesenteroides* are frequently reported as SSO's in vacuum packed or MA-packed meat products. *Leuc. carnosum* was reported as the SSO in vacuum packed sliced ham in a study by Björkroth *et al.*, 1998 *(78)* and Samelis *et al.*, 2006 *(85)* reported that *Leuc. carnosum* was the dominating bacteria in whole cooked hams in Greece. Different *Leuconostoc* spp. were moreover associated with spoilage of vacuum packed Vienna sausages by Dykes *et al.*, 1994 *(86)* whereas Laursen *et. al.*, 2009 *(81)* reported that *Leuc. carnosum* was present in 6 of 8 samples of vacuum- or MA-packed pork products from different manufacturers on the 'sell by date'.

In another study Samelis *et al.*, 2000 *(51)* investigated the development of spoilage in different types of vacuum packed cooked and sliced meat products. All products were obtained from the same factory and were therefore subjected to the same initial flora. However, during storage the different products selected for different spoilage bacteria. The whole muscle products, ham and turkey breast, had a higher water activity and a lower salt content compared to the emulsion type products tested in the study *(51)*. The lower microbial hurdle of the whole muscle products selected for growth of *Leuconostoc* spp. compared to other types LAB *(51, 85)*. *Leuc. mesenteroides* was found to be the dominating bacteria of both sliced ham and turkey breast when stored in vacuum. However, storage of the sliced ham samples in atmospheric air favored growth of mainly *Leuc. carnosum* on the expense of *Leuc*. *mesenteroides (51)*. The results of Samelis *et al.*, 2000 *(51)* moreover showed that the vacuum packed emulsion type meat products, which had an increased hurdle for microbial growth, were dominated by other types of LAB, primarily *Lb. sakei*. The result of this study therefore suggests that Leuconostoc spp. are most relevant as spoilage bacteria in meat products with a low hurdle for microbial growth *(51)*.

Spoilage by *Leuconostoc* spp. packed in vacuum or MA leads to souring of the product and the development of 'fermenting' odor *(51, 85)*. However, there is only limited information available regarding the VOC's contributing to the spoilage by *Leuconostoc* spp. Nevertheless, Borch & Molin, 1989 reported the formation of D-lactic acid, acetic acid and ethanol by 3 *Leuconostoc* strains in aerobic batch cultures *(87)* whereas Vermeiren *et al.*, 2005 *(80)* reported the formation of propionic acid and lactic acid under anaerobic conditions. An overview of the production of metabolites by *Leuc. carnosum* under aerobic and anaerobic conditions is given in Table 4. As mentioned the formation of off-odors is not the only way that spoilage can become apparent. *Leuconostoc* spp. has been shown to be responsible for both greening and formation of gas and ropy slime *(51, 72, 84)*. *Leuc*. *carnosum* ssp. has moreover been reported as a potential bio-protective culture in cooked and sliced meat products due to the ability to produce bacteriocins and to inhibit the growth of *Listeria monocytogenes (82, 83, 88)*. The role of *Leuc. carnosum* in cooked and sliced meat products therefore appears to vary between strains, product types and packing conditions.

***Carnobacterium* spp.** *C. maltaromaticum* (previously *C. piscicola*) and *C. divergens* are often found as part of the microbial flora in fresh meats, processed meat products and seafood stored at refrigerated temperatures *(89)*. Both *Carnobacterium* species have furthermore been found in raw meats stored in vacuum packing, MA-packing and under aerobic conditions *(89)*. In a study by Vasilopoulos *et al.*, 2008 *(3) C. divergens* was reported as an important part of the spoilage flora of MA-packed artisan-type cooked ham, which contains low level of additives. Laursen *et al.*, 2009 *(81)* moreover found *C. divergens* in 3 of 8 samples of pork products from different manufacturers on the 'sell by date'. Though *Carnobacterium* spp. are often found in processed meat products they appear to be outgrown by more acid tolerant LAB species such as *Lb. sakei* and *Leuc. mesenteroides* during storage *(85, 89)*. Moreover, no studies has been found clearly showing that *Carnobacterium* spp. was responsible for spoilage of a given cooked and sliced meat product. However, *Carnobacterium* spp. appear to have higher spoilage potential in raw meats, fish and seafood than in processed meat products *(89-91)*.

The formation of VOC's by *C. maltaromaticum* has been studied in some detail. Larrouture-Thiveyrat *et al.*, 2003 *(92)* illustrated the ability of *C. piscicola* (now *maltaromaticum*) to metabolize leucine to primarily 3-methylbutanoic acid when used as a starter culture in sausage fermentation. Borch & Molin, 1989 *(87)* reported the formation of acetic acid and acetoin by *C. piscicola* (now *maltaromaticum*) in an aerobic batch culture at 25 °C and pH 6. Laursen et al. 2006 *(90)* measured the production of metabolites after inoculation of different *Carnobacterium* spp. on peeled MA-packed (50 % $CO_2$, 30 % $N_2$, 20 % $O_2$) shrimp. This study showed that tested strains of *C. maltaromaticum* and *C. divergens* were able to produce acetic acid, 2- and 3-methylbutanal, 2- and 3-methylbutanol, 2,3-butandione (diacetyl) and several other alcohols and ketones. An overview of the production of volatile metabolites by *C. maltaromaticum* reported in the existing literature is given in Table 4.

**Table 4. Overview of the production of selected metabolites by *B. thermosphacta*, *Leuc. carnosum*, *C. maltaromaticum* and *Pseudomonas* spp. reported in the literature. The production of metabolites under aerobic and anaerobic conditions is given a list of the precursors of the selected metabolites is also provided.**

| Compound | Precursor | *B. thermosphacta* | | *Leuc. carnosum** | | *C. maltaromaticum*[a] | | *Pseudomonas* spp. | |
|---|---|---|---|---|---|---|---|---|---|
| | | Aerobe[b,c] | Anaerobe[d] | Aerobe[b] | Anaerobe[d] | Aerobe[b,e] | Anaerobe[f] | Aerobe[g] | Anaerobe |
| **diacetyl** | Glucose[b] | X | | | | X | | X | |
| **acetoin** | Glucose[b] | X | | | | X | | X | |
| **3-methylbutanal -ol,-acid** | leucine[h] | X | | | | X | X | | |
| **2- methylbutanal -ol, -acid** | Isoleucine[h] | X | | | | X | | | |
| **2-methylpropanal -ol, acid** | Valine[h] | X | | | | | | | |
| **dimethyl disulfide** | Methionine[h] | | | | | | | X | |
| **dimethyl trisulfide** | Methionine[h] | | | | | | | X | No growth |
| **ethyl-esters** | Glucose[g] | | | | | | | X | |
| **formic acid** | Glucose | X | | | | | | | |
| **propionic acid** | Glucose | | | | X | | | | |
| **acetic acid** | Glucose[b] | X | X | X | | X | | | |
| **lactic acid** | Glucose | | X | X | X | X | | | |
| **ethanol** | Glucose | | X | X | | | | | |
| **1-Hexanol** | Glucose | | | | | | | X | |
| **1-Pentanol** | Glucose | | | | | X | | X | |

[a] Previously known as C. *piscicola*. [b] Borch & Molin, 1989 *(87)*. [c] Dainty & Hibbard, 1983 *(93)*. [d] Vermeiren *et al.*, 2005 *(80)*. [e] Laursen *et al*., 2006 *(90)*. [f] Larrouture *et al*., 2000 *(94)*. [g] Dainty *et al*., 1984 *(95)*. [h] Yvon & Rijnen, 2001 *(96)*. * The aerobic metabolites are detected for *Leuconostoc* spp. in general and not for *Leuc. carnosum* in specific.

### 4.3.2 *Brochothrix thermosphacta*

*B. thermosphacta* is a facultative anaerobic bacterium which grows well at refrigerated temperatures. *B. thermosphacta* is furthermore frequently responsible for spoilage of both raw meat and cooked and sliced meat products *(2, 49, 50)*. Though *B. thermosphacta* is able to ferment sugars in MA- or vacuum packed meat products it grows faster and causes heavier spoilage under aerobic conditions. Dainty and co-workers have made interesting studies of the aerobic metabolism of *B. thermosphacta* growing on raw meat and laboratory media. These studies report the formation of several different off-odors by *B. thermosphacta (76, 93, 97)*. Acetoin and diacetyl, which have sweet and butter-like odors, are main metabolites of the aerobic

glucose metabolism of *B. thermosphacta*. 3-Methylbutanal and 2-methylbutanal, which derive from degradation of leucine and isoleucine respectively, are also important aerobic metabolites of *B. thermosphacta (50, 69, 76, 93)*. The alcohols and carboxylic acid corresponding to 2- and 3-methylbutanal are also frequently found in cooked and sliced meat products spoiled by *B. thermosphacta* under aerobic conditions *(50, 76, 93).* Furthermore, Stanley *et al.*, 1981 *(50)* reported 2-methylpropanol, acetic acid and 2,3-butandiol as metabolites of *B. thermosphacta* in sliced luncheon meat products under aerobic conditions. According to Vermeiren *et al.*, 2005 the main metabolites of *B. thermosphacta* from glucose under anaerobic conditions was lactic acid and ethanol *(80)*. An overview of the formation of metabolites by *B. thermosphacta* under aerobic and anaerobic conditions is given in Table 4.

Due to the storage of cooked and sliced meat products in vacuum or MA-packing the growth of *B.* thermosphacta can be suppressed by LAB *(5)*. Under anaerobic conditions LAB are often able to outgrow *B. thermosphacta (51, 83)*. *B. thermosphacta* is furthermore more sensitive to nitrite than LAB *(2)*. However, the risk of spoilage by *B. thermosphacta* increases during storage at the consumers. Here the packages of cooked and sliced meat product are opened and the protective atmosphere is destroyed. This increase the growth rate and spoilage potential of *B. thermosphacta* markedly due to production of acetoin and diacetyl, which are probably the most characteristic off-odors associated with *B. thermosphacta*.

### 4.3.3 *Pseudomonas* spp.

Gram-negative and aerobic *Pseudomonas* spp. play an important role in the spoilage of aerobically stored fresh meat products. The most important species of *Pseudomonas* in meats are *Ps. fragi*, *Ps. flourescens* and *Ps. ludensis (69)*. When glucose and oxygen are available they are able to outgrow most other bacteria at refrigerated temperatures *(2, 97)*. This was also indicated in Figure 12. When the *Pseudomonas* spp. reach a population of $10^{7-8}$ CFU/g the production on slime and off-odors become apparent *(49)*. However, in cooked and sliced meat products which are commonly packed in MA or vacuum the *Pseudomonas* spp. are not able to grow and compete with the remaining spoilage flora. The relatively high level of salt in cooked and sliced meat products is furthermore not favorable for growth of *Pseudomonas* spp. *(2)*. However, in cases of high oxygen permeability of the packing film or after package opening by the consumers *Pseudomonas* spp. could play a role in cooked and sliced meat products. In PAPER III sliced saveloy samples were inoculated with *Chryseomonas luteola*, which is a facultative anaerobic bacteria belonging to the *Pseudomonas* genus. This bacterium could therefore be very interesting in relation to spoilage of cooked and sliced meat products. The *C. luteola* strain used in PAPER III was isolated from a meat product and was moreover able to cause sensory spoilage in sliced saveloy.

The aerobic metabolism by *Pseudomonas* spp. yield a wide range of metabolites of which some are shown in Table 4. The formation of VOC's from the growth of different *Pseudomonas* spp. on raw beef was studied by Dainty *et al.* 1984 *(95)*. The most characteristic of these are the ethyl-esters which could give raise to fruity off-odors in the meat products. When glucose is depleted and the metabolism switch to degradation of free amino acids the formation of sulfur compounds,

including dimethyl disulfide and dimethyl trisulfide are produced. The production of sulfur compounds, which generally have cabbage-like and putrid off-odors, are often associated with spoilage by *Pseudomonas* spp. *(95, 97)*.

## 4.4 Summary of microbial changes in cooked and sliced meat products

In this chapter it was shown that the development of microbial spoilage in cooked and sliced meat products is a complex issue which depends on several factors. The pH-value, the water activity, the level of preservatives and the availability of nutrients are determined by the composition of the product and are moreover all factors which contribute to select for a specific spoilage flora. The storage temperature and composition of the surrounding atmosphere are moreover very important factors for the composition of the specific spoilage flora in a given cooked and sliced meat product.

In MA- and vacuum packed meat products LAB are often the dominating flora and the development of spoilage caused by *Leuc. carnosum* and *C. maltaromaticum* were described as examples of this. *B. thermosphacta* and *Pseudomonas* spp. are moreover often associated with spoilage of cooked and sliced meat products, particularly under aerobic conditions where these bacteria are able to produce potent off-odors.

# 5. Modeling spoilage of cooked and sliced meat products

The ability to accurately predict the shelf-life and the development of spoilage of a given cooked and sliced meat product would be of great value to manufacturers, retailers and consumers. The development of shelf-life models for different meats and meat products has therefore received a lot of attention. This chapter reviews some of the existing literature dealing with the modeling of shelf-life and spoilage in cooked and sliced meat products using different types of spoilage indicators.

## 5.1 Approaches to the modeling of spoilage

As described in chapter 4 the processes leading to microbial spoilage in meat products are highly complex and depend on several intrinsic and extrinsic factors. A good shelf-life model should be able to account for the factors which are relevant for the shelf-life of a given meat product, and translate them to a simple and understandable measure. This is not an easy task and the development and validation of a predictive shelf-life model is therefore a complex process which demands a lot of experimental work.

There are different approaches towards the development of shelf-life models, which are based on different methods of defining and measuring the characteristics of an unacceptable product. Measurement and prediction of microbial growth is probably the most widespread method applied in predictive shelf-life modeling *(59, 68, 98)*. However, the possibility of using chemical indicators for the spoilage of cooked and sliced meat products has been reported is some studies *(70, 81, 99)*. The relation between the shelf-life period, growth of the total microbial flora, growth of the specific spoilage organisms and the formation of an unspecified chemical spoilage index is illustrated in Figure 14. The figure illustrates that the shelf-life period can be defined either as the time needed to reach a critical growth level of the SSO's, or as the time needed to reach a critical concentration of the chemical spoilage index. The figure moreover shows that the SSO's not necessarily are a predominant part of the initial microbial flora but that they gradually outgrow the other bacteria in the product during storage. The chemical spoilage index could potentially be all types of compounds which are affected by the presence of the SSO's. For example volatile and non-volatile metabolites of microbial growth and their precursors, which include sugars and amino acids. The development of a shelf-life model based on measurements of a chemical spoilage index in cooked and sliced meat products would require the use of accurate methods with low detection limits.

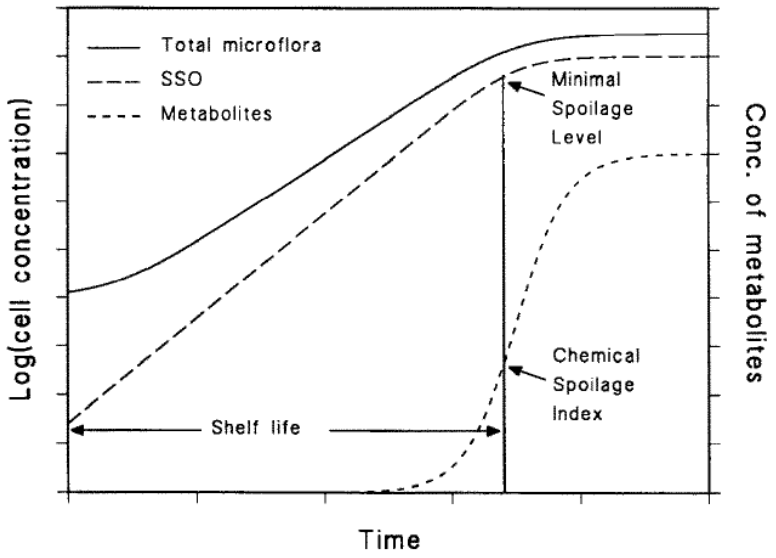


**Figure 14. Illustration of the growth of the total microbial flora, the specific spoilage organisms (SSO) and the production of microbial metabolites which can be used as chemical spoilage index with storage time. Adapted from Dalgaard 1993 *(100)*.**

## 5.2 Shelf-life modeling based on microbial growth responses

The modeling of shelf-life in meat products based on data describing microbial growth responses at different conditions can be divided into three classes. The primary models describe the changes in a microbial population as a function of time at particular environmental conditions. The secondary models describe the relation between the parameters of the primary model and a number of environmental factors. Tertiary models are computer based tools which integrate both primary and secondary models and account for the response of microbial populations to variation of multiple factors *(59, 101)*. Several different primary and secondary equations or models exist to which the growth of microbial populations in meat products can be fitted. These equations or models can be used to describe the growth of the total population or the growth of the SSO in a given meat product. The publications by Devlieghere *et al.*, 1999 *(98)* and Kreyenschmidt *et al.*, 2010 *(102)* are good examples of the application of microbial response models for shelf-life predictions of cooked and sliced meat products.

Kreyenschmidt *et al.*, 2010 *(102)* combined primary and secondary models to predict the shelf-life of MA-packed cooked and sliced ham obtained from a specific meat processing facility. First it was established that LAB were the SSO's of the product and a LAB count of $10^7$ CFU/g was used as the upper limit of an acceptable product. Via sensory analyses it was established that $10^7$ CFU/g corresponded well with the onset of sensory spoilage *(102)*. The model included temperatures ranging from 2-15 °C and it was validated using sliced ham stored at dynamic temperature programs within the same temperature range. Different types of primary and secondary models were used to describe the data but the best result was obtained when using a two step approach combining the modified Logistic model as the primary model and the Arrhenius equation as the secondary model *(102)*. The relative growth rate was estimated at the different temperatures in

the primary models and the temperature dependency of the relative growth rate was modeled with the secondary models. Within the tested temperature range, the model developed by Kreyenschmidt *et al.*, 2010 *(102)* was able to predict the remaining shelf-life of MA-packed cooked and sliced ham from a specific processing facility, which was spoiled by a specific microbial flora dominated by LAB. However, the model would be sensitive to changes in the composition of the packing gas, the product composition and changes in the microbial flora *(102)*. It is therefore probable that the model would not give accurate shelf-life predictions of a different product from the same processing facility. Though this product would have the same initial flora, the differences in product composition would result in a different hurdle for microbial growth which could select for different LAB as the SSO's.

Devlieghere *et al.*, 1999 combined primary and secondary models to describe the growth of *Lactobacillus sake* which was argued to be a relevant SSO in meat products. Growth of *L. sake* was tested at varying temperatures (4-12 °C), water activities (0.986-0.962) and concentrations of dissolved $CO_2$ (0-2411 ppm) *(98)*. The *L. sake* growth curves for the model were developed in modified brain hart infusion broth, whereas the validation of the model was done on aseptically sliced cooked ham inoculated with *L. sake*. The shelf-life was defined as the time needed to reach a *L. sake* level of $10^7$/g *(98)*. The finished model was able to predict the shelf-life, measured as *L. sake* growth, in cooked ham at different storage temperatures, water activities and levels of dissolved $CO_2$. However, the model would not be valid for other SSO in meat products *(98)*. Though *L. sake* is a highly relevant spoilage bacteria in cooked and sliced meat products a change in concentration of salt, nitrite or other additives could select for other spoilage bacteria *(51)*. Therefore other models should be made to account for different spoilage bacteria. The model presented by Devlieghere *et al*., 1999 *(98)* furthermore does not account for the effect of interactions between *L. sake* and other bacteria in the spoilage flora on the growth rate.

Even though Devlieghere *et al*., 1999 *(98)* included several environmental factors into the shelf-life model it is still only applicable for cooked and sliced meat product in which L. sake is responsible for spoilage. This model is a good illustration of the challenges associated with the development of a broadly applicable shelf-life model based microbial growth responses. According to Pin & Baranyi, 1998 *(103)* modeling based on microbial growth responses is more straightforward when dealing with pathogen models because they only need to account for a limited number of organisms. In cooked and sliced meat products a wide range of bacteria has the potential to cause spoilage depending on a variety of factors, and this has implications for the development of predictive models based on microbial growth.

## 5.3 Modeling of spoilage by the use of chemical markers

As described in chapter 4 a high microbial count does not always equal spoilage. Furthermore, a significant delay, between the time when the maximum microbial population is reached and the onset of spoilage, has been reported in cooked and sliced meat products *(2, 74)*. The spoilage of cooked and sliced meat products is moreover usually not caused directly by the presence of the

SSO's in high numbers. It is rather the production of metabolites such as organic acids, off-flavors, gas and slime which is the direct reason that the product is perceived as spoiled *(2)*. Many of the microbial metabolites, which have potential as spoilage index, are moreover produced by several of the bacteria that are relevant in relation to meat spoilage (see Table 4). The use of a chemical spoilage index for the development of predictive shelf-life models could therefore be a shortcut towards the development of models which are applicable for a broader spectrum of spoilage bacteria. Traditional microbial methods are furthermore relatively slow due the time needed for incubation whereas measurements of a chemical spoilage index potentially could be done using fast and on-line/at-line analyses instead *(70)*. Such measurements could potentially provide a fast and early warning of later occurring quality problems. An example of the use an at-line method for detection of spoilage markers is given in PAPER IV, where PTR-MS is tested for detection of VOC's with a potential as chemical markers for spoilage.

Mataragas *et al.*, 2007 *(70)* used a multivariate approach to develop a predictive spoilage model of cooked and sliced ham. The model was based on measurements of both microbial (LAB count) and chemical variables (L-lactate, D-Lactate, Acetate, Glucose and pH) in samples subjected to different combinations of storage time and temperature. The samples were split into 3 groups based on the results of the described measurements using hierarchical cluster analysis and *k*-means clustering. The cluster analysis ensured that the selected groups of samples had minimal variability within the groups and maximum variability between the groups *(70)*. The 3 clusters corresponded to 3 different levels of spoilage of the samples. Hence, samples stored short time at low temperature ended in group 1 and samples stored long time at high temperature ended in group 3. The group number (1, 2 or 3) of the samples was used as the Y-variable, which was to be predicted, in a PLS-model where the microbial and chemical spoilage indicators were included as X-variables. In this PLS-model it was possible to predict the level of spoilage in cooked and sliced ham, according to the 3 spoilage groups, based on knowledge of the LAB plate count and the level of selected chemical variables *(70)*.

Stolzenbach *et al.*, 2009 *(99)* investigated the development of spoilage in the typically Danish processed meat product 'rullepølse'. The 'rullepølse' samples were stored in MA-packing which was breached at different stages of the experiment. This study aimed to relate the microbial metabolites acetic acid, lactic acid and α-ketoisocaproic acid with sensory quality. α-Ketoisocaproic acid is a precursor of 3-methylbutanal and the corresponding alcohol and acid *(48)*. Stolzenbach *et al*., 2009 *(99)* moreover used PLS-models to investigate the relation between selected sensory variables and the microbial metabolites, which were tested as potential chemical shelf-life markers. However, no clear relation between sensory deterioration and the microbial metabolites were found, though sensory deterioration was observed in some of the 'rullepølse'-samples during storage *(99)*. The microbial formation of acetic acid and α-ketoisocaproic varies with the genus of LAB *(99)*. It is therefore possible that the α-ketoisocaproic could be correlated with spoilage if another type of LAB were dominating the spoilage flora of the samples investigated in this study.

## 5.4 Studies relating microbially produced volatile organic compounds with spoilage

In this PhD-project volatile organic compounds were used as chemical markers for spoilage. The link between odor and quality of cooked and sliced meat products is well established and has been investigated in several studies *(2, 37, 79, 104)*. There has moreover been some focus on the relation between the microbial formation of VOC's and the shelf-life or consumer acceptability of different meat products.

Pham *et al*., 2008 *(104)* studied the sensory profile of 8 types of American dry-cured ham, and furthermore linked the consumer acceptability of these products with the aroma composition measured with solid phase microextraction coupled with GC-MS. PCA was used to relate specific VOC's with consumer acceptability. Based on the PCA-model it was concluded that VOC's such as 3-methylbutanal, 2-heptanone, 2-butanone, methanethiol and hexanal were linked with low consumer acceptability whereas compounds such as 4-methyl-2-methoxyphenol and 2-methoxyphenol were linked with high consumer acceptability *(104)*. The purpose of the study was mainly descriptive. However, the relation between the specific VOC's and consumer acceptability could probably be developed into a model for prediction of the consumer acceptability of American dry cured ham based on the aroma composition.

In Leroy *et al*., 2009 *(79)* the development of spoilage in MA-packed (70 % $N_2$, 30 % $CO_2$) artisan-type cooked and sliced ham, at different storage temperatures, was investigated using static headspace extraction coupled with GC-MS and phase microextraction coupled with GC-MS. Spoilage was defined as the point where microbial growth exceeded $10^6$ CFU/g. 3-Methylbutanol was found to give the highest response in the GC-MS measurements and it was moreover found to be related with the increase in biomass. 3-Methylbutanol was therefore concluded to have potential as an indicator of spoilage of MA-packed artisan-type ham. The VOC's ethanol, methanethiol, dimethyl disulphide, acetoin and acetic acid were furthermore ascribed to microbial metabolism.

The spoilage metabolism of different *Carnobacterium* spp. and *B. thermosphacta* inoculated on MA-packed (50 % $CO_2$, 30 % $N_2$, 20 % $O_2$) shrimp stored a 5 °C for 10 days was evaluated by Laursen *et al*., 2006 *(90)*. This was done with high pressure liquid chromatography, dynamic headspace coupled with GC-MS and sensory analysis. After 10 days storage the samples were divided in 3 classes based on their sensory acceptance, and the percentage of rejected samples was linked with the microbial production of metabolites using a PLS-model. This PLS-model showed that there was a good correlation between the predicted and the measured percentage of shrimp samples rejected by the sensory assessors. Diacetyl, isoleucine and tyramine all had a high impact on the model and were therefore important factors for the sensory rejection of shrimp inoculated with *Carnobacterium* spp. and *B. thermosphacta (90)*. However, several other interesting volatile and nonvolatile microbial metabolites were found in this study.

## 5.5 Evaluation of different approaches to the modeling of spoilage and shelf-life

Based on the studies reviewed above it appears that the models using microbial growth responses to predict the shelf-life of cooked and sliced meat products are the most successful at present. Although these models are only valid under specific conditions they are able to predict the shelf-life of cooked and sliced meat products measured as growth of the SSO's. However, the use of microbial growth as a measure of spoilage is associated with some difficulties. As mentioned a high microbial count does not always equal spoilage and the SSO's can moreover change depending on the product composition and the composition of the flora of the production facility.
The use of a chemical spoilage index for prediction of shelf-life would therefore be an interesting alternative. As reviewed above, interesting attempts have been made to use different chemical markers to index the sensory quality of cooked and sliced meat products. However, this type of spoilage modeling needs to be developed further in order to be applicable in practice. The present PhD-project contributes to this by investigating whether VOC's have potential as a chemical spoilage index in cooked and sliced meat products. The relevance of VOC's as spoilage indicators has previously been shown in the literature *(50, 79, 90)*.

# 6. Identification and application of chemical markers for shelf-life

In the experimental work of this PhD-project the quality changes of sliced saveloy during storage have been measured using mainly sensory profiling, GC-MS measurements and microbial plate counts. The results of the experimental work will be summarized and discussed in this chapter.

## 6.1 Introduction to the experimental work

All of the studies briefly reviewed in chapter 5 share similar characteristics with the experimental work of this PhD-project. This could be in relation to the purpose of modeling and describing quality changes, the analytical methods applied or the use of chemometrics for the data processing. The results of the excising literature are therefore important when evaluating the relevance and the validity of the results presented in PAPER I-IV.

**Methods**. In this PhD-project sensory evaluation of the sliced saveloy samples was used as a measure of eating quality in PAPER II and PAPER III. The advantage of using sensory evaluations is that they directly characterize the impact of different attributes of the product on the human senses *(70, 99)*. The disadvantage of sensory evaluations is that they are costly, time consuming and difficult to use as a routine measurement. Therefore the search for alternative measurements of product quality is highly relevant *(70)*. As mentioned in chapter 5 microbial plate counts as well as sensory evaluations have been used to represent of product quality in the existing literature *(98, 99, 102)*. In this PhD-project sample extracts were spread on different microbial growth mediums in PAPER II and PAPER III to provide the total microbial count, the LAB count, the *B. thermosphacta* count and the *Pseudomonas* spp. count. These plate counts were included to account for microbial quality of the product, and to support the observations made in the sensory evaluations and the GC-MS measurements. However, the sensory evaluations were used as the main measure of product quality in the experimental work of this PhD-project.
Dynamic headspace extraction coupled with GC-MS measurements were used to characterize the saveloy samples throughout the experimental work of this PhD-project. There is an obvious link between the odor and the quality and acceptability of foods. As described in chapter 4 and chapter 5 there is moreover a relation between the development of spoilage in cooked and sliced meat products and the production of volatile metabolites by microorganisms. In this PhD-project GC-MS measurements of the aroma composition were used to describe the quality changes in cooked and sliced meat products during the shelf-life period. The link between the aroma composition and sensory evaluations was studied in PAPER II and PAPER III in order to investigate the use of VOC's as chemical markers for the sensory quality of cooked and sliced meat products. In the GC-MS-results presented in the following, the VOC's 2-methylbutanal and 3-methylbutanal were eluting together from the GC-column. These compounds were quantified together and will

therefore be referred to as 2- and 3-methylbutanal. Moreover, 2-methylbutanol and 3-methylbutanol were quantified together and will be referred to as 2- and 3-methylbutanol.
Rapid on-line methods for measurement of VOC's, such as PTR-MS, adds new perspectives to the use of VOC's as chemical markers for sensory shelf-life. With the PTR-MS instrument it is possible to measure VOC's directly at the meat processing facility and obtain the results of the measurements almost instantaneously. This could facilitate early on-line/at-line detection of potential shelf-life problems in a given batch of meat product. However, this presumes that markers for the development of spoilage can be measured shortly after slicing and packing. The use of PTR-MS for measurement of VOC's in cooked and sliced meat products was therefore investigated in PAPER IV.

**Experimental setup**. Similar experimental setups were used in all 4 papers. In PAPER I-III samples were initially stored at constant temperature for 3 weeks in MA-packing (70 % $N_2$, 30 % $CO_2$). In fourth and final week of storage some of the saveloy samples in PAPER I-III were subjected to consumer simulated storage (CSS). Manufacturers of cooked and sliced meat products normally guarantee the quality of products which have been stored at constant temperatures of 5 °C during the shelf-life period. Moreover, it is normally recommended that packages are opened for a maximum of 2-4 days before consumption. However, as described in an unpublished study from Danish Meat Research Institute (DMRI)-Teknologisk *(64)*, the consumers tend to store the products at fluctuating temperatures. In this study Danish consumers were given packages of cooked and sliced meat products equipped with thermologgers. These thermologgers were returned to DMRI-Teknologisk after the product was consumed. Based on data from the thermologgers the average temperature profile was found to be: 12.7 hours at 5 °C, 9.8 hours at 12 °C and 1.5 hours at 20 °C *(64)*. Part of the storage at the consumers would moreover be in open packages. CSS was therefore defined as the combination of package opening and the temperature fluctuations described above. CSS was incorporated into the experiments to account for the actual microbial stress which cooked and sliced meat products are subjected to within the shelf-life period. Temperature fluctuations and package opening could lead to a loss of eating quality within the shelf-life period. This would lead to unsatisfied consumers and reduce the chance of repurchase of the specific product. In PAPER IV CSS was not part of the experimental setup. However, in this experiment saveloy samples were stored for a period of 6 weeks instead of the 4 weeks used in PAPER I-III.

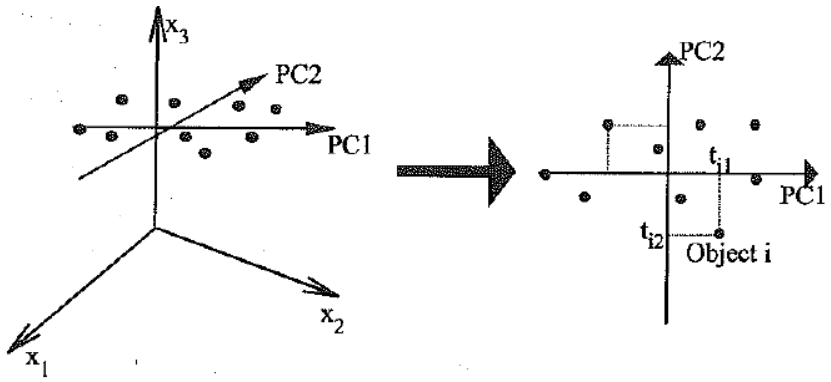


**Figure 15. Illustration of the basic principle of principal component analysis (PCA), which is to enhance the relevant structures in a complex data set. The figure is adapted from Esbensen, 2002 *(105)***

**Chemometric data processing**. The link between the sensory evaluations and the measurements of the aroma composition was investigated using chemometric tools such as PCA and PLS. These chemometric tools are ideal for investigation of complex data matrices as those obtained from the experimental work of this PhD-project. As an example, the GC-MS data matrix from PAPER II consisted of the peak areas of 60 VOC's from 30 saveloy samples. PCA is able to simplify and enhance the relevant structures of such matrices. This is done by decomposing the original data structure and replacing it with a new structure consisting of principal components (PC's ) as illustrated in Figure 15 *(105)*. Here $x_1$, $x_2$ and $x_3$ represents variables (VOC's) from the original data matrix. PC1 and PC2 are calculated from an algorithm which ensures that they account for the two main directions of variation in the original dataset. Additional PC's can be added, if needed, to account for the variation in the original data matrix *(105)*. PLS is able to relate two sets of data (X and Y) using principles similar to those of PCA. However, in PLS the PC's are calculated with the purpose of explaining the variation of the Y using the relevant structures of the X-matrix. The resulting PLS-models are able to predict values of the Y based on known values of the X-matrix *(106)*. In this project PLS-models were used to relate sensory descriptors (Y) with GC-MS data (X). PLS-models need to be validated in order to assure that the model is valid for other samples than those used to calculate the model *(107)*. In this PhD-project this was achieved using random subset validation. Here several sub-models are calculated leaving randomly chosen sample subsets out of the calculation. The final PLS-model is an average of the sub-models, and the root mean square error of cross validation (RMSECV) is a measure of the prediction error of the validated model *(107, 108)*.

## 6.2 Factors affecting the aroma composition of sliced saveloy (PAPER I)

PAPER I was an initial investigation of the factors affecting the composition of VOC's in the headspace of sliced saveloy. The investigated effects included the slicing conditions (commercial vs. experimental), the storage temperature (5°C, 8°C) during the initial 3 weeks of the experiment and the use of temperature fluctuations during the fourth and final week of the experiment. 5 experimental series were constructed, as shown in Table 5, and statistically compared in pairs in order to study the impact of the different effects on selected VOC's. GC-MS measurements of the composition of VOC's were performed at week 3, week 3+3 days, week 3+5 days and week 3+7 days.

**Table 5. An overview of experimental series included in PAPER 1.**

| Series | Slicing | Initial Temp. (3 weeks) | Package opening in week 4 | Temp. fluctuations in week 4 |
|---|---|---|---|---|
| 1 | Exp. | 5 °C | No | No |
| 2 | Exp. | 5 °C | Yes | No |
| 3 | Exp. | 5 °C | Yes | Yes |
| 4 | Com. | 5 °C | Yes | Yes |
| 5 | Com. | 8 °C | Yes | Yes |

Commercial slicing compared to experimental slicing (series 3 vs. series 4) resulted in a significant increase in 2-and 3-methylbutanal, which was used as an indicator of microbial activity. Hexanal, which was used as an indicator of lipid oxidation was found in decreased amounts in the commercially sliced samples. The effect of initial storage at 8 °C compared to 5 °C on the content of VOC's in the sample headspace was investigated in commercially sliced saveloy samples (series 4 vs. series 5). The level of 2- and 3- methylbutanal was significantly affected by the initial storage temperature. After the 3 weeks initial storage, the samples initially stored at 8 °C contained a higher amount of 2- and 3-methylbutanal compared to the samples stored at 5 °C. However, during the fourth week of storage the level of 2- and 3-methylbutanal decreased for samples initially stored at 8°C. The level of dimethyl disulphide, which can also be microbially produced, was significantly higher after initial storage at 8 °C compared to initial storage at 5 °C. The effect of temperature fluctuations during the fourth week of storage was studied by comparing the experimental series 2 and 3. The introduction of temperature fluctuations resulted in an increase in the level of 2- and 3-methylbutanal during the fourth week of storage compared to samples stored at 5 °C throughout the experiment. The general level of terpenes was also significantly increased due to storage at fluctuating temperatures.

Overall, this initial study showed that commercial slicing, fluctuating temperatures and an increased initial storage temperature increased the level of the microbially produced 2- and 3-methylbutanal in sliced saveloy. This suggests that these factors are important for the microbial activity and the development of spoilage in cooked and sliced meat products.

## 6.3 Identification of chemical markers for the sensory shelf-life of industrially sliced saveloy (PAPER II)

The purpose of PAPER II was to identify VOC's with potential as chemical markers for the sensory quality of saveloy. This was done based on a sample set consisting of sliced saveloy from 3 different slicing locations denoted A, B, and C. An overview of the experimental setup is given in Table 6. The use of different slicing locations and different initial storage temperatures created a set of samples with a large variation in the sensory scores, the level of microbial contamination and the aroma profile.

**Table 6. Experimental setup of PAPER II. Samples were sliced at 3 locations, A, B and C and stored initially for 3 weeks at either 5 °C or 8°C. Measurements of the aroma composition with GC-MS, the sensory profile and microbial plate counts were done at 5 measure points in the fourth week of storage.**

| Time | Location | Ini. Temp. | GC-MS | Sensory | Microbiology |
|---|---|---|---|---|---|
| **Week 3** | A, B, C | 5 C° | X | X | X |
| | | 8 °C | X | X | X |
| **Week 3+3 days CSS** | A, B, C | 5 C° | X | X | X |
| | | 8 °C | X | X | X |
| **Week 3+5 days CSS** | A, B, C | 5 C° | X | X | X |
| | | 8 °C | X | X | X |
| **Week 3+7 days CSS** | A, B, C | 5 C° | X | X | X |
| | | 8 °C | X | X | X |
| **Week 4 (Closed packages)** | A, B, C | 5 °C | X | X | X |
| | | 8 °C | X | X | X |

PLS-models were used to model the sensory descriptors meaty odor and sour&old odor (Y-variables) based on the GC-MS measurements (X-variables). Based on the PLS-models, the 10 VOC's which had the highest impact on the sensory descriptors were selected. The selection of the VOC's was based on the numeric value of their regression coefficients to the relevant sensory descriptor in the PLS-models. The final PLS-models of meaty odor and sour&old, which included only the 10 selected VOC's, had relatively low prediction errors (RMSECV) and captured the vast majority of the variation in the sensory descriptors. The main characteristics of the final PLS-models are shown in Table 7. Using these PLS-models it was possible to predict the scores in meaty odor and sour&old odor of a given saveloy sample based on the peak areas of the 10 selected VOC's.

**Table 7. Characteristics of the PLS-models predicting meaty odor and sour&old odor in PAPER II.**

| | Meaty odor | Sour&old Odor |
|---|---|---|
| **Variation in sensory evaluation** | 1.5-4.4 | 2.4-11.7 |
| **RMSECV** | 0.5 | 1.5 |
| **$R^2$ (Predicted vs. measured)** | 0.61 | 0.72 |
| **PC's** | 2 | 2 |
| **Y-explained** | 71 % | 80.5 % |

The predicted vs. measured plots of the PLS-models describing meaty odor and sour&old odor are shown in Figure 16. These plots illustrate the ability of the PLS-models to predict the sensory scores of the individual saveloy samples in the experiment. The samples from location B (green symbols) measured after package opening generally had lower scores in meaty odor and higher scores in sour&old odor compared to the remaining samples. However, as seen in Figure 16 it was also these samples which had the highest deviation between predicted and measured values of the sensory descriptors. The predicted scores of meaty odor and sour&old odor of sample 5B7 (5°C, location B, Week 3+7 days) was particularly off target. From Figure 16 it is moreover seen that samples from location A (red symbols) showed very limited signs of spoilage during the storage period whereas samples from location C (blue symbols) reached relatively high scores in sour&old odor after the introduction of CSS in the fourth week of the experiment. The sour&old odor of 5 samples from location B and C (8C7, 8C5, 5C7, 5B3, 8BK) with intermediate scores in sour&old odor was moreover under-predicted by the model. This suggests that there is still room for optimization of the developed PLS-model. The models could be optimized by increasing the number of samples used in the model significantly and moreover by fitting individual PLS-models for each of the 3 slicing locations.

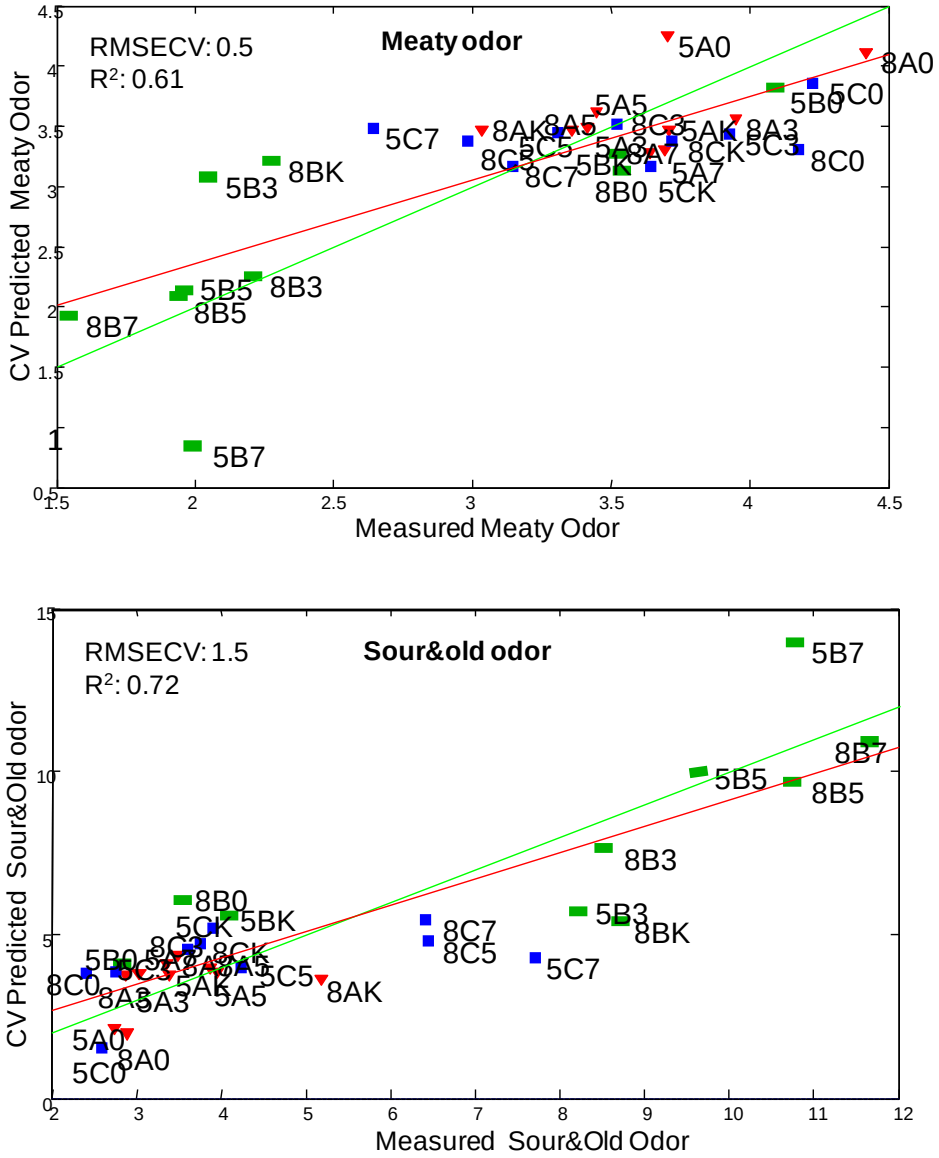


**Figure 16. Predicted vs. measured plot of the final PLS-models of meaty odor (top) and sour&old odor (bottom). The denotation accounts for the initial temperature (5 or 8 °C), the slicing location (A, B or C) and the days of storage after package opening (0, 3, 5 or 7). k represents samples stored 4 weeks in closed packages. Samples from location A are represented by red symbols whereas samples from location B and C are represented by green and blue symbols respectively. The red line in the figure represents the actual relation between the predicted and measured values of the sensory descriptor whereas the green line represents the perfect relation between predicted and measured values.**

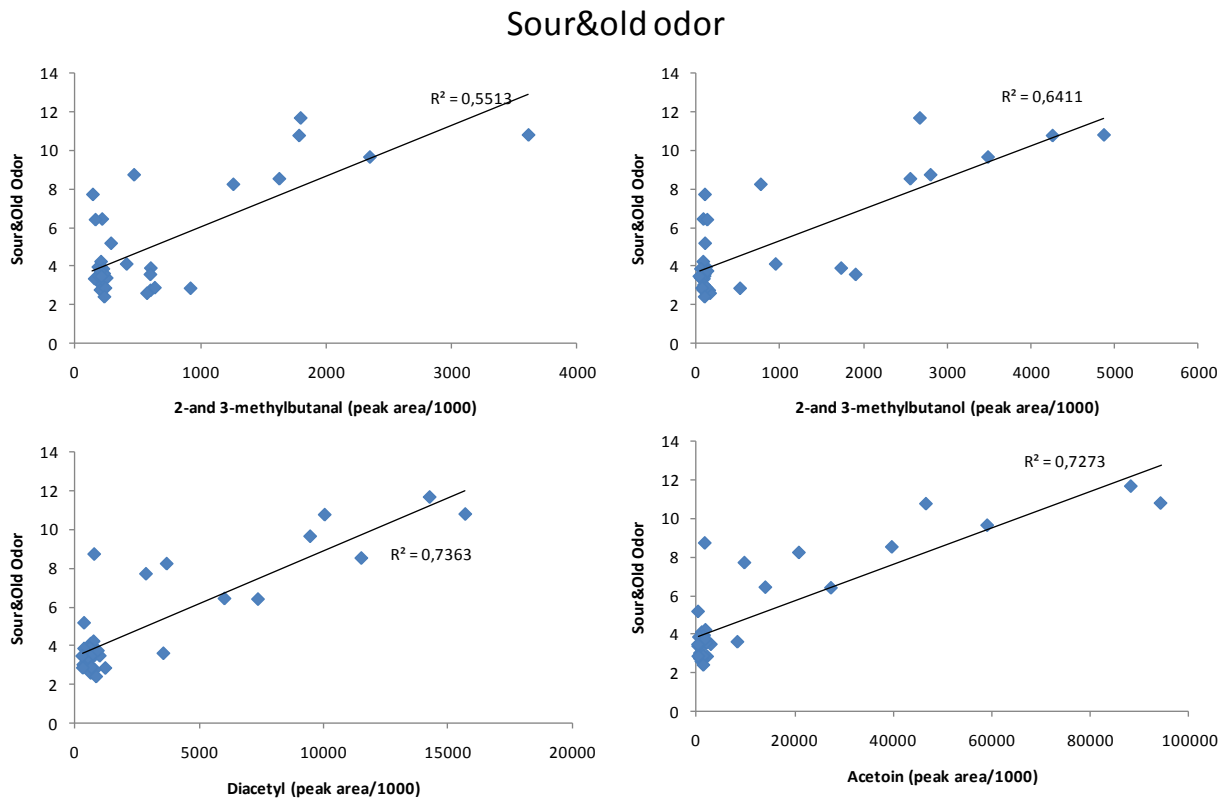


**Figure 17. Sour&old odor plotted against the peak area of the identified chemical markers (2- and3- methylbutanol, 2 and 3-methylbutanal, acetoin and diacetyl).**

The VOC's acetoin, diacetyl, 2- and 3-methylbutanal and 2- and 3-methylbutanol were found to have the highest regression coefficients towards the sensory scores of meaty odor and sour&old odor. These VOC's were therefore concluded to be chemical markers for the sensory shelf-life of saveloy. In order to study the relation between the identified chemical markers and the sensory descriptor sour&old odor these are plotted against each other in Figure 17. From this figure it is seen that acetoin and diacetyl generally were better correlated to the sour&old odor than 2- and 3- methylbutanal and 2- and 3-methylbutanol. All the suggested chemical markers have previously been related to spoilage of luncheon meat products by *B. thermosphacta* in a study by Stanley *et al*., 1981 *(50)*. 3-methylbutanol was moreover related to microbial growth in artisan-type sliced ham by Leroy *et al*., 2009 *(79)*.

## 6.4 Formation of VOC's in saveloy inoculated with potential spoilage bacteria (PAPER III)

In order to learn more about the microbial formation of the VOC's, which were found to have potential as chemical markers for spoilage in PAPER II, sliced saveloy samples were inoculated with either *B. thermosphacta*, *C. luteola*, *Leuc. carnosum* or *C. maltaromaticum*. An uninoculated control series and a 1:1:1:1 mixture of 4 potential spoilage bacteria was also included. An overview

of the analyses done on each of the 6 experimental series at different sampling times during the experiment is given in Table 8.

From Table 8 it is seen that the aroma composition was studied at regular intervals during the 4 weeks expected shelf-life period. This was done in order to investigate the early changes in the aroma composition caused by inoculation of spoilage bacteria, and to find out whether these changes were characteristic for the development of spoilage. However, no significant change in the peak area of the identified chemical markers acetoin, diacetyl, 2- and 3-methylbutanal and 2- and 3- methylbutanol was found between day 1 and week 1 for any of the investigated spoilage bacteria. This was hypothesized to be because the bacteria were in the lag phase and their metabolic activity therefore was limited. Further investigations could be directed towards testing whether the sensitivity of the dynamic headspace sampling and the GC-MS measurement could be improved in order to achieve an early detection of changes in chemical the markers caused by spoilage bacteria.

**Table 8. An overview of the experimental setup of PAPER III. The 6 series of saveloy samples were subjected to analysis as described below.**

| Time | GC-MS | Sensory | Microbiology | Free fatty acids | Free amino acids |
|---|---|---|---|---|---|
| **Day 1** | X | X | X | X | X |
| **Week 1** | X | | | | |
| **Week 2** | X | | | | |
| **Week 3** | X | X | X | X | X |
| **Week 3+3 days CSS** | X | X | X | X | X |
| **Week 3+7 days CSS** | X | X | X | X | X |
| **Week 4 (closed packs)** | X | X | X | X | X |

Inoculation of the sliced saveloy samples with the 4 potential spoilage bacteria resulted in different aroma profiles and different sensory profiles during storage. The differences in the sensory profile of the 6 experimental series are illustrated in the 'spider web' plots in Figure 18. *B. thermosphacta*, *C. luteola* and *C. maltaromaticum* were responsible for the highest degree of sensory spoilage in the saveloy samples. However, as seen in Figure 18, the 3 bacteria affected the sensory profiles differently. Spoilage as a result of *C. luteola* inoculation was characterized by a high increase in old odor and a moderate increase in sour odor. These changes were observed after the saveloy packages were subjected to CSS in the final week of the experiment (week 3+3 days and week 3+7 days). However, samples inoculated with *C. luteola* and stored in closed packages for 4 weeks were also moderately spoiled. Spoilage by *B. thermosphacta* and *C. maltaromaticum* resulted in increases in butter odor, old odor and sour odor in week 3+3 days and week 3+7 days. Inoculation with *C. maltaromaticum* moreover resulted in sensory spoilage after storage for 4 weeks in closed packages, and as seen in Figure 18 the sensory scores of these samples were similar to those observed in the samples subjected to CSS. However, saveloy samples inoculated with *B. thermosphacta* and stored for 4 weeks in closed packages only showed

very limited signs of sensory spoilage. The sensory profile of samples inoculated with the mixture of all 4 spoilage bacteria was very similar to the profile of the samples inoculated with *Leuc. carnosum*. This indicated that *Leuc. carnosum* dominated the mixed spoilage flora. Inoculation with *Leuc. carnosum* only resulted in limited sensory spoilage in form of a small increase in sour odor.

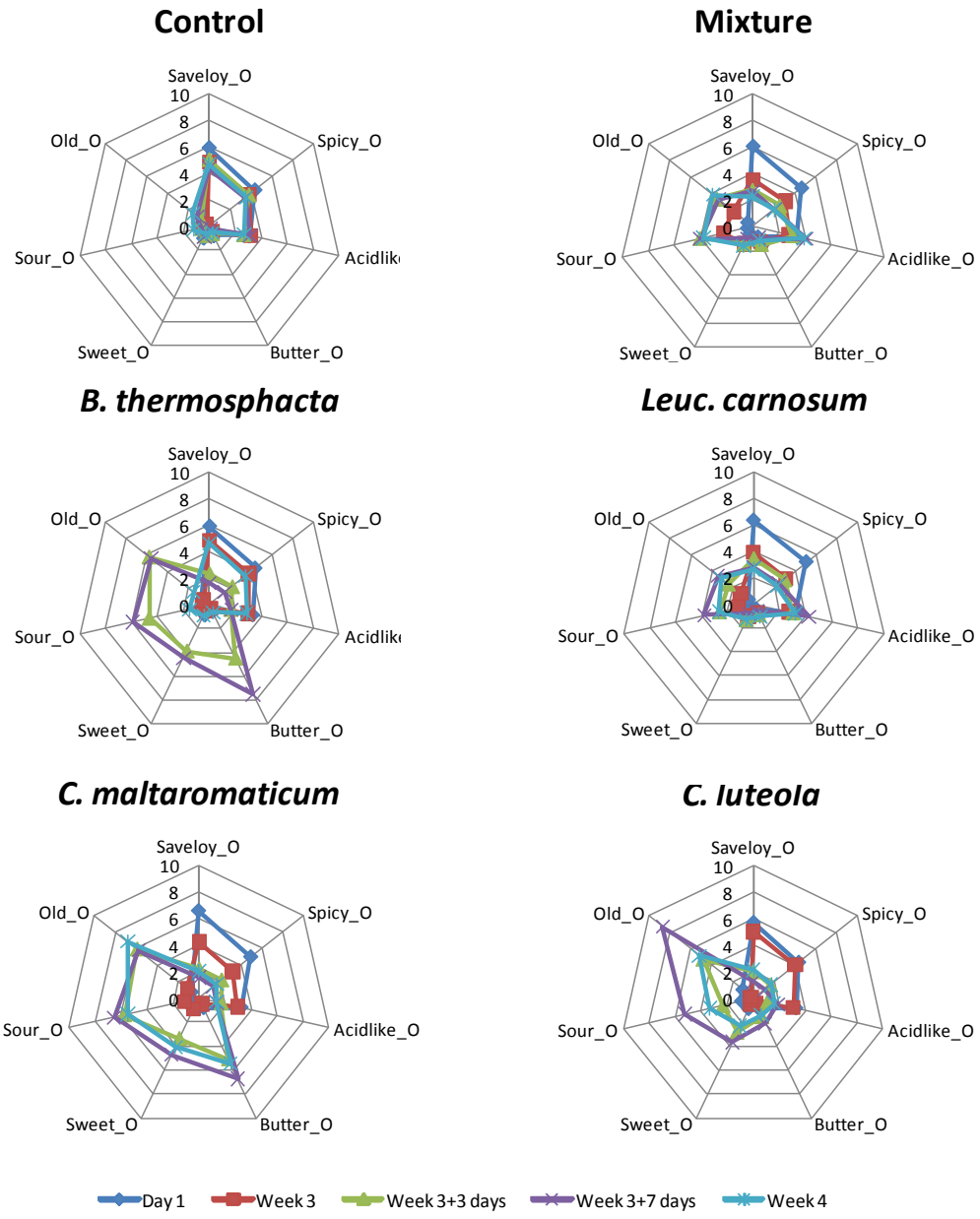


**Figure 18. Spider web plots providing an overview of the sensory profile of the six experimental series measured at day 1, week 3, week 3+3 days, week 3+7 days and week 4.**

**Table 9. The characteristics of the PLS-models predicting the sensory descriptors based on the 42 VOC's**

| Y-variable | Saveloy odor | Spicy odor | Butter-like odor | Sweet odor | Sour odor | Old odor |
|---|---|---|---|---|---|---|
| **Variation in sensory descriptor** | 1.3-6.6 | 1.1-5.1 | 0.3-7.5 | 0.4-4.7 | 0.2-6.5 | 0.2-8.7 |
| **RMSECV** | 1.02 | 0.68 | 0.93 | 0.88 | 1.24 | 1.59 |
| **$R^2$** | 0.69 | 0.68 | 0.81 | 0.63 | 0.63 | 0.57 |
| **PC's** | 2 | 2 | 3 | 2 | 2 | 2 |
| **Y-explained** | 80.1 | 77.3 | 87.3 | 74.0 | 73.1 | 70.8 |

The changes in 6 sensory descriptors were modeled based on the peak area of the 42 identified VOC's using PLS. This resulted in 6 PLS-models which accounted for different aspects of sensory spoilage. The characteristics of these PLS-models are shown in Table 9.

The 10 most important VOC's for each of the sensory descriptors were identified based on the numeric value of the regression coefficient towards the individual VOC's. Acetoin, diacetyl and 2- and 3-methylbutanol were among the 4 most important VOC's for all 6 sensory descriptors, and are therefore of general importance for the development of microbial spoilage in cooked and sliced meat products. 2- and 3- methylbutanal was the most important VOC for the development of sweet odor and butter-like odor and was moreover among the ten most important VOC's for the remaining sensory descriptors. 2-Methylpropanol was furthermore among the 5 most important VOC's to all the 6 sensory descriptors. Dimethyl disulphide, dimethyl trisulfide, acetic acid and octanal also frequently appeared among the 10 most important VOC's. These results confirmed the role of acetoin, diacetyl, 2-and 3-methylbutanol and 2- and 3-methylbutanal as chemical markers for the development of spoilage in cooked and sliced meat products. A link between 2-methylpropanol and sensory spoilage was moreover found in this experiment. *B. thermosphacta*, *C. luteola* and *C. maltaromaticum* were all found to produce acetoin, diacetyl, 2- and 3-methylbutanol and 2- and 3-methylbutanal in varying amounts. However, these compounds were not produced in samples with *Leuc. carnosum.* This corresponds well with the sensory changes observed after inoculation of these bacteria on sliced saveloy. These findings moreover correspond well with the results of PAPER II and observations made in the previous literature *(50, 79, 90)*.

The predicted vs. measured plots of from the PLS-models predicting saveloy odor and sour odor based on the 42 VOC's are shown in Figure 19. Sour odor and saveloy odor represents 2 opposite ways of viewing the quality changes in sliced saveloy. Sour odor described an increase off-odors with storage time whereas saveloy odor described a decrease in the positive odor attributes of fresh saveloy. However, as seen in Figure 18, the saveloy odor of the uninoculated control samples was relatively constant. The decrease in saveloy odor is therefore indirectly caused by an increase in microbially produced off-odors, which mask the VOC's contributing to the pleasant odor to the product. As seen in Figure 19 there is generally good correlation between the predicted and measured scores of saveloy odor and sour odor. However, for both descriptors the sensory score

of the sample inoculated with *B. thermosphacta* (Bt3+7) is significantly over- or under- predicted. This sample contained the highest amount of diacetyl, acetoin and 2- and 3- methylbutanol of all the samples in the experiment. This could suggest that the PLS-models have problems handling very high levels of the chemical markers.

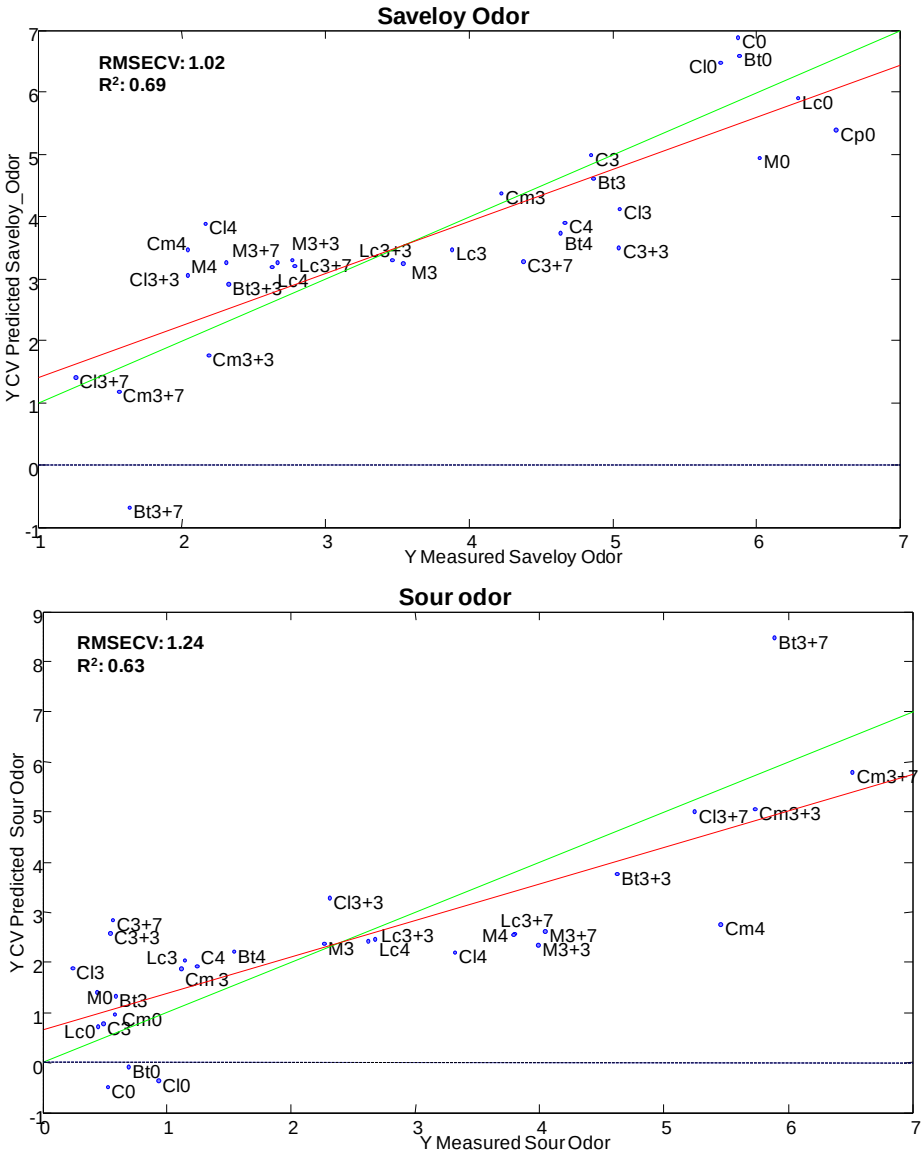


**Figure 19. Predicted vs. Measured plots for the PLS-models of saveloy odor (top) and sour odor (bottom). The following denotation is used C (control samples), M (Mixture samples), Bt (*B. thermosphacta*), Cl (*C. luteola*), Lc (*Leuc. carnosum*), Cm (*C. maltaromaticum*), 0 (day 1), 2 (week 3), 3+3 (week 3+3 days with CSS), 3+7 (week 3+7 days with CSS), 4 (week 4). The red line in the figure represents the actual relation between the predicted and measured values of the sensory descriptor whereas the green line represents the perfect relation between predicted and measured values.**

As seen in Table 8 the experiment also included measurements of free fatty acids (FFA's) and free amino acids (FAA's) which are precursors of VOC's in meat products. The percentage of FFA's in the saveloy samples was determined by extraction of the fat fraction followed by titration with sodium hydroxide, whereas the FAA's measurement was done by extraction followed by derivatization and GC-MS measurement. However, there was no significant effect of the inoculation of spoilage bacteria on the percentage of FFA's in the saveloy. This could indicate that the tested spoilage bacteria did not metabolize FFA's in this experiment. Leucine and valine, which are precursors of 3-methylbutanal and 2-methylpropanol respectively, were among the 6 FAA's found in the saveloy samples. The control series generally contained significantly higher amounts of FAA's compared to the inoculated series. However, no differences in the content of the FAA's between the 5 inoculated series were found, though these series had different sensory profiles. Therefore no clear relation between the investigated aroma precursors and the development of spoilage was found, and the measurements of FFA's and FAA's did not contribute to the understanding of the development of spoilage in this experiment.

## 6.5 At-line detection of chemical markers for shelf-life with PTR-MS (PAPER IV)

PTR-MS was studied as an alternative method for measurement of the VOC's contributing to spoilage of cooked and sliced meat products. As discussed earlier this method has some advantages and disadvantages compared to GC-MS. However, PTR-MS is a robust instrument which can provide rapid on-line measurements of VOC's, and it therefore has potential for applications in the meat industry. In PAPER IV it was tested whether PTR-MS was a useful tool for measurement of changes in the aroma composition of cooked and sliced meat products during storage. This was done with specific focus on the chemical markers for sensory spoilage identified in PAPER II and on the ability of PTR-MS to detect these markers in a complex sample matrix.
The experiment was divided in two. In the first and main experiment the changes in industrially manufactured saveloy was studied in a 6 week storage period with PTR-MS, GC-MS and in a small sensory study as shown in Table 10. The second experiment included PTR-MS measurements of 4 types of cooked and sliced meat products in the middle of their shelf-life period and again near the expiry date after the packages were opened in the final week of storage.

**Table 10. Overview of the experimental setup of the first part of the experiment with PTR-MS, GC-MS and sensory measurements of sliced saveloy in a 6 weeks storage period.**

| Time | PTR-MS | GC-MS | Sensory |
|---|---|---|---|
| **Day 1** | X | X | X |
| **Week 3** | X | | |
| **Week 3+4 days opened** | X | | |
| **Week 3+6 days opened** | X | | |
| **Week 4** | | X | X |
| **Week 4+4 days opened** | | X | X |
| **Week 4+6 days opened** | X | X | |
| **Week 5** | X | X | X |
| **Week 5+4 days opened** | X | | |
| **Week 5+6 days opened** | X | | |

In the first part of the experiment PTR-MS measurements of the industrially manufactured saveloy were compared to the sensory evaluations and the GC-MS measurements. From the GC-MS measurements it was seen that that the main change in the saveloy samples during the first 5 weeks of the experiment was a loss in the peak area of VOC's contributing to the fresh odor of the product. This was for example seen as a decrease in the peak area of several of the terpenes found in the product along with a decrease in compounds like 2-butanone and hexanal which were produced in thermal lipid oxidation. In the PTR-MS measurements m/z 137 and 81 were assigned to monoterpenes in general, and these two masses were found to decrease significantly with storage time. The fragmentation of aldehydes and alcohols during PTR-MS measurement made it difficult to assign a specific m/z-ratio to the VOC's formed in lipid oxidation. However, m/z 57, which is a typical alcohol fragment *(23)*, was found to decrease with storage time. Hexanal contributes mainly to m/z 83 and m/z 55 where the latter is a general aldehydes fragment *(23)*. The level of m/z 83 decreased slightly during the first 5 weeks, as seen with GC-MS measurements, but started to increase again at week 5+4 days. The fragmentation of ketones during PTR-MS measurement is very limited and the most abundant mass of 2-butanone was therefore m/z 73, which is the protonated molecular ion. 2-Butanone, measured as m/z 73, was found to decrease significantly with storage time when measured with PTR-MS *(23)*.

The headspace of the saveloy samples only showed limited signs of microbially induced changes during storage since no significant increase in microbially produced VOC's was found in the GC-MS and PTR-MS measurements. Of the chemical markers suggested in PAPER II and III only acetoin and 2- and 3- methylbutanol were detected in the GC-MS measurements. However, these compounds were only a minor part of the total aroma in saveloy samples even in the packages measured with GC-MS in week 4+4 days opened and week and week 4+6 days opened. In the PTR-MS measurements signs of microbial activity were seen in week 5+4 days opened and week 5+6 days opened. Here 1 of 5 repetitions was separated from the remaining in a PCA model due to high levels of m/z 71 and 87 along with other masses (see PAPER IV). M/z 87 is the protonated

molecular ion (M+1) of diacetyl and 2- and 3-methylbutanal, whereas m/z 71 is a fragment of 3 methylbutanol *(23)*.

Overall, there was good correspondence between the PTR-MS and GC-MS measurements. As mentioned only limited amounts of microbially produced VOC's were found in the industrially produced saveloy samples tested in this experiment, and these samples therefore appeared to be more resistant to spoilage compared to the saveloy samples used in PAPER II and III. This could be due to a high hygienic standard of the tested processing facility, which would result in a low 'inoculation' level of the samples. Furthermore, no temperature fluctuations were applied to the saveloy samples in this experiment, which could slow down the microbial growth rate significantly. The industrial recipe used in this experiment could moreover contain a higher level of preservation agents, compared to the recipes used in PAPER I-III. This would also slow down the growth rate of the spoilage.

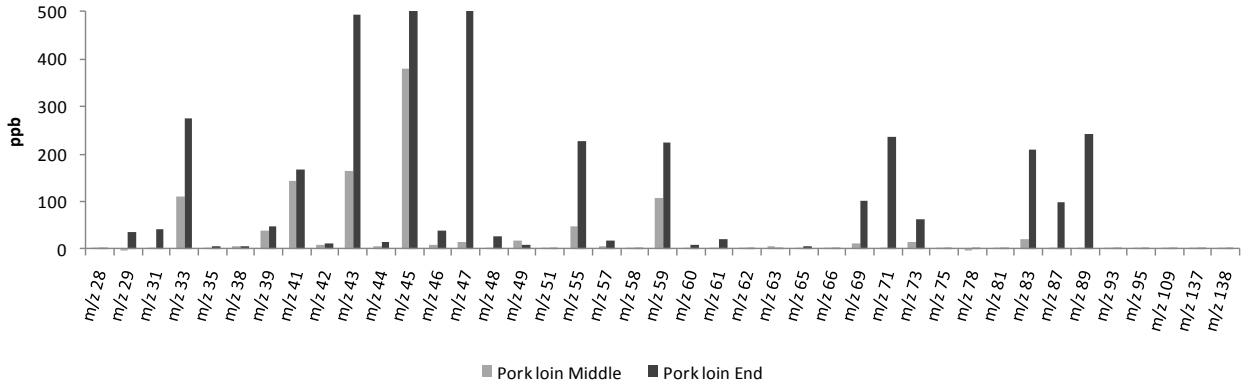


**Figure 20. Overview of PTR-MS measurements of cooked pork loin in the middle and the end of the shelf-life period. Note that the masses 43, 45, and 47 in this figure are cut off at 500 ppb, and were present in higher amounts than indicated.**

In the second part of the experiment cooked and sliced pork loin, sandwich ham and the Danish products 'rullepølse' and 'jægerpølse' were purchased in a supermarket and measured twice during their remaining shelf-life period. The samples were measured in the middle of their 4 week shelf-life period without prior package opening and again near their expiry date after being stored in open packages for 1 week. Compared to the saveloy samples, the 4 types of cooked and sliced meat product were likely to have been subjected to temperature fluctuations during distribution and storage in the supermarkets. Unlike the saveloy samples, the 4 types of cooked and sliced meat product showed clear signs of microbial spoilage in the PTR-MS measurements. This is illustrated in Figure 20 where the difference in the content of selected masses, between the middle and the end of the shelf-life period, is shown for cooked pork-loin. Masses 69, 71, 87 and 89 were among those found to increase significantly between the measurements. M/z 89 is the protonated molecular ion of acetoin whereas a fragment of 2- and 3-methylbutanal contributes to m/z 69. M/z 71 and 87 were assigned above. Together masses 69, 71, 87 and 89 account for the chemical markers for sensory spoilage identified in PAPER II. However, it should be considered

that other VOC's are likely to contribute to these masses. Ethyl acetate, which was found in the saveloy samples, also contributes to m/z 89 whereas several aldehydes contribute to m/z 69. Nevertheless, the clear increase in these masses in the pork loin samples was associated with spoilage. Clear off-odors were moreover registered when preparing the samples, of especially pork loin and 'rullepølse', for measurement.
This experiment showed that PTR-MS was a suitable method for measurement of VOC's in cooked and sliced meat products. Though fragmentation makes the data processing challenging it was possible to detect spoiled samples via specific masses.

## 6.6 Discussion and evaluation of the results

The outcome of this PhD-project can roughly be divided into 3 main results.

- Chemical markers for the sensory quality of cooked and sliced meat products have been identified.
- PLS-models able to predict sensory quality based on these chemical markers have been developed.
- PTR-MS was successfully tested as a tool for at-line measurement of the identified chemical markers.

In the following section the strengths and weaknesses of these results will be discussed and evaluated.

**Identification of chemical markers**. Part of the objective of this PhD-project was to identify chemical markers for the sensory quality of cooked and sliced meat products. This was also the purpose of PAPER II where the peak areas of acetoin, diacetyl, 2- and 3-methylbutanal and 2- and 3-methylbutanol, via PLS-modeling, were found to be closely related to the sensory descriptors meaty odor and sour&old odor. The relation between these VOC's and the development of sensory spoilage in saveloy samples was confirmed in PAPER III. In this paper the inoculation of sliced saveloy with *B. thermosphacta*, *C. maltaromaticum* and *C. luteola* was found to have a negative impact on the sensory profile. This negative impact was related to the formation of acetoin, diacetyl, 2- and 3-methylbutanal, 2- and 3-methylbutanol and 2-methylpropanol in PLS-models. Several of the studies in the existing literature, which were discussed earlier, confirm the relevance of the highlighted VOC's in development of microbial spoilage *(2, 79, 90, 93, 104)*. This obviously adds further to the credibility of the results seen in PAPER II and PAPER III. Acetoin, diacetyl, 2- and 3-methylbutanal, 2- and 3- methylbutanol and 2-methylpropanol were therefore concluded to be chemical markers for sensory quality of cooked and sliced meat products.
The use of CSS in the experimental setup has had some effect on the outcome of PAPER II and PAPER III. The production of the suggested chemical markers was markedly increased in samples subjected to CSS during the fourth week of the experiments compared to samples stored in closed packages throughout the experiments. CSS was used because it was considered highly relevant in order to get a realistic picture of the development of spoilage in cooked and sliced meat products.

This implies that the chemical markers identified in PAPER II and PAPER III account for the development of spoilage in saveloy samples stored at consumer simulated conditions rather than the formation of spoilage under the conditions recommended by the manufacturers. The VOC's suggested as spoilage markers do therefore not necessarily account for the gradual souring, which is often observed in meat products stored in vacuum or MA-packing throughout the shelf-life period *(2, 65)*. Under these circumstances compounds such as lactic acid and other organic acids could be probable chemical marker for spoilage.
However, acetoin, diacetyl, 2- and 3-methylbutanal, 2- and 3- methylbutanol and 2-methylpropanol were generally able to account well for the development of spoilage in sliced saveloy samples stored as described in PAPER II and PAPER III. The results of these two papers moreover showed that the identified chemical markers were able to account for the spoilage of saveloy caused by 'real' factory floras as well as samples inoculated with monocultures of different spoilage bacteria. The development of spoilage in the 4 different types of cooked and sliced meat products studied in PAPER IV was furthermore related to an increase in 'PTR-MS masses' corresponding to diacetyl, acetoin, 2- and 3- methylbutanal and 2- and 3- methylbutanol. Overall, the results of this PhD-project indicate that the suggested chemical markers can be used to predict sensory spoilage caused by a wide range of the of the SSO's relevant in cooked meat products. The results moreover show that the chemical markers are valid in different types of cooked and sliced meat products.

**Development of models for sensory quality of cooked and sliced meat products**. A practical application of the identified chemical markers in the meat industry would involve the development of a model able to predict the sensory quality of a given product based on these chemical spoilage markers. In this PhD-project PLS-models were used in an attempt to achieve this in PAPER II and PAPER III. However, these experiments were designed mainly to describe the development of spoilage and to identify the chemical markers for sensory shelf-life which were discussed above. The slicing of the samples at different locations and inoculation with different spoilage bacteria ensured that spoilage caused by different SSO's was incorporated in the PLS-models. This means that the samples included in the data material of PAPER II and PAPER III cover a wide range of the spoilage which is expected to be encountered in cooked and sliced meat products in practice. However, the number of samples included in the experiment, in order to account for this variation, is relatively low. The development of a shelf-life model for practical applications in cooked and sliced meat products should be based on a much higher number of samples and on a thorough validation using test set samples. Based on the results of PAPER II, it could furthermore be considered to limit the amount of variation included in this shelf-life model. This could be achieved by developing shelf-life models for the individual processing facilities rather than constructing a generally valid model. This approach was used with some success by Kreyenschmidt et al. 2010 *(102)*. A clear definition of spoilage based on the sensory evaluations should furthermore be developed for an easy classification of the saveloy samples into a limited number of spoilage categories. Measurements of consumer acceptability, rather than a complete

sensory profile, could therefore be a simpler way to express the level of spoilage in a given saveloy sample.

When considering the above the PLS-models developed in PAPER II and PARER III should be seen as a preliminary attempt to model the sensory evaluation of saveloy samples based on the composition of VOC's found in the sample headspace. The details of these PLS-models, including the RMSECV and the coefficient of determination ($R^2$) of the predicted vs. measured plot, are seen in Table 7 and Table 9. In the developed PLS-models VOC's were able to capture the main variation of the sensory descriptors using between 2 and 3 PC's. When considering the high level of variation between the samples included in the PLS-models from PAPER II and PAPPER III the resulting RMSECV of these models was acceptable. The PLS models made in PAPER II had particularly good RMSECV compared to the observed span of the sensory scores. However, as seen in Figure 16 and Figure 19, the predicted sensory score did not correspond well with the measured sensory score for all samples, and the predictive ability of the PLS-models could therefore still be improved.

The PLS-models presented in PAPER II and PAPER III are able to predict the sensory score of a given sample based on measurements of specific VOC's. These models can be used directly to control the present sensory quality of a product at various stages during the shelf-life period. This would be useful in the supermarkets where measurements of the chemical markers could predict whether the actual shelf-life of a given meat product corresponds with the expected shelf-life. The developed PLS-models would therefore provide a tool for quality assurance of cooked and sliced meat products displayed for sale in the supermarkets. Further research in this application could be directed towards developing sensors in the packing material, which would react to high levels of these chemical markers in the sample headspace.

**Early detection of the chemical markers**. Another part of the objective of this PhD-project was to investigate whether VOC's could be used in the meat industry for early detection of sensory changes in cooked and sliced meat products. In this context the ability of the PLS-models to predict the current sensory quality of cooked and sliced meat products, as described above, is of limited use. Models able to predict the future sensory quality of a given product should therefore be developed for practical applications in the meat industry. Such models could provide an early warning of a decrease in the shelf-life of a given batch of meat product. However, In order to achieve this, the PLS-models developed in PAPER II and PAPER III need to be reconstructed.

Based in the data described in PAPER III this was done by combining the GC-MS measurements from day 1 with the sensory scores obtained in week 3+7 days with CSS. A PLS-model based on this combination of the data would theoretically be able to predict the sensory quality of the product at the end of the shelf-life period based on the GC-MS measurements from the beginning of the shelf-life period. However, due to the reconstruction of the dataset from PAPER III the vast majority of the samples were excluded, and only one sample from each of the six experimental series remained. The PLS-models calculated based on these data are therefore mainly of theoretical interest. Nevertheless, the resulting PLS-model showed that there was no obvious

relation between the GC-MS measurements from day 1 and the sensory scores in from week 3+7 days with CSS. A similar PLS-model showed that measurement of the identified chemical markers at week 3 to some extent were able to predict the sensory scores at week 3+7 days with CSS. However, this model was not able to account for the significant decrease in sensory quality observed for the sample inoculated with *B. thermosphacta* in week 3+7 days with CSS. Therefore this preliminary attempt to predict sensory shelf-life of saveloy samples based on the GC-MS measurements from day 1 and week 3 is concluded to be unsuccessful. However, because of the limited number of samples included in these PLS-models, no general conclusions regarding the use of VOC's as early markers of sensory spoilage should be made based on these results.

The early changes in the aroma composition of sliced saveloy were also studied in PAPER III. GC-MS measurements of the aroma composition were done on day 1 after slicing and inoculation and again after 1 week storage at 5°C. However, no significant change in the peak area of the identified chemical markers was found in the saveloy samples in this period of time. Based on this result it appears to be difficult to achieve shelf-life predictions based on early detection of the identified chemical markers. The microbial flora of cooked and sliced meat products derive from the handling and slicing prior to packing. In the early stages after slicing and packing the microbial population of the product is in the lag phase where it adapts to the new microenvironment. During the lag phase the level of microbial growth is very low. It therefore appears plausible that it is difficult to detect signs of microbial metabolism in the sample headspace shortly after slicing. However, the early changes in the aroma composition of cooked and sliced meat products should be investigated in more detail before a final conclusion regarding the possibility using VOC's for early detection of microbial spoilage can be made.

Different methods of increasing the sensitivity of the measurements of the aroma composition could be tested. The release of VOC's from the saveloy samples in the dynamic headspace sampling could be improved by increasing the total purge volume or by increasing the sampling temperature. The sensitivity of the MS could moreover be improved by the focusing on the detection of single ions corresponding to the identified chemical markers. This would apply for GC-MS- as well as PTR-MS measurements. Acceleration of the microbial processes in the product could moreover be a method of achieving early detection of shelf-life problems in cooked and sliced meat products. This could for example be done by storing the samples at increased temperatures after slicing and packing. Accelerated shelf-life studies could shorten the storage time needed for a detectable increase in the level of microbial metabolites considerably. However, the increased temperatures could also select for spoilage by other bacteria than the expected spoilage flora which would result in a misleading predictions. A small accelerated shelf-life study was done in connection with PAPER IV where saveloy samples were stored at 10 °C and 20 °C for 4 days in MA-packing (data not shown). No significant increase in the peak areas of the indentified chemical markers was observed in the GC-MS measurements as a result of this. This could be partly ascribed to the high stability observed for the saveloy samples tested in PAPER IV. However, this result was not promising for the use of accelerated shelf-life tests as a tool to obtain early detection of spoilage in cooked and sliced meat products.

The experimental work of PAPER III included measurement of the percentage of FFA's and concentration of FAA's in the saveloy samples. FFA's and FAA's are precursors of some of the VOC's found in spoiled meat products, and could therefore also have potential as early indicators of spoilage in cooked and sliced meat products. The amino acids leucine, isoleucine and valine would be of particular interest as they are precursors of the suggested chemical markers 3-methylbutanal, 2-methylbutanal and 2-methylpropanol respectively *(96)*. However, based on the results of PAPER III neither FFA's nor FAA's were suitable as spoilage markers in cooked and sliced meat products. There was no effect of inoculation of the saveloy samples with spoilage bacteria on the percentage of FFA's in the fat fraction. The concentration of FAA's in the saveloy samples was moreover not able to explain the differences in spoilage level caused by the 4 tested spoilage bacteria. Stolzenbach et al. 2009 *(99)* and Laursen et al. 2009 *(81)* tested α-ketoisocaproic acid, which is an intermediate product in the conversion of leucine to 3-methylbutanal, as a spoilage marker in Danish 'rullepølse'. However, in correspondence with the results of PAPER III, these studies did not find a clear relation between the aroma precursor α-ketoisocaproic acid and the development of spoilage in 'rullepølse'.
Overall, no final conclusion was reached in this PhD-project regarding the possibility of predicting the sensory shelf-life of cooked and sliced meat products based on measurements made shortly after slicing and packing. However, the results of PAPER III suggested that the possibility of using the identified chemical markers for early detection of spoilage is limited.

**PTR-MS measurements as a tool for at-line measurement of the chemical spoilage markers**. A practical application of a predictive PLS-model based on the identified chemical markers would require the development of a robust, fast and easy applicable tool which could be used by employees in supermarkets or in the meat industry after limited training. The dynamic headspace extractions coupled with GC-MS, which were used to identify the suggested chemical markers in PAPER II and PAPER III, would clearly be unsuited for this application. GC-MS measurements are time consuming due to the chromatographic step, and it moreover requires highly trained personal to operate and maintain the GC-MS instrument. Therefore PTR-MS was tested in PAPER IV as an alternative method for detection of the identified volatile chemical spoilage markers. The PTR-MS technology could possibly be applied in the meat industry as a tool for early measurement of the expected shelf-life of a cooked and sliced meat product. However, this presumes that later occurring sensory changes in cooked and sliced meat products can be detected in the volatile profile shortly after slicing and packing. Rapid at-line measurements of the aroma profile could be done directly in the packages by connecting a hypodermic needle to the PTR-MS inlet. A simple warning system, triggered by a high level of specific 'PTR-MS masses', could be used to identify batches with an increased risk of premature spoilage. The implementation of such a warning system in the meat industry would decrease the risk of distributing and selling products of decreased microbial quality to the consumers. The warning system would moreover indicate whether the level of microbial contamination from the processing equipment had reached an unacceptable level, and could therefore also be used to indicate that thorough cleaning is needed.

PTR-MS measurements could moreover reveal the possible changes in the level of microbial contamination during a normal production day and hereby assess the quality fluctuation of the output from a given meat processing facility.

The results of PAPER IV showed that PTR-MS was able to account for the general changes in the aroma composition of cooked and sliced meat products during storage. However, these changes were difficult to associate with specific VOC's due to the fragmentation of particularly alcohols and aldehydes. Unfortunately only limited changes in the microbially produced VOC's were observed in the saveloy samples used to compare PTR-MS with GC-MS. Therefore it was not possible to compare GC-MS measurements of spoiled saveloy samples with corresponding PTR-MS measurements. However, PTR-MS measurements of cooked pork loin and 'jægerpølse', which showed clear signs of microbial spoilage, contained significantly increased levels of masses 69, 71, 87 and 89. These masses were assigned to the identified chemical markers for sensory shelf-life acetoin, diacetyl, 2- and 3- methylbutanal and 2- and 3- methylbutanol. Fragmentation of the identified chemical markers during PTR-MS measurement will have some impact on the sensitivity of the PTR-MS measurement because large percentages of these compounds are not accounted for in m/z 69, 71, 87 and 89. This indirectly decreases the ability of PTR-MS to detect the chemical markers in a complex food matrix. The contribution of other compounds to masses 69, 71, 87 and 89 will moreover mask changes in the level of the chemical markers and therefore make the interpretation of these measurements challenging.

Therefore further studies relating PTR-MS measurements of spoiled cooked and sliced meat products with sensory evaluations and GC-MS measurements would be very interesting in terms of gaining more knowledge of the relation between specific 'PTR-MS-masses' and microbial spoilage. However, as previously discussed the results of this PhD-project indicate that it could be difficult to predict spoilage of cooked and sliced meat products based on early measurements of the aroma composition. Nevertheless, further studies of potential applications of the PTR-MS technology in the meat industry would still be interesting. PTR-MS could possibly be used to study the formation of microbially produced VOC's from biofilm or other accumulating organic material on the processing equipment. Such measurements could potentially be used as an indicator of the hygienic standards of a given meat processing facility.

# 7. Conclusion

This PhD-project has investigated quality changes in cooked and sliced meat products during storage using mainly measurements of the aroma composition, the microbial composition and the sensory profile. The main focus has been to identify VOC's which could be used as chemical markers for the sensory quality of cooked and sliced meat products. It was hypothesized that such markers could be developed into a tool for early detection of sensory spoilage which would enable manufacturers of cooked and sliced meat products to identify batches with an increased risk of premature spoilage.

In PAPER II and PAPER III PLS-models were used to describe changes in the sensory evaluations of sliced saveloy based on GC-MS measurements of the aroma composition. In PAPER II saveloy samples were sliced at 3 different locations whereas the saveloy samples in PAPER III were inoculated with monocultures or a mixture of the potential spoilage bacteria *B. thermosphacta*, *C. maltaromaticum, C. luteola* and *Leuc. carnosum*. The PLS-models developed in PAPER II and PAPER III showed that the microbially produced VOC's acetoin, diacetyl, 2- and 3-methylbutanal, 2- and 3-methylbutanol and 2-methylpropanol were closely related to the sensory changes in sliced saveloy during storage. These VOC's were therefore concluded to be chemical markers for the sensory quality of cooked and sliced meat products. These results were obtained in studies which included saveloy samples from slicing locations with different spoilage floras, and samples inoculated with different spoilage bacteria which are relevant in meat products. This suggests that the VOC's identified as chemical markers for sensory quality are valid for at wide range of the spoilage bacteria encountered in cooked and sliced meat products in practice.

The changes in the aroma composition of saveloy shortly after slicing and packing were investigated in PAPER III. However, no significant changes in the suggested chemical markers were found between day 1 and week 1 after slicing and packing. An optimization of the dynamic headspace extraction and the GC-MS analysis was suggested for improvement of the sensitivity of the measurements of the aroma composition. Accelerated shelf-life experiments were moreover suggested to shorten the time needed for a detectable change in the aroma composition. However, based on the results of PAPER III the potential of VOC's as early markers for sensory changes in cooked and sliced meat products seems limited.

PTR-MS is an interesting technique for at-line/on-line measurement of VOC's, which is faster and easier to use in a production environment than traditional GC-MS measurements. PTR-MS was therefore tested as an at-line tool for detection of the chemical markers for sensory quality in PAPER IV. Here the ability of PTR-MS to account for quality changes in industrially sliced saveloy was compared to corresponding GC-MS measurements. Fragmentation of particularly alcohols and aldehydes during PTR-MS measurements made it somewhat complicated to interpret the resulting mass spectra. Nevertheless, a good correspondence between measurement of the changes in the aroma composition with PTR-MS and GC-MS was found. PTR-MS measurements made on samples of cooked pork loin and 'rullepølse' close to their expiry date moreover showed that these samples contained high levels the 'PTR-MS masses' 69, 71, 87 and 89. These masses were assigned

to the suggested chemical markers acetoin, diacetyl, 2- and 3-methylbutanal, 2- and 3-methylbutanol. Based on these observations PTR-MS was concluded to be a suitable method for detection of quality changes in cooked and sliced meat products.

Beside the specific results presented above the experimental work of this PhD-project has also contributed to the general knowledge of the microbial spoilage of cooked and sliced meat products. Quality changes caused by potential spoilage bacteria in cooked and sliced meat products have been characterized using sensory profiling and GC-MS measurements of their production of VOC's in PAPER III. The importance of the composition of the recontaminating flora from the processing facility for the production of off-odors and the development of sensory spoilage was described in PAPER II. PAPER II and PAPER III moreover illustrated the significant effect of consumer simulated storage on the production of off-odors and on the extent of the sensory deterioration of cooked and sliced meat products.

Overall, the results of this PhD-project showed that specific VOC's were closely related to the development of microbially induced sensory spoilage in cooked and sliced meat products. More research is needed to clarify the early changes in the aroma composition of these products. However, based on the results obtained in this PhD-project the possibility of using VOC's as early markers for sensory spoilage in the meat industry appears to be limited.

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# Paper I

## Effect of slicing and storage on the aroma composition of saveloy

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# PE8.21 Effect of Slicing and Storage on the Aroma Composition of Saveloy 205.00

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***Abstract*—The effect of commercial slicing, consumer simulated storage and temperature of initial storage on the composition of volatile organic compounds (VOC) in the headspace of saveloy was investigated using dynamic headspace extractions and GC-MS measurements. Both commercial slicing and temperature loads, for simulation of consumer storage, increased the level of 2- and 3-methylbuthanal. This indicates that these factors increase the microbial activity and decrease product shelf life. Initial storage of saveloy at 8ºC compared to 5ºC also increased the amount of 2- and 3-methylbuthanal and furthermore raised the level of dimethyldisulfide. This also indicates a decrease in eating quality. The results of this experiment suggest that the composition of VOC's from the headspace of saveloy can be used as an indicator of product shelf life.**

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***Index Terms*—Aroma composition, saveloy, storage conditions, shelf life, VOC.**

## I. INTRODUCTION

Changes in the composition of VOC's in the headspace of meat products are related to changes in eating quality and therefore also to product sustainability [1, 2]. The aroma impression that reaches the consumer is a result of chemical and microbial induced reactions that occur in the time span from production to consumption. These reactions are influenced by a number of factors which are important for product shelf life.
In this paper it is investigated how some of these factors (time, temperature, temperature loads and package opening) influence the composition of VOC's present in the headspace of saveloy. Furthermore sausages sliced and packed under experimental conditions are compared with sausages which were sliced and packed in a commercial scale. The results of this paper will be first step towards identifying VOC's which can be used as chemical markers for product shelf life.

## II. MATERIALS AND METHODS

*Production and slicing*
The saveloys were produced at the Danish Meat Research Institute (DMRI) and contained: 25% fat, 10% protein, 1% Collagen, 56% water plus spices and additives. The mixture was stuffed in sterile plastic casings and steam pasteurized at 80ºC for 50min, reaching a core temperature of 75ºC. After 10min cooling by water sprinkling, the products were kept at 2ºC overnight.
After one week the saveloys were cut in 2mm thick slices and sealed in packages of 100g in modified atmosphere (MA, 70% $N_2$, 30% $CO_2$). The sausages were sliced under experimental condition in DMRI's pilot plant and in a commercial production facility. The packages were placed in a chill cabinet, at temperatures and conditions as described below.

*Treatments*
Samples sliced under experimental (Exp) conditions were subject to 3 different treatments after 3 weeks of storage at 5ºC. Exp1: Stored 1 week at 5ºC, Exp2: The packages were opened allowing atmospheric air to enter, then reclosed with a lit and stored 1 week at 5ºC, Exp3: The packages were opened, then reclosed and stored 1 week with temperature loads in a repeated daily pattern shown to represent storage in a consumer situation (5ºC for 12.7h, 12ºC for 9.8h and 20ºC for 1.5h) [3]. Samples sliced in a commercial (Com) facility were subject to 2 treatments. Com1: Stored for 3 weeks at 5ºC, the opened and reclosed and stored 1 week with temperature loads, Com2: Stored for 3 weeks at 8ºC, then opened and reclosed and stored 1 week with temperature loads. The aroma analysis was performed 4 times during the experiment for all the different treatments: 3weeks, 3weeks+3days, 3weeks+5days and 3weeks+7days. For each combination of treatment and measure point a new package of saveloy was used. 3 repetitions of each measurement were made.

*Aroma extraction*
The aroma composition of the saveloys was measured by dynamic headspace using traps containing 73mg Tenax TA (60-80 MESH) and 100mg carbograph 1 TD

(Llantrisant, UK). 25g of sample was chopped, placed in a closed system and conditioned in a water bath at 30ºC for 10min. The samples were then purged with a $N_2$-flow at 60mL/min for 15min. The $N_2$ flow passed the trap which retained the volatiles released from the sample. All traps were back purged with $N_2$ (20mL/min) for 5 min in order to remove water from the trap.

For each day of measurement a mixture of aroma standards corresponding to the compounds chosen for quantification were loaded on traps. The traps were loaded with different concentrations (20-500ppm) of the standards, and were used to generate a standard curve for quantification purposes.

*Desorption and GC-MS analysis*

The traps was thermally desorbed at 240ºC for 10min with a helium flow of 20ml/min using an ATD 400 automatic thermal desorption system (Perkin Elmer, Bucks, UK). The volatiles were cryofocused on the ATD-cold-trap at -30ºC, and subsequently desorbed from the cold-trap at 250ºC for 5min with a helium flow of 10mL/min and an outlet split ratio of 1:10. The temperature of the transfer line to the gas chromatograph was 200ºC.

Further analysis of the volatiles was preformed with GC-MS. The GC was equipped with a HP-INNOWax column (30 m x 0.25mm with 0.25µm film thickness, Agilent 19091N-133) and operated with following parameters: carrier gas, helium; column pressure, 7.6 psi; oven programme, 35 ºC for 5min, from 35ºC to 110ºC at 10ºC/min, from 110ºC to 260ºC at 20ºC/min and 260ºC for 10min. The MS was equipped with a quadrupole mass analyser (Agilent Technologies, Palo Alto, USA). The mass selective detector was operated in the electron impact mode with energy of 70eV and an emission current of 35µA. The MS scanned from 33m/z to 350m/z at a rate of 3scans/s, and simultaneously chosen m/z ratios and selected ions were collected in SIM-mode.

*Data analysis*

18 aroma compounds were selected for quantification based on results from previous experiments [4]. These compounds were: 2- and 3-methylbutanal, hexanal, heptanal, 2-pentylfuran, dimethyldisulfide, dimethyltrisulfide, α-pinene, camphene, β-phellandrene, 3-carene, α-phellandrene, β-myrcine, α-terpinen, limonene, β-phellandrene1, γ-terpinen and myrsticin. These compounds were quantified (in ng/25g of sample) using Chemstation version D.02.00.275 (Agilent Technologies, Palo Alto) for calculation of standard curves. 3-carene, limonene and α-pinene were the only terpenes included in the standard mixture. The remaining terpenes were quantified using standard curves from α-pinene or limonene. 2- and 3-methylbutanal co-eluted from the GC and it was chosen to quantify these compounds together.

The effect of slicing environment, temperature loads, time- and temperature- of storage was analysed using mixed models in SAS version 9.13 (SAS statistical systems, SAS institute, Cary, USA). The analysis was done by comparing the relevant treatments. Time was considered a continuous variable and repetition was considered as random effect.

## III. RESULTS AND DISCUSSION

The effect of the 5 different treatments on VOC's extracted from saveloy sausages are shown Table 1. Only the most relevant of the quantified compounds are included in the table and quantification results are not included in the paper.

*Effect of commercial slicing vs. slicing at DMRI*

Slicing at commercial conditions compared with experimental conditions (Exp3 vs. Com1, table 1) had significant effect for the level of 2- and 3-methylbutanal and hexanal

The quantified amount of 2- and 3-methylbutanal was significantly higher for commercially sliced sausages than for sausages sliced at DMRI. 2- and 3-methylbutanal are produced by some bacteria and can be used as an indicator of microbial growth [5, 6]. Under experimental conditions it was possible to minimize microbial contamination which could explain the observed differences in 2- and 3-methylbutanal concentration. The slicing environment therefore appears to play an important role for product sustainability.

The amount of hexanal, which is known to be a lipid oxidation product [2, 7], was lowest in commercially sliced saveloy (table 1). Moreover a decrease in hexanal level was observed throughout the fourth week of storage for both Exp3 and Com1. The hexanal level therefore seems to have peaked during the first 3 weeks after slicing, which could imply that it has been further oxidised to yield other volatiles.

*Effect of consumer simulated storage*

The temperature loads under consumer simulated storage had significant effect on the quantified amount of 2- and 3-methylbutanal (Exp2 vs. Exp3, table 1). The average storage temperature is increased considerably by the temperature loads during the fourth week of storage. This could cause an increase in microbial activity and explain the elevated level of 2 and 3-methylbutanal compared to the level in saveloys kept at 5ºC during the fourth week.

The terpenes, which derive from the spices in the saveloy [8], were also affected by temperature loads. The level of 3-carene, α-pinene, α-terpinen, and limonene all increased when applying temperature loads in the fourth week compared to storage at 5ºC.

The temperature reached 20ºC for 1.5 hours each day. This could change the texture of the saveloy and favor the release of terpenes from the sample matrix explaining the raise in terpene level.

*Effect of temperature during the first 3 weeks of storage*

The amount of 2- and 3-methylbutanal was higher in the saveloy samples measured 3 weeks after slicing and packaging when stored at 8ºC compared to samples stored at 5ºC (Com1 vs. Com2, table 1). However, a decrease in 2- and 3-methylbutanal concentration was observed during the fourth week of storage for samples initially stored at 8ºC. This could indicate that 2- and 3-methylbutanal at 8ºC has been further degraded. Temperature of initial storage seems to be important for the rate of 2- and 3-methylbutanal formation and perhaps also for the following degradation.

Hexanal, concentration was lower for the packages stored at 8ºC compared to the ones stored at 5ºC, and moreover a reduction during the fourth week of storage was observed for both temperatures. Again this could indicate that hexanal is further oxidised and that the reaction rate increases with temperature of storage.

For saveloy stored at 8ºC there was a significant rise in dimethyldisulfide compared to sausages stored at 5ºC (Com1 vs. Com2 in table 1). This compounds is not detected in most of the other samples, and since Com2 is the most extreme treatment in the experiment this could imply that these compounds are formed late in the shelf life period. This corresponds well with the findings of Withfield, 1998 [9]. Dimethyldisulfide has been shown to be produced by *Staphylococcus carnosus* in dry sausages and increase with high inoculation levels [6]. Their odours have furthermore been described as onion-like, cabbage-like and putrid [10]. It therefore seems likely that these compounds contribute negatively to the aroma of saveloy.

## IV. CONCLUSION

The aroma composition in the headspace of saveloy was influenced by all of the investigated factors.

Slicing commercially compared to slicing at DMRI increased the level of 2- and 3-methylbutanal. This indicates a raise in microbial activity which is expected to shorten the shelf life period. Storing the samples with temperature loads, simulating a consumer situation, also increased the level of 2 and 3-methylbutanal which indicates an acceleration of microbial spoilage. Applying temperature loads furthermore increased the level of terpenes released from the product. Increasing the initial storage temperature from 5ºC to 8ºC increased the rate of 2- and 3-methylbutanal and dimethyldisulfide formation and hereby decreasing the shelf life period.

GC-MS measurements coupled with sensory and microbial measurements would help understanding to which extend the observed changes in headspace composition affects the eating quality. However the result of this study indicates that the composition of volatiles in the headspace of saveloy can be used as an indicator of product shelf life.

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**Table1.** The effect of the investigated factors on the quantified amount of selected aroma compounds

| Compound | Exp3 vs. Com1 | | Com1 vs Com2 | | Exp2 vs. Exp3 | |
|---|---|---|---|---|---|---|
| | slicing | time | temp | time | loads | time |
| 2/3-methylbutanal | <0.001 | ns | 0.013 | 0.015 | <0.001 | ns |
| dimethyldisulfide | ns | <0.001 | 0.012 | 0.022 | ns | 0.011 |
| hexanal | 0.007 | <0.001 | <0.001 | 0.002 | ns | 0.020 |
| 3-carene | ns | ns | Ns | ns | 0.004 | ns |
| α-pinene | ns | ns | Ns | ns | 0.010 | ns |
| α-terpinen | ns | ns | Ns | ns | 0.018 | 0.032 |
| limonene | ns | ns | Ns | ns | <0.001 | ns |

Exp3 vs. Com1 shows the effect of slicing at DMRI vs. slicing commercially. Com1 vs. Com2 shows the effect of initial storing saveloy at 5ºC vs. storing at 8ºC. Exp2 vs. Exp3 shows the effect of storing saveloy at 5ºC vs. applying temperature loads during the fourth week of storage. Effects were considered significant for $p<0.05$.

# Paper II

## Identification of chemical markers for the sensory shelf-life of saveloy

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# Identification of chemical markers for the sensory shelf-life of saveloy

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ABSTRACT

The aroma composition, the microbial composition and the sensory profile were measured in sliced saveloy samples packed in modified atmosphere (MA). The main objective was to identify aroma compounds with potential as chemical markers to identify the sensory changes of saveloy. The 60 aroma compounds isolated from the saveloy samples by dynamic headspace extraction and measured by Gas Chromatography Mass Spectrometry (GC-MS) were used to model the sensory attributes sour&old odour and meaty odour using partial least squares regression (PLS). 2- and 3-methylbutanal, 2- and 3-methylbutanol, acetoin and diacetyl were found to have the highest impact on both sour&old odour and meaty odour of the samples. The results show that these four aroma compounds have high potential as chemical markers for the sensory shelf-life of sliced and MA-packed saveloy.



## 1. Introduction

The link between sensory shelf-life and microbial activity in cooked and sliced meat products packed in modified atmosphere (MA) is well established (Borch, KantMuermans, & Blixt, 1996; Mataragas, Skandamis, Nychas, & Drosinos, 2007; Samelis, Kakouri, & Rementzis, 2000; Vermeiren, Devlieghere, De Graef, & Debevere, 2005). The cooking process eliminates practically all microorganisms and recontamination during slicing and packing is therefore largely responsible for the eventual spoilage of the product (Laursen, Byrne, Kirkegaard, & Leisner, 2009; Samelis, Kakouri, Georgiadou, & Metaxopoulos, 1998; Samelis et al., 2000). The rate of spoilage depends on the composition of the bacterial flora along with processing factors such as the oxygen permeability of the packing material and temperature fluctuations during transport and storage (Nychas, Skandamis, Tassou, & Koutsoumanis, 2008). Cooked and sliced meat products typically have a shelf-life of three to six weeks before they are spoiled by microbial formation of off-odours, gas and slime (Borch et al., 1996; Mataragas, Drosinos, Vaidanis, & Metaxopoulos, 2006). The typical spoilage flora of cooked and sliced meat products consists of a mixture of lactic acid bacteria (LAB) possibly in combination with *Brochothrix Thermosphacta* and *Pseudomonas* spp. (Borch et al., 1996; Stanley, Shaw, & Egan, 1981).

The growth of spoilage organisms and their production of off-odours have been studied in different cooked meat products (Leroy, Vasilopoulos, Van Hemelryck, Falony, & De Vuyst, 2009; Samelis et al., 1998; Vermeiren et al., 2005). Under anaerobic conditions LAB such as *Lactobacillus sakei*, *Leuconostoc carnosum* and *Carnobacterium divergens* dominate the spoilage flora and typically cause production of sour and acid off-odours and off-flavours when growth has reached a population of $10^7$–$10^8$ CFU/g (Borch et al., 1996; Dainty & Mackey, 1992; Holzapfel, 1998; Leroy et al., 2009; Vermeiren et al., 2005). In the presence of oxygen the diversity of volatile compounds from microbial metabolism increases and their odour becomes increasingly offensive (Borch et al., 1996; Stanley et al., 1981; Vermeiren et al., 2005). Oxygen furthermore favours growth of *pseudomonas* spp. and *Brochothrix thermosphacta* which will interfere with the LAB under these conditions. This could lead to formation of diacetyl and acetoin, which are well known off-odours in meat products, produced by the aerobic metabolism of glucose by *B. thermosphacta* and some LAB (Bartowsky & Henschke, 2004; Borch & Molin, 1989; Dainty & Mackey, 1992; Stanley et al., 1981; Vermeiren et al., 2005).

The odour is among the first quality attributes registered when opening a package of sliced meat products and therefore volatile organic compounds (VOC's) have potential as early markers for consumer acceptability. In the present study the relation between the formation of VOC's and shelf-life of saveloy was investigated. The VOC's were measured by dynamic headspace extraction coupled with Gas Chromatography Mass Spectrometry (GC-MS) whereas product shelf-life was measured by sensory profiling. The measurements were done in the fourth week of storage where the storage conditions were set to simulate the conditions at the consumers.

## 2. Materials and methods

### 2.1. Production and slicing of saveloy

The saveloy was produced in the pilot plant at Danish Meat Research Institute (DMRI) using a recipe containing approximately: 40% shank

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and belly meat, 20% pork trimmings, 5% pork fat, 26.3% water, 4% potato starch, 2% soy-isolate 1% nitrite salt, 0.6% spices, 0.3% phosphate, 0.7% vacuum salt and 0.1% sodium ascorbate. The sausage mince was stuffed in sterile plastic casings and steam pasteurised at 80 °C for 50 min, reaching a core temperature of 75 °C. After 10 min cooling by water sprinkling, the products were kept at 2 °C overnight. The saveloy was cut into 2 mm slices and packed in 100 g APET/PE-peel trays with an oxygen transfer rate (OTR) of 15 mL/m$^2$ (24 h, 1 atm, 23 °C), and sealed with a PETP12/PE-peel film with an OTR of 5 mL/m$^2$. The MA packaging gas consisted of 70% $N_2$ and 30% $CO_2$. The product-headspace relation in the package was approximately 1:1.2.

### 2.2. Experimental setup

The saveloy was sliced as described above at DMRI and at two industrial slicing facilities. The three slicing locations were denoted A, B and C. After slicing and packing in MA, the samples were stored at either 5 °C or 8 °C for 3 weeks. During the fourth and final week of the experiment the storage conditions were set to describe consumer simulated storage (CSS). This included package opening and introduction of a temperature programme which has previously been shown to represent storage by Danish consumers (5 °C for 12.7 h, 12 °C for 9.8 h and 20 °C for 1.5 h, Blom-Hansen, unpublished results). As a control of the CSS a series of saveloy samples from each slicing location was furthermore kept in closed packages at the initial storage temperature during the fourth week of the experiment. The saveloy samples were subjected to analyses after: 3 weeks, 3 weeks + 3 days, 3 weeks + 5 days, 3 weeks + 7 days and 4 weeks. Note that 3 weeks + 7 days and 4 weeks were actually the same day of the experiment but the samples were stored under different conditions. The analyses included measurements of the aroma composition with GC-MS, a sensory profiling and measurements of the microbial composition. Three replicate measurements for each combination of factors were performed for each of the analyses. Furthermore, separate packages of saveloy were used for each replicate in an analysis. In the figures in this paper the samples are denoted according to the initial temperature (5 or 8), the slicing location (A, B or C) and the days of storage after package opening (0, 3, 5 or 7). For the unopened samples analysed after 4 weeks k is used as time indicator. An overview of the experimental setup is provided in Fig. 1.

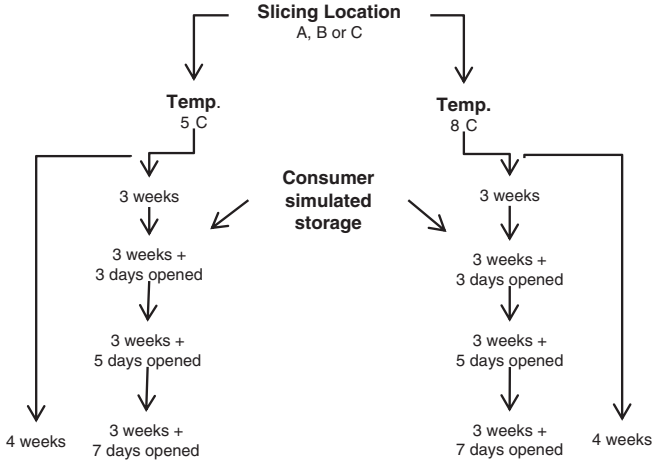


**Fig. 1.** Overview of the experimental design. The samples were sliced at three different locations (A, B and C) and opened after 3 weeks storage at either 5 °C or 8 °C. After package opening the samples were stored with temperature loads during the fourth week of the experiment. Measurements were performed after 3 weeks, 3 weeks + 3 days, 3 weeks + 5 days, 3 weeks + 7 days. A series of samples were not opened, kept at the initial storage temperature and measured after 4 weeks.

### 2.3. Aroma extraction

The aroma composition of the saveloy samples was measured by dynamic headspace extraction using traps containing 73 mg Tenax TA (60–80 MESH, Markes International Ltd., Llantrisant, UK) and 100 mg carbograph 1 TD (Markes International Ltd., Llantrisant, UK). 25 g of sample was coarsely chopped, placed in a 500 mL closed glass container and conditioned in a water bath at 30 °C for 10 min. The samples were then purged with a $N_2$-flow of 60 mL/min for 15 min. The $N_2$ flow was let through the trap which retained the volatiles released from the sample. All traps were back purged with a $N_2$ flow of 60 mL/min for 5 min in order to remove water from the trap.

### 2.4. Desorption and GC-MS analysis

The traps were thermally desorbed at 240 °C for 10 min with a helium flow of 20 ml/min using an ATD 400 automatic thermal desorption system (Perkin Elmer, Waltham, MA, USA). The volatiles were cryofocused on the ATD-cold-trap at −30 °C, and subsequently desorbed from the cold-trap at 250 °C for 5 min with a helium flow of 10 mL/min and an outlet split ratio of 1:10. The temperature of the transfer line to the gas chromatograph was 200 °C.

Further analysis of the volatiles was performed with GC-MS. The 6890 N GC system (Agilent Technologies, Santa Clara, CA, USA was equipped with an HP-INNOWax column (30 m × 0.25 mm with 0.25 μm film thickness, Agilent 19091 N-133) and operated with the following parameters: carrier gas, helium; column pressure, 7.6 psi; oven programme, 35 °C for 5 min, from 35 °C to 110 °C at 10 °C/min, from 110 °C to 260 °C at 20 °C/min and 260 °C for 10 min. The system was equipped with a 5973 network mass selective detector (Agilent Technologies, Santa Clara, CA, USA) which was operated in the electron impact mode with energy of 70 eV and an emission current of 35 μA. The MS scanned from 33 m/z to 350 m/z at a rate of 3 scans/s.

The retention times were standardised using the Kovats retention index (KI) calculated from GC-MS runs of a C5-C15 alkane standard (Air Liquide, Paris, France). Furthermore GC-MS runs of Tenax traps spiked with dilutions of the following compounds were used for identification purposes: 2-methylbutanol and hexanal (Merck KGaA, Darmstadt, Germany). 3-methylbutanal and acetoin (ChemService inc., West Chester, Pennsylvania, USA), 1-octen-3-ol and 1-hexanol (Sigma-Aldrich, St. Louis, Missouri, USA), octanal (Bie & Berntsen, Herlev, Denmark), toluene (Honeywell Ridel deHaen, Seelze, Germany).

### 2.5. Sensory analysis

The sensory analysis was done using a six person sensory panel with previous experience in assessing meat products. The sensory panel had furthermore passed a training programme based on ISO 8585–1 and ASTM STP758. During training sessions four odour descriptors and eleven taste and texture descriptors were chosen for the assessment. However, after 3 weeks and 5 days of storage some of the saveloy samples were rejected for taste/flavour and texture evaluation by the sensory panel due to spoilage. Together with the staff of the sensory laboratory it was agreed to assess the saveloy samples using only the odour descriptors for the rest of the experiment. As a consequence of this only the odour descriptors are available throughout the experiment. The odour descriptors were: Meaty odour, Sour & Old odour, Spicy odour and Acidic odour. The attributes were assessed on a 15 cm unstructured line scale. The samples were placed in closed glass containers at room temperature before being served. Three replicates of each combination of factors were presented to the panellist in random order and assessed by each panellist twice. Furthermore the sensory panel was calibrated before each session using a reference saveloy sample which had been stored at −1 °C since production.

### 2.6. Microbial analysis

For the microbial analysis approximately 25 g of sample was placed in a sterile filter back, diluted ten times in 0.85% salt water with 0.1% peptone using a dilumat 3 (AES Laboratoire, Bruz Cedex, France) and homogenised 1 min with a stomacher 400 (Seward, Worthing, UK). Appropriate 10-fold dilutions of the sample extracts were made using a Dilucup serial dilution system (Lab Robot Products, Stenungsund, Sweden) and plated on the four following growth mediums: brain heart infusion ager (BHI-agar, Oxoid Ltd., Basingstoke, UK), streptomysin and thallous acetate agar (STA-agar, Oxoid Ltd., Basingstoke, UK) with STA selective supplement, pseudomonas agar base with cetrimide, fucidin and cephalosporin agar supplement (CFC-agar, Oxoid Ltd., Basingstoke, UK) and an all purpose medium with tween containing polymyxin (APT-agar, Merck KGaA, Darmstadt, Germany) with a cover layer of APT-agar. All plates were incubated for 5 days at 20 °C.

### 2.7. Data analysis

Chemometric analysis was done using the PLS Toolbox (version 5.2.2, Eigenvector Research inc., Wenatchee, WA, USA). The PLS toolbox is running in the MATLAB environment (version 7.6.0.324, The Matworks inc., Natick, MA, USA). All partial least squares regression (PLS) models and principal component analyses (PCA) were validated using random subset validation.

The least significant difference (LSD) for the sensory scores and microbial counts was calculated using an LSD-test in the R-software (version 2.10.1, The R foundation of statistical computing).

#### 2.7.1. Processing of GC-MS data

The GC-MS data was processed using the MSD Chemstation software (D.01.02.16, Agilent Technologies, Santa Clara, CA, USA). The peaks in the chromatograms were initially integrated using the RTE integrator. The chromatographic shifts in the data were handled with peak alignment based on retention time using the in-house ALIGNE software (DMRI Teknologisk, Roskilde, Denmark). This facilitated a comparison of peak areas across samples. Due to co-elution some peaks could not be quantified individually. Therefore 2-methylbutanal and 3-methylbutanal were quantified together at a KI of 915, and 2-methylbutanol and 3-methylbutanol were quantified together with a KI of 1205. Diacetyl and pentanal also eluted together and were quantified with a KI of 975. However, an analysis of this peak showed that the contribution of pentanal was relatively constant through the experiment. Therefore the area increase of this peak was ascribed mainly to diacetyl.

Three different methods were used for identification of the chromatographic peaks. The mass spectra of the peaks were compared with mass spectra of potential matching compounds using the NIST/EPA/NIH mass spectral library (V.1.7a, Agilent Technologies, Santa Clara, CA, USA). Secondly the mass spectra and KI were in some cases compared with GC-MS measurements of authentic standards. Moreover, in order to support the identity of the chromatographic peaks their KI was compared with the KI of potential matching compounds using the C20M column from internet database www.flavornet.org (Acree & Arn, 2004).

## 3. Results and discussion

### 3.1. Initial reduction and analysis of the GC-MS data

A total of 94 peaks in the GC-MS chromatograms were integrated and subsequently aligned across the different chromatograms. Several of the peaks were specific to a single slicing location or time point and were therefore considered unsuitable as chemical markers. Peaks which were present in less than 2/3 of the samples at each time point were therefore removed from the data. This left 60 volatile compounds in the dataset.

An overview of the distribution of the saveloy samples according to their content of the 60 aroma compounds is provided by a PCA model from which the bi-plot (principal component (PC) 1 vs. PC2) is shown in Fig. 2. PC1 and PC2 together account for approximately 70% of the variation in the data. The bi-plot demonstrates good separation of the samples according to time and location. PC1 separates the samples according to storage time and CSS. Only samples measured after 3 weeks storage without opening have positive scores on PC1 whereas samples measured after 3 weeks + 3 days, 3 weeks + 5 days, 3 weeks + 7 days and 4 weeks have negative scores. On PC2 only samples from location B have positive scores whereas samples from location A and C have negative scores. This indicates that the main causes of variation in the experiment are storage time and CSS on PC1 (58.2%) and slicing location on PC2 (11.4%). The aroma compounds, denoted by their KI, are distributed in the bi-plot according to their loadings. Most of the aroma compounds are located in a large cluster with scores around zero on PC2 and positive scores on PC1. Another smaller cluster of aroma compounds in the bi-plot has positive scores on PC2 and numerically low scores on PC1 and is located near samples from location B stored for 3, 5 and 7 days after the onset of CSS.

### 3.2. Sensory changes during storage of saveloy

Data from the sensory assessment of the saveloy samples is shown in Table 1. The sour&old odour scores of the saveloy samples ranged from 2.4 in samples from location C (8 °C) measured after 3 weeks to 11.7 in samples from location B (8 °C) measured after 3 weeks + 7 days. In samples from location B and C there was a significant increase in sour&old odour after the onset of CSS for both of the initial storage temperatures. In samples from location A there was no effect of CSS on sour&old odour. In samples from location B and C the sour&old odour was furthermore significantly higher after 3 weeks + 7 days compared to samples stored in MA-packaging for all 4 weeks. This shows that the availability of oxygen, induced by package opening and the temperature programme increased the rate of spoilage in saveloy samples from location B and C. However, a significant increase in sour&old odour was also observed during the fourth week of storage in the samples kept in modified atmosphere (3 weeks vs. 4 weeks) from location A and B stored at 8 °C. This means that during the fourth week a decrease in sensory quality was also observed in samples which were not subjected to CSS.

For saveloy samples initially stored at 5 °C the meaty odour decreased significantly with CSS and storage time for locations B and C but was stable in samples from location A. In packages stored at 8 °C the decrease in meaty odour induced by CSS and storage time was significant for all three locations. From Table 1 it was seen that the sensory scores for meaty odour ranged from 1.5 to 4.4. This is a relatively small difference compared to the changes in sour&old odour. However, the assessors were more in agreement about the meaty odour scores of the saveloy samples which therefore lowered the LSD values.

There were no significant changes caused by package opening, CSS and storage time on the sensory attributes spicy odour and acid-like odour. These attributes were therefore not of interest in relation to the modelling of the quality changes of saveloy in this experiment. In contrast meaty odour and sour&old odour proved to be useful parameters for assessment of quality changes and shelf-life of saveloy. As seen from Table 1 both attributes change markedly in the saveloy samples during the experiment, which is useful when modelling quality changes.

### 3.3. Microbial changes during storage of saveloy

Table 2 shows the development in the total microbial count (BHI-agar), the LAB count (APT-agar), the *Brochothrix* spp. count (STA-agar)

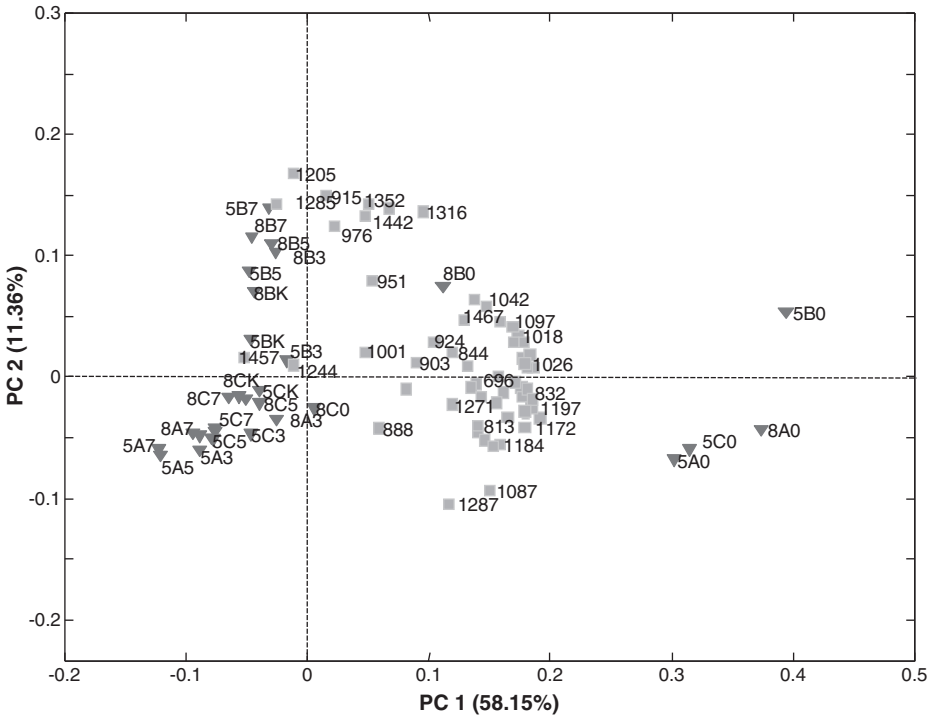


**Fig. 2.** Bi-plot of the PCA model including all samples and the 60 aroma variables. Saveloy samples (triangles) are labelled with a code including the initial storage temperature (5 °C or 8 °C), slicing location (A, B or C) and days of storage after package opening (0, 3, 5, 7 and k) where k represents 4 weeks in closed packages. The volatile compounds (squares) are labelled with their Kovats retention index.

and the *Pseudomonas* spp. count (CFC-agar) during storage for location A, B and C and storage at 5 °C, 8 °C followed by CSS.

A significant increase in the total microbial count caused by CSS and storage time was observed in the saveloy samples from location A, B and C stored at 5 °C plus samples from location A stored at 8 °C. In saveloy samples from location B and C initially stored at 8 °C there were no significant effects of package opening and storage time on the total bacterial count. However, these samples had already reached microbial counts above $10^7$ CFU/g after three weeks of storage in closed packages. This result illustrates the importance of keeping sliced meat products sufficiently chilled during storage and distribution.

A comparison of the sensory scores in Table 1 and the microbial counts in Table 2 shows that high microbial counts did not necessarily result in sour&old scores in the high end of the scale (above 7.5). This suggests that the composition of the microbial flora is of great importance to the sensory quality of the product. The number of *B. thermosphacta*, *Pseudomonas* spp. and LAB present in the product is therefore important in relation to the type and degree of spoilage in the product.

From Table 2 it is seen that the saveloy samples from location A and C were dominated by LAB. *Pseudomonas* spp. and *B. thermosphacta* were also found in samples from location A and C but their growth was often below the limit of detection. In samples from location B the introduction of CSS after 3 weeks of storage caused P*seudomonas* spp., *B. thermosphacta* and LAB to reach high numbers. Therefore all three types of bacteria could contribute to the spoilage of samples from location B. Overall samples from location B appeared to have a more diverse microbial flora than location A and C. This diversity could very well explain the increased level of sour&old odour in samples sliced at location B compared to samples from location A and C.

**Table 1**
Shows the averaged results of the sensory analysis of the saveloy samples for each location and measure point. The samples were assessed using the four attributes: Sour&old odour, meaty odour, spicy odour and acid-like odour. Letters a, b, c and d in the same column, indicate samples with significantly different sensory scores based on calculations of the LSD-value.

| | Location A | | Location B | | Location C | |
|---|---|---|---|---|---|---|
| | 5 °C | 8 °C | 5 °C | 8 °C | 5 °C | 8 °C |
| *Sour&Old Odour* | | | | | | |
| 3 weeks | 2.7 | $2.9^{b}$ | $2.8^{c}$ | $3.6^{c}$ | $2.6^{b}$ | $2.4^{b}$ |
| 3 weeks + 3 days | 3.0 | $2.9^{b}$ | $8.2^{b}$ | $8.5^{b}$ | $2.7^{b}$ | $3.6^{b}$ |
| 3 weeks + 5 days | 3.9 | $3.9^{ab}$ | $9.6^{ab}$ | $10.8^{ab}$ | $4.2^{b}$ | $6.4^{a}$ |
| 3 weeks + 7 days | 3.3 | $3.5^{b}$ | $10.8^{a}$ | $11.7^{a}$ | $7.7^{a}$ | $6.4^{a}$ |
| 4 weeks | 3.3 | $5.2^{a}$ | $4.1^{c}$ | $8.7^{b}$ | $3.9^{b}$ | $3.7^{b}$ |
| *Meaty Odour* | | | | | | |
| 3 weeks | 3.7 | $4.4^{a}$ | $4.1^{a}$ | $3.5^{a}$ | $4.2^{a}$ | $4.2^{a}$ |
| 3 weeks + 3 days | 3.4 | $3.9^{ab}$ | $2.1^{b}$ | $2.2^{b}$ | $3.9^{ab}$ | $3.5^{bc}$ |
| 3 weeks + 5 days | 3.4 | $3.4^{bc}$ | $2.0^{b}$ | $1.9^{b}$ | $3.3^{bc}$ | $3.0^{c}$ |
| 3 weeks + 7 days | 3.7 | $3.6^{bc}$ | $2.0^{b}$ | $1.5^{b}$ | $2.6^{c}$ | $3.1^{bc}$ |
| 4 weeks | 3.7 | $3.0^{c}$ | $3.5^{a}$ | $2.3^{b}$ | $3.6^{a}$ | $3.7^{ab}$ |
| *Spicy Odour* | | | | | | |
| 3 weeks | 3.5 | 3.7 | 3.5 | 3.4 | 3.7 | 3.8 |
| 3 weeks + 3 days | 3.8 | 4.0 | 3.6 | 4.0 | 3.9 | 4.0 |
| 3 weeks + 5 days | 4.1 | 4.4 | 3.6 | 4.0 | 4.4 | 3.6 |
| 3 weeks + 7 days | 3.9 | 4.3 | 3.0 | 2.2 | 3.8 | 3.6 |
| 4 weeks | 4.2 | 3.8 | 4.0 | 2.9 | 3.9 | 4.2 |
| *Acid-like odour* | | | | | | |
| 3 weeks | 3.3 | 3.9 | 3.3 | 3.4 | 4.2 | 3.9 |
| 3 weeks + 3 days | 3.5 | 3.8 | 4.0 | 4.2 | 3.9 | 4.1 |
| 3 weeks + 5 days | 3.7 | 4.0 | 4.6 | 5.0 | 4.2 | 3.9 |
| 3 weeks + 7 days | 4.1 | 3.8 | 3.9 | 2.9 | 3.3 | 3.4 |
| 4 weeks | 3.8 | 3.7 | 3.7 | 3.5 | 3.6 | 3.8 |

### 3.4. Modelling of the sensory attributes

Development of a tool for prediction of the rate of spoilage would be of great value to the meat industry. Therefore several studies have already dealt with the modelling and prediction of the shelf-life in different types of sliced meat products. This has been done based on microbial growth and metabolites such as lactic-, acetic- and α-ketoisocaproic acid and their precursors (Devlieghere, Van Belle, & Debevere, 1999; Mataragas et al., 2007; Stolzenbach, Leisner, & Byrne,

**Table 2**
The averaged results of the microbial analysis are shown in log cfu/g. BHI-agar counts the total aerobe bacterial count. ATP-agar selects for LAB. STA-agar selects for *Brochothrix* spp., whereas CFC-agar selects for *pseudomonas* spp. Letters a, b, c and d in the same column, indicate samples with significantly different microbial counts based on calculations of the LSD-value.

| | Location A | | Location B | | Location C | |
|---|---|---|---|---|---|---|
| | 5 °C | 8 °C | 5 °C | 8 °C | 5 °C | 8 °C |
| *Total bacterial count (BHI)* | | | | | | |
| 3 weeks | 2.3* | 3.1$^{c}$ | 7.8$^{c}$ | 8.0 | 5.3$^{b}$ | 7.1 |
| 3 weeks + 3 days | 3.7$^{b}$ | 5.3$^{b}$ | 8.6$^{b}$ | 7.1 | 6.7$^{a}$ | 7.7 |
| 3 weeks + 5 days | 7.0$^{a}$ | 5.5$^{ab}$ | 8.7$^{b}$ | 8.7 | 7.0$^{a}$ | 7.8 |
| 3 weeks + 7 days | 6.8$^{a}$ | 7.3$^{a}$ | 9.4$^{a}$ | 8.8 | 7.8$^{a}$ | 8.0 |
| 4 weeks | 3.1$^{b}$ | 4.3$^{bc}$ | 7.8$^{c}$ | 8.4 | 6.5$^{ab}$ | 7.7 |
| *Lactic acid bacteria (ATP)* | | | | | | |
| 3 weeks | 2.0* | 2.9$^{c}$ | 7.8$^{d}$ | 8.0 | 5.1$^{b}$ | 7.0 |
| 3 weeks + 3 days | 3.1$^{b}$ | 4.5$^{bc}$ | 8.5$^{bc}$ | 8.5 | 6.6$^{a}$ | 7.7 |
| 3 weeks + 5 days | 6.2$^{a}$ | 5.5$^{ab}$ | 8.7$^{ab}$ | 8.6 | 7.0$^{a}$ | 8.0 |
| 3 weeks + 7 days | 6.8$^{a}$ | 6.8$^{a}$ | 9.2$^{a}$ | 8.8 | 6.6$^{a}$ | 7.9 |
| 4 weeks | 2.7$^{b}$ | 3.7$^{c}$ | 7.9$^{cd}$ | 8.5 | 6.5$^{ab}$ | 7.7 |
| *Brochothrix spp. (STA)* | | | | | | |
| 3 weeks | -** | -** | 6.0$^{c}$ | 6.7$^{b}$ | -** | 3.8 |
| 3 weeks + 3 days | -** | -** | 7.6$^{b}$ | 7.6$^{a}$ | 3.2$^{b}$ | 2.3* |
| 3 weeks + 5 days | -** | 3.2* | 8.1$^{ab}$ | 7.7$^{a}$ | 3.4$^{b}$ | - |
| 3 weeks + 7 days | 2.9* | 3.7 | 8.3$^{a}$ | 8.0$^{a}$ | 4.4$^{a}$ | 4.0* |
| 4 weeks | -** | -** | 3.8$^{d}$ | 6.6* | -** | -** |
| *Pseudomonas spp. (CFC)* | | | | | | |
| 3 weeks | -** | -** | 4.3$^{d*}$ | 7.0* | -** | -** |
| 3 weeks + 3 days | -** | 5.0* | 6.5$^{bc*}$ | 7.3* | -** | -** |
| 3 weeks + 5 days | 6.1 | -** | 7.2$^{ab}$ | 8.0 | 3.0 | -** |
| 3 weeks + 7 days | -** | 6.3 | 8.3$^{a}$ | 7.6 | -** | 4.9 |
| 4 weeks | -** | -** | 5.4$^{cd}$ | 6.9 | -** | -** |

*1 or 2 of 5 repetitions was below the limit of detection (LOD = 2 log), LSD was calculated based on the remaining measurements. ** Growth was detected but below the LOD in at least 3 of 5 repetitions.

2009). In this study the modelling of shelf-life by identifying aroma compounds which can be used as chemical markers for sensory changes of saveloy using PLS-regression was studied.

PLS-models predicting the sensory attributes, meaty- and sour&old odour from the 60 aroma compounds were calculated. The ten aroma compounds from the PLS-models of sour&old odour and meaty odour with the highest regression coefficients relative to the standard error are shown in Figs. 3 and 4, respectively. The mean and standard errors of the regression coefficients were calculated based on ten repetitions of the PLS models with two PC's and five randomly selected samples left out in each of the models. In this way uncertain and unsystematic aroma compounds are excluded and important and systematic aroma compounds maintained. An overview of the KI and identification of the aroma compounds with the highest regression coefficients to meaty- and sour&old odour is provided in Table 3.

### 3.4.1. PLS-model of sour&old odour

Of the ten aroma compounds, shown in Fig. 3, 2- and 3-methylbutanal, 2- and 3-methylbutanol, acetoin and diacetyl had the highest regression coefficients and were all positively correlated with sour&old odour. These compounds are all well established microbial metabolites which have previously been associated with microbial activity (Dainty & Hibbard, 1983; Larrouture-Thiveyrat, Pepin, Leroy-Setrin, & Montel, 2003; Whitfield, 1998). A PLS-model of sour&old odour including the ten aroma compounds with the highest regression coefficient as X-block was made. The validated root mean square error of calibration (RMSECV) of this PLS-model was 1.5. This should be related to the sensory scores of sour&old odour of the saveloy samples which ranged from 2.4 to11.7. The scores and loadings from the two component PLS-model are shown in the bi-plot in Fig. 5. The model

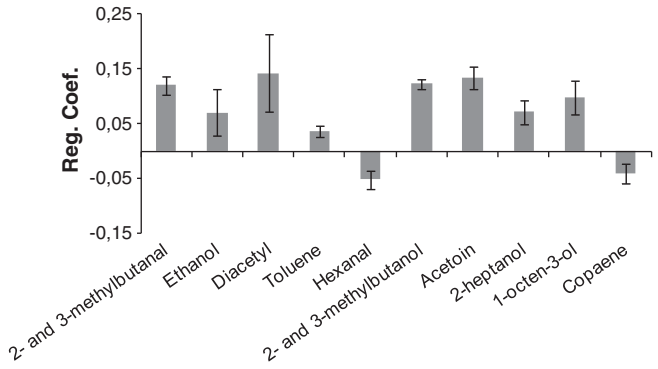


**Fig. 3.** From the PLS-model of sour&old odour including the 60 aroma components the regression coefficients of the two component model were calculated based on ten repetitions of the PLS-model using random subsets cross-validation with five data splits and five iterations. The ten aroma components with the numerical highest regression coefficients, when the standard errors were subtracted, are shown.

accounts for 80.5% of the variation in sour&old odour in the data. The bi-plot further confirms the high impact of 2- and 3-methylbutanal, 2- and 3-methylbutanol, acetoin and diacetyl. These compounds are closely related to a cluster of saveloy samples from location B analysed after package opening which had high scores in sour&old odour. Hexanal and copaene are negatively related to sour&old odour and these compounds are clustered with the saveloy samples analysed after 3 weeks. The bi-plot in Fig. 5 furthermore contains a third cluster of samples with negative scores on PC1 and positive scores on PC2. This cluster mainly contains samples from location A and C analysed 3, 5, and 7 days after package opening plus samples analysed after 4 weeks storage in closed packages. Though these samples had sour&old scores ranging from low to medium they were not separated in the PLS-model. However, as seen in Fig. 5, the PLS-model of sour&old odour was able to divide the saveloy samples into three clusters according to their sour&old odour. Furthermore, the model differentiated well between the samples from location B with high scores in sour&old odour and the samples measured at 3 weeks with low scores in sour&old odour.

### 3.4.2. PLS model of meaty odour

Fig. 4 showed that the microbial metabolites 2- and 3-methylbutanal, 2- and 3-methylbutanol, acetoin and diacetyl also had the highest regression coefficients towards meaty odour. However, in opposition to the PLS-model of sour&old odour these four aroma compounds were negatively correlated to meaty odour. A two component PLS-model was calculated including the ten aroma compounds with the highest regression coefficients, shown in Fig. 4, as X-block. This model had a

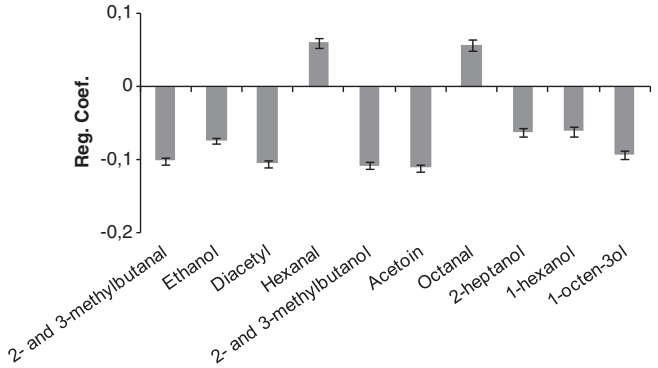


**Fig. 4.** From the PLS-model of meaty odour including the 60 aroma components the regression coefficients of the two component model were calculated based on ten repetitions of the PLS-model using random subsets cross-validation with five data splits and five iterations. The ten aroma components with the numerical highest regression coefficients, when the standard errors were subtracted, are shown.

**Table 3**
An overview of the volatile compounds which had the highest regression coefficients in the PLS-models of meaty odour and sour&old odour. The Kovats retention index is shown along with the method of the identification. The identification was based on either compound standards (S), comparison of KI with www.flavournet.org (L) or by using the NIST MS library v. 1.7a (N).

| Compound | KI | ID | Sour&old | Meaty |
|---|---|---|---|---|
| 2 and 3-methylbutanal | 915 | L-N-S | X | X |
| Ethanol | 929 | L-N | X | X |
| Diacetyl | 976 | L-N-S | X | X |
| Toluene | 1042 | L-N | X | |
| Hexanal | 1087 | L-N-S | X | X |
| 2- and 3-methylbutanol | 1205 | L-N-S | X | X |
| Acetoin | 1285 | L-N-S | X | X |
| Octanal | 1287 | L-N-S | | X |
| 2-heptanol | 1316 | N-S | X | X |
| 1-hexanol | 1352 | L-N-S | | X |
| 1-octen-3-ol | 1442 | N-S | X | X |
| Copaene | 1488 | L-N | X | |

RMSECV of 0.5. This measure of the prediction error should be related to the sensory assessments of meaty odour which ranged from 1.5 to 4.4. Fig. 6 shows the bi-plot of the two component PLS-model of meaty odour which explained approximately 71% of the variation in the meaty odour assessments. The distribution of the aroma compounds and saveloy samples is similar to that observed for sour&old odour in Fig. 5. 2- and 3-methylbutanal, 2- and 3-methylbutanol, acetoin and diacetyl are related to the samples from location B analysed after package opening, which had low scores in meaty odour. Hexanal and octanal were found in high levels in the cluster of samples from week 3 which generally had the highest scores in meaty odour. The bi-plot from the PLS model of meaty odour shown in Fig. 6 contains a large cluster of samples with positive scores on PC1 and negative scores on PC2. This cluster contains samples with medium to high scores in meaty odour which were not well separated by the PLS-model. The cluster is furthermore comparable to the cluster of samples with low to medium scores in sour&old odour observed in Fig. 5. Nevertheless, the PLS-model of meaty odour was able to differentiate between the saveloy samples with relatively high scores in meaty odour from week 3 and the samples with low scores in meaty odour from location B analysed after the onset of CSS.

There are several similarities between PLS-models of sour&old and meaty odour. The four aroma compounds with the highest regression coefficients to meaty odour and sour&old odour are identical and furthermore eight of the ten most important aroma compounds shown in Figs. 3 and 4 are the same. This suggests that sour&old odour and meaty odour are correlated and therefore roughly describe the same variation in the product only with opposite sign.

### 3.5. Evaluation of the identified chemical markers

From the PLS-models of meaty odour and sour&old odour it is apparent that 2- and 3-methylbutanal, 2- and 3-methylbutanol, acetoin and diacetyl are important to the sensory quality of sliced saveloy. The development of the peak areas corresponding to these compounds in saveloy samples from location A, B and C initially stored at 5 °C is shown in Fig. 7.

2- and 3-methylbutanal and their corresponding alcohols 2- and 3-methylbutanol are produced by the breakdown of some amino acids, including leucine and valine, by several LAB and *B. thermosphacta* (Larrouture-Thiveyrat et al., 2003; Masson, Hinrichsen, Talon, & Montel, 1999; Pin, de Fernando, & Ordonez, 2002; Smit, Engels, & Smit, 2009; Tjener, Stahnke, Andersen, & Martinussen, 2004). The acceptability of their odour seems to depend on the product type in question. In meat products like dried fermented sausages or dry-cured hams, they are considered an important part of the aroma (Ruiz, Ventanas, Cava, Andres, & Garcia, 1999; Stahnke, 1995; Tjener et al., 2004). However, in other studies 3-methylbutanol and 3-methylbutanal have been negatively associated with consumer acceptability of artisan-type ham and American dry-cured ham respectively (Leroy et al., 2009; Pham et al., 2008). In the present study 2- and 3-methylbutanal and 2- and 3-methylbutanol were negatively associated with the eating quality of

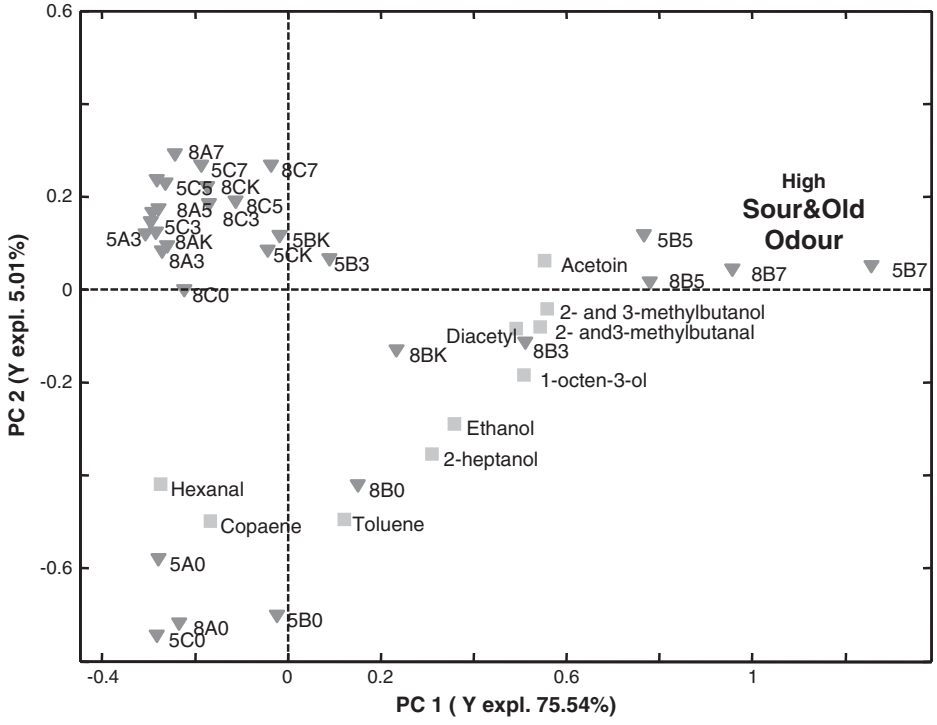


**Fig. 5.** Bi-plot from the final two component PLS-model of sour&old odour. The ten aroma compounds (squares) with the highest regression coefficients towards sour&old odour were used as X-block. The saveloy samples (triangles) are labelled with a code including the initial storage temperature (5 °C or 8 °C), slicing location (A, B or C) and days of storage after package opening (0, 3, 5, 7 and k) where k represents 4 weeks in closed packages.

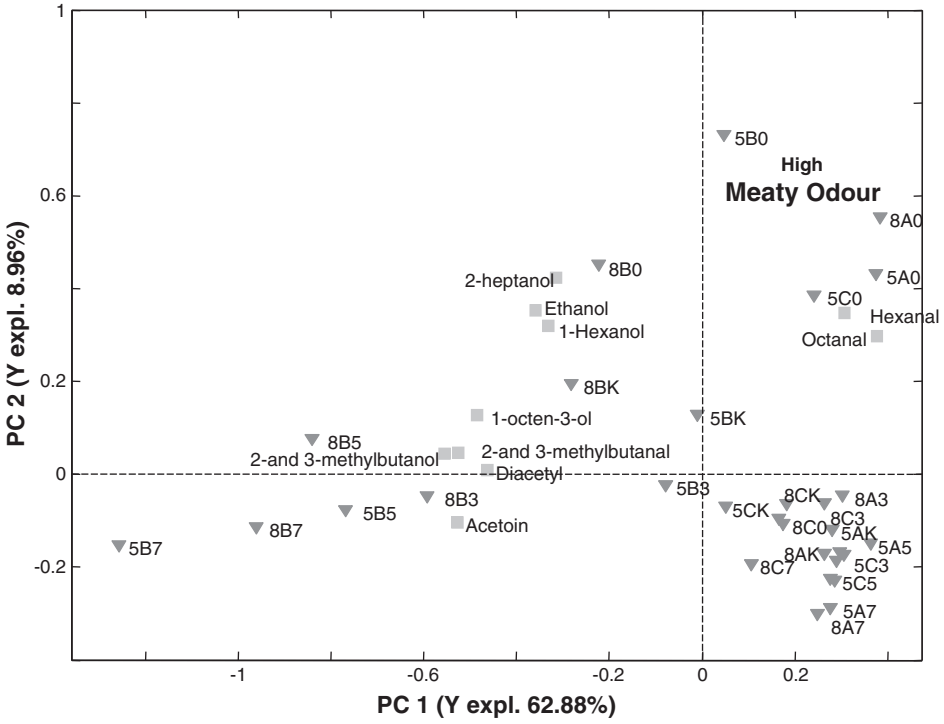


**Fig. 6.** Bi-plot from the final two component PLS-model of meaty odour. The ten aroma compounds (squares) with the highest regression coefficients towards meaty odour were used as X-block. Saveloy samples (triangles) are labelled with a code including the initial storage temperature (5 °C or 8 °C), slicing location (A, B or C) and days of storage after package opening (0, 3, 5, 7 and k) where k represents 4 weeks in closed packages.

saveloy. From Fig. 7 it is seen that the peak areas corresponding to 2- and 3-methylbutanal and 2- and 3-methylbutanol developed differently depending on slicing location. However, in samples from location B a considerable increase in 2- and 3-methylbutanal and 2- and 3-methylbutanol was observed from day three to seven after package opening. This increase corresponds well with the sensory evaluations of samples from location B and with the PLS-models of meaty- and sour&old odour. Overall, the results of this study combined with observations made by other research groups show that 2- and 3-methylbutanal and 2- and 3-methylbutanol have a high impact on the eating quality of various meat products. These compounds are therefore concluded to be suitable as chemical markers for the sensory shelf-life of saveloy.

Diacetyl and acetoin have previously been described as important contributors to the spoilage of different meat products (Dainty & Hibbard, 1983; Dainty & Mackey, 1992; Pin et al., 2002; Stanley et al., 1981). Both compounds, which have buttery odours, are major products of the aerobic metabolism of glucose by *B. thermosphacta*. They are furthermore produced by some LAB including different *Carnobacterium* spp. and *Lactobacillus* spp. (Bartowsky & Henschke, 2004; Borch & Molin, 1989). In this experiment both *B. thermosphacta* and LAB were present in high numbers in several of the samples with high scores in sour&old odour (Table 1 and 2). From Fig. 7 it is seen that the area of the GC-MS peaks corresponding to diacetyl and especially acetoin increased markedly from day three to seven after package opening in samples from location B. This compares well with the observed increase in sour&old odour of these samples. The level of diacetyl and acetoin in samples from location A and C was relatively low compared to location B. However, in samples from location C there was a tendency towards an increase in the acetoin level after package opening though the standard deviation at week 3 + 7 days was high. These observations clearly suggest that acetoin and diacetyl are produced in the microbial spoilage processes during the storage of saveloy. In combination with the information gained from the literature it is apparent that diacetyl and acetoin are useful as chemical markers for the sensory shelf-life of saveloy in this experiment.

### 3.6. Potential applications of the PLS-models

The PLS-models of both meaty odour and sour&old odour, presented in this study, had relatively low RMSECV compared to the range of their sensory scores. Both models moreover explained the main part of the sensory variation using two PC's. The PLS-models were furthermore able to divide the samples into three groups according to their sensory scores based on ten selected aroma compounds. This included a clear differentiation between saveloy samples with poor sensory evaluation and samples with positive sensory evaluations.

This result is promising in relation to the development of a shelf-life model based on measurement of selected aroma compounds. However, the presented PLS-models need thorough validation and further refinement before being applied in the meat industry. This refinement would include adapting the PLS-models to a single slicing location whereas the present models account for the rather large variation between three different slicing locations. The models should furthermore be expanded to include samples from the beginning of the shelf-life period. Upon successful validation and refinement PLS-models as presented here could be developed into a useful quality assurance tool in the production of cooked and sliced meat products. This tool would involve measurements of 2- and 3-methylbutanal, 2- and 3-methylbutanol, acetoin and diacetyl. A single measurement of these chemical markers, made shortly after slicing and packing, could provide an estimate of the shelf-life period of a given batch of saveloy. Recontamination of the product during slicing and packing is moreover the major cause of spoilage in cooked and sliced meat products. An unexpected low shelf-life estimate could therefore indicate insufficient cleaning of the process equipment. Measurements of the chemical markers could therefore also serve as indicators

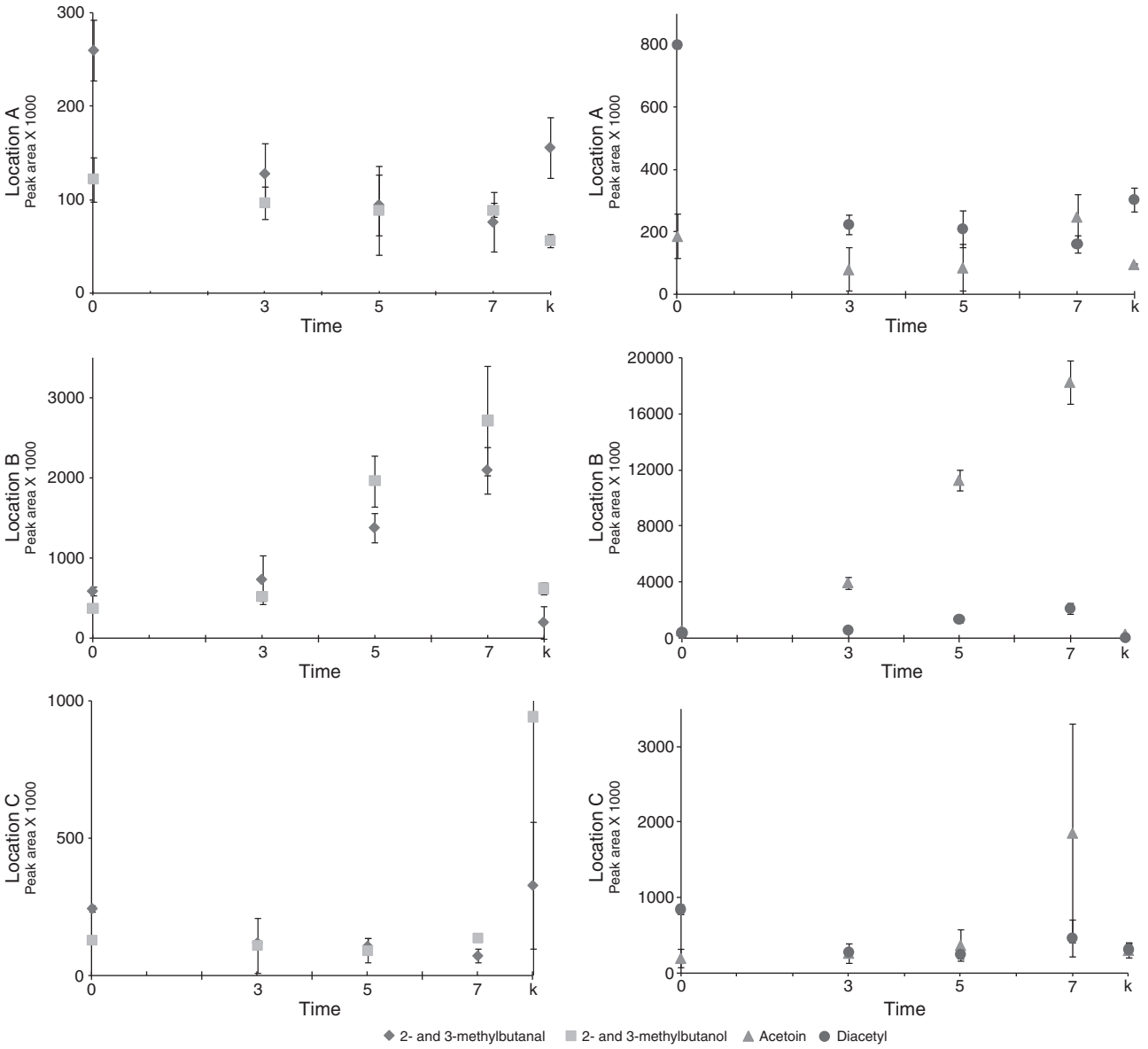


**Fig. 7.** Peak areas and standard deviations of diacetyl, acetoin, 2- and 3-methylbutanal and 2- and 3-methylbutanol from saveloy samples stored at 5 °C for the initial 3 weeks. The peak areas are shown for location A, B and C separately. The scale of the Y-axis is different for each location. The time scale on the X-axis shows the storage time in days after package opening plus three weeks initial storage where k denotes four weeks storage without package opening.

of the current hygiene conditions. Furthermore, specific electronic noses for detection of the identified chemical markers could be incorporated in an intelligent packaging system for detection of spoiled packages by retailers and consumers.

## 4. Conclusion

The results showed that CSS significantly increased the sour&old odour of saveloy samples from slicing locations B and C and moreover decreased the meaty odour of saveloy samples from all three locations. PLS-regression was used to model the relation between the sensory attributes and the aroma composition of the samples. The PLS-models showed that the microbial metabolites 2- and 3-methylbutanal, 2- and 3-methylbutanol, acetoin and diacetyl were closely related to the changes of the sensory attributes meaty- and sour&old odour. These aroma compounds were therefore concluded to be chemical markers for the sensory shelf-life of sliced saveloy. The results should be of interest to the meat industry which would benefit from being able to predict the development in sensory quality throughout the shelf-life. However, the development of a reliable shelf-life model based on the suggested chemical markers needs further refinement of the PLS-models presented in this study.

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# Paper III

## Investigation and modeling of spoilage in saveloy samples inoculated with four potential spoilage bacteria

**In preparation for submission**

**Investigation and modeling of spoilage in saveloy samples inoculated with four potential spoilage bacteria**

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**Abstract**

Sliced saveloy samples were inoculated with monocultures of four potential spoilage bacteria and studied during the expected shelf-life period. The main objective was to study and model the resulting changes in the sensory quality and the aroma composition during the expected shelf-life period. The sensory changes in the saveloy samples were modeled based on the aroma composition using partial least squares regression (PLS). The changes in 6 sensory descriptors were found to be closely related to the amount of diacetyl, acetoin, 2- and 3-methylbutanol, 2- and 3-methylbutanal and 2-methylpropanol in the samples. These compounds are important chemical markers for sensory shelf-life of saveloy. Furthermore, *Brochothrix thermosphacta*, *Chryseomonas luteola*, *Carnobacterium maltaromaticum* were found to have high potential for spoilage of cooked and sliced meat products.

Keywords: Microbial spoilage, Aroma composition, Sensory profiling, PLS-model.

## Introduction

The modeling of shelf-life is a concept of great interest to both meat researchers and the meat industry *(1-4)*. The development of predictive models based on early detection of chemical markers for shelf-life would enable the meat industry to react to changes in sensory quality appearing before the product reaches the consumers. Aroma compounds have potential as chemical markers for shelf-life of cooked and sliced meat products. This was demonstrated in a previous study where four microbially produced aroma compounds were closely linked with the sensory evaluation of saveloy *(5)*.

The microbial spoilage of meat products has been intensively studied and the microbial production of aroma compounds has often been related to the eating quality of cooked and sliced meat products *(3, 4, 6, 7)*. The microbial flora of these products usually derives from slicing and handling at the production facility after heat processing *(8-10)*. The composition and spoilage potential of the in-house microbial flora is therefore an important factor for the development in the sensory quality of these products during storage *(1, 2, 5)*.

The spoilage flora of cooked and sliced meat products is typically composed of lactic acid bacteria (LAB) in combination with *B. thermosphacta* and *Pseudomonas* spp. *(5, 6, 11)*. *B. thermosphacta* is an important spoilage organism in meat products and is well known for production of diacetyl and acetoin under aerobic conditions *(7, 12, 13)*. *Pseudomonas* spp. have been associated with spoilage of meat products due to their rapid growth under aerobic conditions and production of sulfur compounds with low odor threshold values *(4, 6, 14)*. The group LAB refers to a huge group of bacteria of which several genera are important in foods *(15)*. Evidently the spoilage potential of different LAB varies greatly. Several studies have reported specific LAB as the main cause of spoilage of a particular cooked meat product *(9, 16, 17)*. However, other studies report their potential use in biopreservation of similar meat products *(18)*.

In this study sliced saveloy samples were inoculated with monocultures of 4 potential spoilage bacteria: *Brochothrix thermosphacta*, *Chryseomonas luteola*, *Leuconostoc carnosum* and *Carnobacterium maltaromaticum*. These bacteria were chosen based on previous experience and their role in meat spoilage described in previous studies *(16, 19)*. The main objective of this study was to investigate and model the development in the sensory quality of the saveloy samples based on the production of aroma compounds by the 4 potential spoilage bacteria during a four week storage period. Furthermore, measurements of the microbial composition and the content of aroma precursors: free fatty acids (FFA) and free amino acids (FAA) were also included in order to provide further knowledge of the spoilage processes during storage.

The results of this experiment should contribute to the understanding of the quality changes in sliced saveloy during storage and to the development of a model that can predict spoilage in industrially produced meat products based on specific chemical markers.

## Materials and methods

**Experimental setup.** The experiment included 6 series of sliced and modified atmosphere (MA)-packed saveloy. Four series were inoculated with monocultures of *B. thermosphacta*, *C. luteola*, *Leuc. carnosum* and *C. maltaromaticum*. Furthermore, 1 series was inoculated with a 1:1:1:1 mixture of the included bacteria and one series was an uninoculated control. The 6 experimental series were studied in a 4 weeks storage period. During the first 3 weeks of the experiment the saveloy samples were stored at 5 °C in modified atmosphere packaging (MAP). During the fourth week of the experiment the storage conditions were changed to consumer simulated storage (CSS). The packages were opened and then reclosed with a plastic lid permeable to oxygen. The storage temperatures were furthermore set to follow a daily repeated pattern which has been shown to represent storage by Danish consumers *(20)* (5 °C for 12.7 hours, 12 °C for 9.8 hours and 20 °C for 1.5 hours). The effect of 4 weeks storage in the MAP at 5°C was also studied as comparison to CSS. During the experiment the saveloy samples were analyzed at day 1, week 1, week 2, week 3, week 3+3days with CSS, week 3+7 days with CSS and after 4 weeks in MAP at 5 °C. The analyses included measurements of the aroma composition, a sensory profiling, measurements of the microbial composition and measurements of aroma precursors: FFA, FAA. Three replicate measurements for each combination of factors were performed for each of the analyses. Separate packages of saveloy were used for each replicate.

**Production and slicing of saveloy.** The saveloy was produced at the Danish Meat Research Institute (DMRI) using the same recipe and procedure as described in Holm et al. 2012 *(5)*. The saveloy was cut in 1 cm slices of approximately 25 g and inoculated with potential spoilage bacteria. Two saveloy slices were placed in each package which was sealed and packed in a MA consisting of 70% $N_2$ and 30% $CO_2$ as described in Holm et al. 2012 *(5)*.

**Culture development and inoculation.** Frozen cultures (- 80 °C) of *B. thermosphacta* (4618), *C. luteola* (4616), *L. carnosum* (4010), *C. maltaromaticum* (4318) were obtained from the culture collection at DMRI. The cultures were transferred to brain heart infusion (BHI) bouillon (Oxoid Ltd., Basingstoke, UK) and kept for three days at 20°C, and afterwards spread on BHI agar and kept for five days at 20 °C. A single colony was transferred to BHI bouillon and kept at 20 °C for four days before inoculation. Each saveloy slice was inoculated with 0.1 mL diluted BHI bouillon which, based on previous tests, was expected to contain between 50-100 colonies of the relevant bacteria. The mixture of all four bacteria was prepared just before inoculation and contained between 50-100 colonies of each of the four bacterial species.

**Dynamic headspace extraction of aroma compounds and GC-MS analysis.** The extraction of aroma compounds from the saveloy samples was done using dynamic headspace sampling and traps packed with Tenax and Carbograph (Markes International Ltd., Llantrisant, UK). The dynamic headspace extraction and the subsequent GC-MS analyses is described in Holm et al. 2012 *(5)*. However, in the present method the $N_2$ flow used to back purge the traps was set to 20 mL/min

for 5 min. Measurements of the aroma composition were done at: day 1, week 1, week 2, week 3, week 3+3 days, week 3+7 days and week 4. The retention times were standardized using the Kovats linear retention index (LRI) calculated from GC-MS runs of a C5-C15 alkane standard (Air Liquide, Paris, France). GC-MS runs of Tenax traps spiked with authentic standards of specific aroma compounds were furthermore done for identification purposes (See Table 1).

**Sensory analysis.** The sensory profiling was carried out as described in Holm et al. 2012 *(5)* using a trained panel of eight assessors. During training sessions the panel selected 7 sensory descriptors related to odor. These were saveloy odor, spicy odor, acid-like odor which are all positively associated with eating quality and butter-like odor, sweet odor, sour odor and old odor which all are negatively associated with eating quality. Butter-like odor was selected based on reference of acetoin whereas spicy odor was selected based on a sample of the spice mixture used for saveloy production. The remaining descriptors were selected by evaluating saveloy samples at different stages of the shelf-life period. The sensory analysis was performed at sampling time: day 1, week 3, week 3+3 days, week 3+7 days and week 4.

**Microbial analysis.** The analysis of the microbial composition of the saveloy samples was carried out as described in Holm et al. 2012 *(5)*. Four different growth substrates were used. BHI (Oxoid Ltd., Basingstoke, UK) was used for the total microbial count. Streptomysin and Thallous Acetate agar (STA, Oxoid Ltd., Basingstoke, UK) with STA selective supplement was used to detect B. *thermosphacta*. Pseudomonas agar base with Cetrimide, Fucidin and Cephalosporin agar supplement (CFC, Oxoid Ltd., Basingstoke, UK) was used to detect *C. luteola*. All Purpose medium with Tween containing polymyxin (APT, Merck KGaA, Darmstadt, Germany) with a cover layer of APT was used to detect LAB. Saveloy samples were spread on BHI at day 1, week 3, week 3+3 days, week 3+7 days and week 4 whereas the specific growth media was only used at day 1, week 3+7 days and week 4.

**Free fatty acid analysis.** For determination of the percentage of FFA in the fat fraction of the saveloy samples two slices (about 50 g) were blended (Grinomix GM200 Retsch, Düsseldorf, Germany) and heated in a microwave oven for 3x30 seconds at 780 W. After each 30 seconds the blended saveloy was stirred. The melted fat fraction was separated from the remaining sample by centrifugation (5 minutes, 3500 rpm, Omnifuge 2.0 RS, Heraeus Sepatech, Hanau, Germany). 2 g melted fat was mixed with 20 mL ethanol: chloroform solution (2:1). The percentage of FFA in the sample was determined by titration with 0.1 N sodium hydroxide using phenolphthalein as indicator. The molecular weight of oleic acid (282 g/mol) was used to represent the FFA in the calculations. Determination of FFA was done on sampling time: day 1, week 3, week 3+3 days, week3+7 days and week 4.

**Free amino acid analysis.** The concentration of FAA in the saveloy samples was determined by a GC-MS method. 2.5 g saveloy was taken from a cross-section of the 1 cm thick slice and added 10 mL 25 mM sodium phosphate buffer with pH 6.0. The mixture was homogenized for 30 seconds at

28.6x1000 rpm using a kinematica polytron equipped with a 12 mm tip (PT 3000, Kinematica AG, Lucerne, Switzerland). The mixture was centrifuged 10 min at 4000 G (Omnifuge 2.0 RS, Heraeus Sepatech, Hanau Germany) and the resulting supernatant was centrifuged another 5 minutes at 14000 G (Biofuge15, Heraeus Sepatech, Hanau Germany). The final supernatant was filtered through a 0.45 µm syringe filter (Frisenette, Knebel Denmark).

The preparation of the saveloy extract for GC-MS measurement was adapted from Glastrup et. al. 2009 *(21)* with some modification. 150 µL of the saveloy extract was mixed with four reagents: 150 µL norvaline solution which was used as internal standard; 200 µL methanol/pyridine solution (4:1) was added for catalyzation purposes; 25 µL methyl chloroformate (MCF) was added as derivatization reagent; 500 µL 1% MCF in chloroform which acted as solvent. The solution was carefully mixed after adding each reagent.

The samples were then injected on G1800A GCD system equipped with a DB XLB column (15 m x 0.250 mm with a film thickness of 0.25 µm, Agilent Technologies, Santa Clara, CA, USA). The GC-MS system was operated with the following parameters: injection temperature; 250 °C, injection volume; 2.0 µL; split flow 10:1; carrier gas: helium (1.5 mL/min), column pressure of 7.6 psi, Oven program; from 90 °C to 240 °C at 6 °C/min with a final holding time of 5 min. The mass selective detector operated in the electron impact mode at 70 eV and the MS scanned from 70 m/z to 250 m/z. For quantification purposes an amino acid standard solution with concentrations ranging from 0.2-5 mM was measured using the procedure described above (Sigma-Aldrich, St. Louis, Missouri, USA) Measurements of FAA in the saveloy extract were done at sampling time: day 1, week 3, week 3+3 days and week 3+7 days.

**Data processing.** The GC-MS data was processed using the MSD Chemstation software (D.01.02.16, Agilent Technologies, Santa Clara, CA, USA). An integration method was setup for calculation of peak areas based on selected target and qualifier ions. Due to co-elution and similar mass spectra some peaks were not quantified individually. 2-methylbutanal and 3-methylbutanal were quantified together at an LRI of 924. 2-methylbutanol and 3-methylbutanol were quantified together with an LRI of 1197. Diacetyl and pentanal were quantified with an LRI of 970. However, an analysis of this peak showed that pentanal was relatively constant throughout the experiment whereas diacetyl was responsible for the observed increase in the area of this peak.

Three different methods were used for identification of the chromatographic peaks. The mass spectra of the peaks were compared with mass spectra of potential matching compounds using the NIST/EPA/NIH mass spectral library (V.1.7a, Agilent Technologies, Santa Clara, CA, USA). The LRI of the chromatographic peaks was moreover compared with the LRI of potential matching compounds using the C20M column from internet database www.flavornet.org *(22)*. The maximum difference in LRI accepted for a potential match was 50 units. The mass spectra and LRI were furthermore compared with GC-MS measurements of authentic standards of compounds expected to be relevant for the shelf-life of saveloy.

Statistical analysis was performed in JMP v. 8.0.1 (SAS institute, Cary, North Carolina, USA). For each analysis ANOVA models were built describing the relevant variables using storage time, experimental series and 'storage time *x* experimental series' as fixed effects. For the sensory descriptors the ANOVA models furthermore included assessor as a fixed effect. Based on the ANOVA models Tukey Honest Significant Different (HSD) tests were done to find significant differences between the levels of the relevant variables.

Chemometric analysis was done using the PLS Toolbox (version 5.2.2, Eigenvector Research inc., Wenatchee, WA, USA). The PLS toolbox is running in the MATLAB environment (version 7.6.0.324, The Matworks inc., Natick, MA, USA). All partial least squares regression (PLS) models and principal component analyses (PCA) were validated using random subset validation with 9 iterations of each model and 5 data splits.

**Table 1: An overview of aroma compounds extracted the saveloy samples for each experimental series: Control (c), B. *thermosphacta* (Bt), C *luteola* (Cl), Leuc. *carnosum* (Lc) and C. *maltaromaticum* (Cm) and the Mixture series (M). The linear retention index (LRI) is shown along with a compound code and the target ion used for quantification. The following abbreviations are used for the identification methods: The NIST mass spectral data base (N), comparisons of the observed LRI with LRI from flavornet.com (L), GC-MS runs of authentic standard compounds (S). Letters *a* to *d* are used to indicate significant differences in the peak area of the aroma compounds between experimental series based on a Tukey HSD test.**

| Nr. | code | Compound name | LRI | T-Ion | ID method | C | Bt | Cl | Lc | Cm | M |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | A1 | Ethanal | 700 | 44 | L-N | a | bc | bc | b | d | cd |
| 2 | K1 | Acetone | 811 | 43 | N | a | bc | ab | d | cd | d |
| 3 | K2 | 2-butanone | 901 | 43 | L-N | a | b | b | c | b | c |
| 4 | A2 | 2- and 3- methylbutanal | 924 | 41 | S-L-N[II] | c | b | c | c | a | c |
| 5 | Ac1 | Isopropyl alcohol/ethanol | 928 | 45 | S-L | a | b | b | a | b | a |
| 6 | K3/A3 | Diacetyl/pentanal | 970 | 43 | S-L-N[III] | c | a | b | c | b | c |
| 7 | T1 | α-pinene | 1012 | 93 | S-L-N[IV] | a | ab | ab | ab | ab | b |
| 8 | T2 | α-thujene | 1018 | 93 | N | a | ab | ab | b | bc | c |
| 9 | T3 | Camphene | 1050 | 93 | L-N | | | | | | |
| 10 | S1 | Dimethyl disulfide | 1060 | 94 | S-L-N[IV] | b | b | a | b | b | b |
| 11 | A4 | Hexanal | 1073 | 44 | S-L-N[I] | a | a | a | b | a | c |
| 12 | T4 | β-pinene | 1085 | 93 | L-N | a | ab | abc | bc | bc | c |
| 13 | Ac2 | 2-methyl-1-propanol | 1073 | 43 | S-L-N[III] | c | b | a | c | c | c |
| 14 | T5 | Sabinene | 1102 | 93 | L-N | | | | | | |
| 15 | T6 | 3-carene | 1131 | 93 | S-L-N[IV] | a | ab | ab | ab | bc | c |
| 16 | T7 | α-phellandrene | 1148 | 93 | L-N | a | ab | bc | b | bc | c |
| 17 | T8 | β-Myrcene | 1153 | 93 | L-N | | | | | | |
| 18 | T9 | α-terpinene | 1163 | 121 | L-N | a | a | ab | ab | bc | c |
| 19 | K4 | 2-Heptanone | 1173 | 43 | S-L-N[IV] | a | ab | ab | c | b | d |
| 20 | A5 | Heptanal | 1175 | 70 | S-L-N[IV] | a | a | a | ab | a | b |
| 21 | T10 | Limonene | 1181 | 68 | S-L-N[IV] | a | b | bc | bc | c | d |
| 22 | T11 | Eucalyptol | 1173 | 43 | L-N | | | | | | |
| 23 | Ac3 | 2- and 3-methylbutanol | 1197 | 55 | S-L-N[I] | c | ab | bc | c | a | c |
| 24 | F | 2-pentylfuran | 1218 | 81 | S-L-N[V] | a | b | b | b | b | c |
| 25 | T12 | γ-terpinene | 1228 | 93 | L-N | a | ab | b | b | bc | c |
| 26 | Ac4 | 3-methylbutenol | 1239 | 41 | N | ab | a | ab | ab | ab | b |
| 27 | Ac5 | 1-Pentanol | 1239 | 42 | L-N | ab | bc | bc | a | c | ab |
| 28 | T13 | p-cymene | 1253 | 119 | L-N | | | | | | |
| 29 | T14 | δ-terpinene | 1253 | 121 | L-N | a | ab | bc | bc | cd | d |
| 30 | K5 | Acetoin | 1269 | 45 | S-L-N[II] | c | a | b | c | a | c |
| 31 | A6 | Octanal | 1273 | 43 | S-L-N[IV] | ab | a | ab | ab | ab | b |
| 32 | Ac6 | 2-heptanol | 1306 | 45 | S-L-N[III] | b | b | b | a | b | a |
| 33 | Ac7 | 1-Hexanol | 1340 | 56 | S- L-N[III] | bc | bc | b | a | b | a |
| 34 | S2 | Dimethyl trisulfide | 1362 | 126 | S-L-N[IV] | b | b | a | b | b | b |
| 35 | A7 | Nonanal | 1383 | 43 | L-N | a | a | ab | a | ab | b |
| 36 | T15 | α-p-dimethylstyrene | 1422 | 117 | L-N | | | | | | |
| 37 | Ac8 | 1-octen-3-ol | 1436 | 57 | S-L-N[III] | ab | a | bc | bc | bc | c |
| 38 | Ca | Acetic acid | 1440 | 45 | S-L-N[III] | a | ab | a | bc | bc | c |
| 39 | T16 | Camphor | 1447 | 95 | L-N | a | a | a | a | a | b |
| 40 | T17 | Linalool | >1447 | 71 | N | a | a | a | a | a | b |
| 41 | T18 | Caryophyllene | >1447 | 133 | N | ab | a | ab | ab | ab | b |
| 42 | T19 | 1-terpinen-4-ol | >1447 | 71 | N | a | a | a | a | a | b |

[I]Merck KGaA (Darmstadt, Germany). [II]Chem Service inc. (West Chester, Pennsylvania, USA). [III]Supplied by Sigma-Aldrich (St. Louis, Missouri, USA). [IV]Acros Organics (Geel, Belgium). [V]Lancaster Synthesis (Windham New Hampshire, USA).

**Results and discussion**

**The aroma composition of the inoculated saveloy samples.** A total of 42 different aroma compounds were found and identified from the headspace of sliced saveloy during the experiment. Of these 19 were classified as terpenes which derive from the spices added to the product *(23)*. Alcohols, ketones and aldehydes were the other major classes of compounds isolated from the saveloy samples. An overview of the volatile compounds found in saveloy is given in Table 1. The table includes the LRI, the target ion used for quantification and a list of the methods used for compound identification. The ANOVA model calculated on the aroma data showed that the fixed effects: experimental series, storage time and 'storage time *x* experimental series' had significant effect on the majority of the aroma compounds. In Table 1 letters *a* to *d* are used to indicate significant differences between different spoilage bacteria for each aroma compound.

**Effect of spoilage bacteria on the formation of aroma compounds.** The results of the Tukey HSD test, shown in Table 1, helped identify aroma compounds that are characteristic for each experimental series. Aroma compounds present in increased levels in the inoculated series compared to the control samples are expected to derive from microbial metabolism.

The microbial metabolites diacetyl, acetoin, 2- and 3-methylbutanol, 2- and 3-methylbutanal are of particular interest in this experiment as they have previously been shown to be closely related to spoilage of different products including saveloy *(5, 11, 19)*. The development in the peak area of these 4 compounds is shown in Figure 1 for saveloy samples inoculated with *B. thermosphacta*, *C. maltaromaticum* and *C. luteola*. For samples inoculated with these three bacteria the production of diacetyl and acetoin increased considerably after the onset of CSS and it peaked at week 3+7 days. Figure 1 furthermore shows that the amount of acetoin and diacetyl found in the saveloy samples after 4 weeks storage in MAP at 5 °C were very low compared to week 3+7 days with CSS. At week 4 the highest amount of acetoin and diacetyl was found in the samples inoculated with C. *luteola*.

2- and 3-methylbutanal was mainly produced by *B. thermosphacta* and *C. maltaromaticum*, whereas 2- and 3-methylbutanol was produced by both *B. thermosphacta*, *C. maltaromaticum* and *C. luteola*. In the *B. thermosphacta* samples the level of 2- and 3-methylbutanal and 2- and 3-methylbutanol increased only after the onset of CSS. In samples inoculated with *C. maltaromaticum* an increase in 2- and 3-methylbutanal was observed between week 2 and week 3 which was prior to package opening. An elevated amount of 2- and 3-methylbutanal was also observed after 4 weeks in MAP for the *C. maltaromaticum* samples. However, the production of both 2- and 3-methylbutanal and 2- and 3-methylbutanol by *C. maltaromaticum* generally appears to be accelerated by CSS. For the *C. luteola* samples 2- and 3-methylbutanol was found in slightly elevated amounts at week 4. However, for samples inoculated with this bacterium production of 2- and 3-methylbutanol was also accelerated by CSS. The observed patterns in the formation of

diacetyl, acetoin, 2- and 3-methylbutanol, 2- and 3-methylbutanal by *B. thermosphacta* and *C. maltaromaticum* corresponds well with observations made in previous studies on raw meat, fermented meat product and shrimp *(7, 19, 24)*.

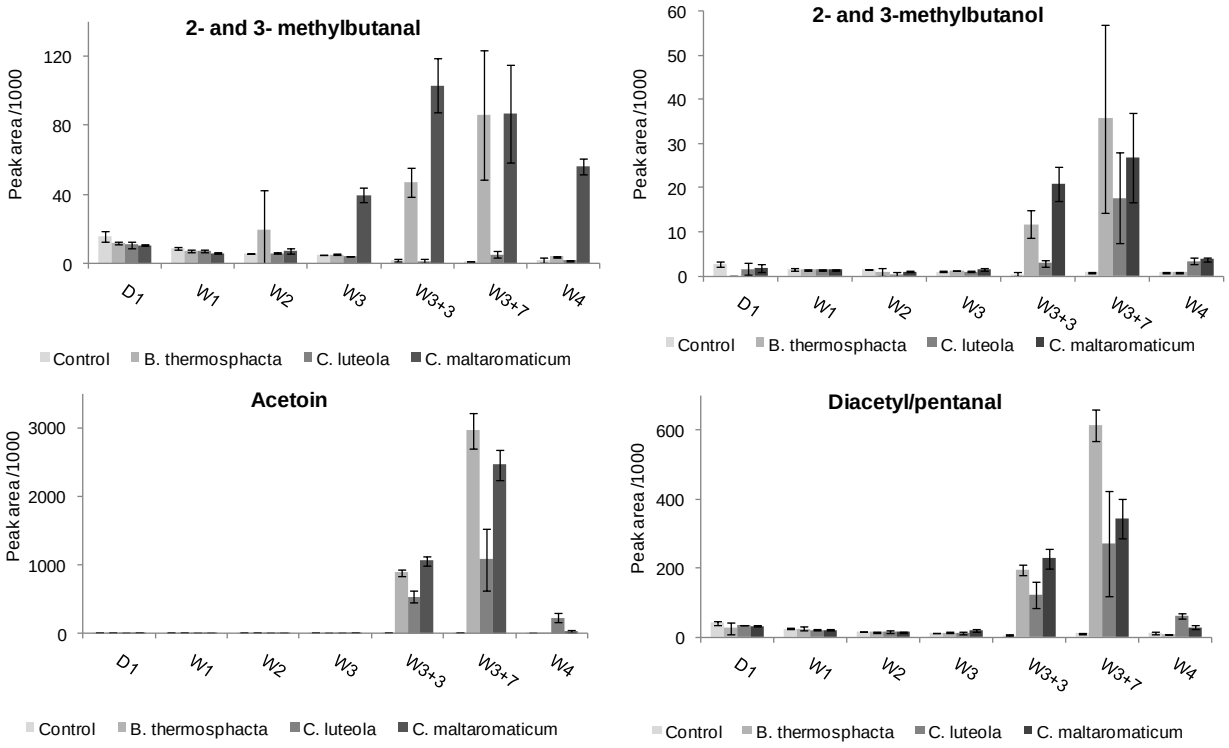


**Figure 1. The peak areas and std of: 2- and 3- methylbutanal, 2- and 3- methylbutanol, acetoin and diacetyl/pentanal are shown for day 1 (D1), week 1 (W1), week 2 (W2), week 3 (W3), week 3+3 days with consumer simulated storage (CSS), week 3+7 days with CSS and week 4 (W4). Only the relevant experimental series are included.**

Table 1 shows that the samples inoculated with *C. luteola* contained significantly higher amounts of dimethyl disulfide (DMDS) and dimethyl trisulfide (DMTS) compared to the other experimental series. However, the standard error on the determination of these compounds was relatively high. The formation of various sulfur compounds, including DMDS and DMTS, by *Pseudomonas* spp, has been reported in previous studies, and these compounds have been associated with spoilage of meat products *(14)*. *C. luteola* and *B. thermosphacta* furthermore produced high amounts of 2-methylpropanol. The peak areas of 2-methylpropanol, DMDS and DMTS are shown in Figure 2 for the relevant experimental series.

From Table 1 it is seen that samples inoculated with *Leuc. carnosum* are characterized by a high content of several straight chain alcohols including ethanol, 1-pentanol, 1-hexanol and 2-heptanol. The samples inoculated with a mixture of all four potential spoilage bacteria also contained elevated amounts these alcohols. This suggests that *Leuc. carnosum* is the dominating bacteria in the Mixture samples. 1-Hexanol was the most characteristic compound in the *Leuc. carnosum*

samples and the Mixture samples. The development of this compound with storage time is seen in Figure 2 for the relevant experimental series.

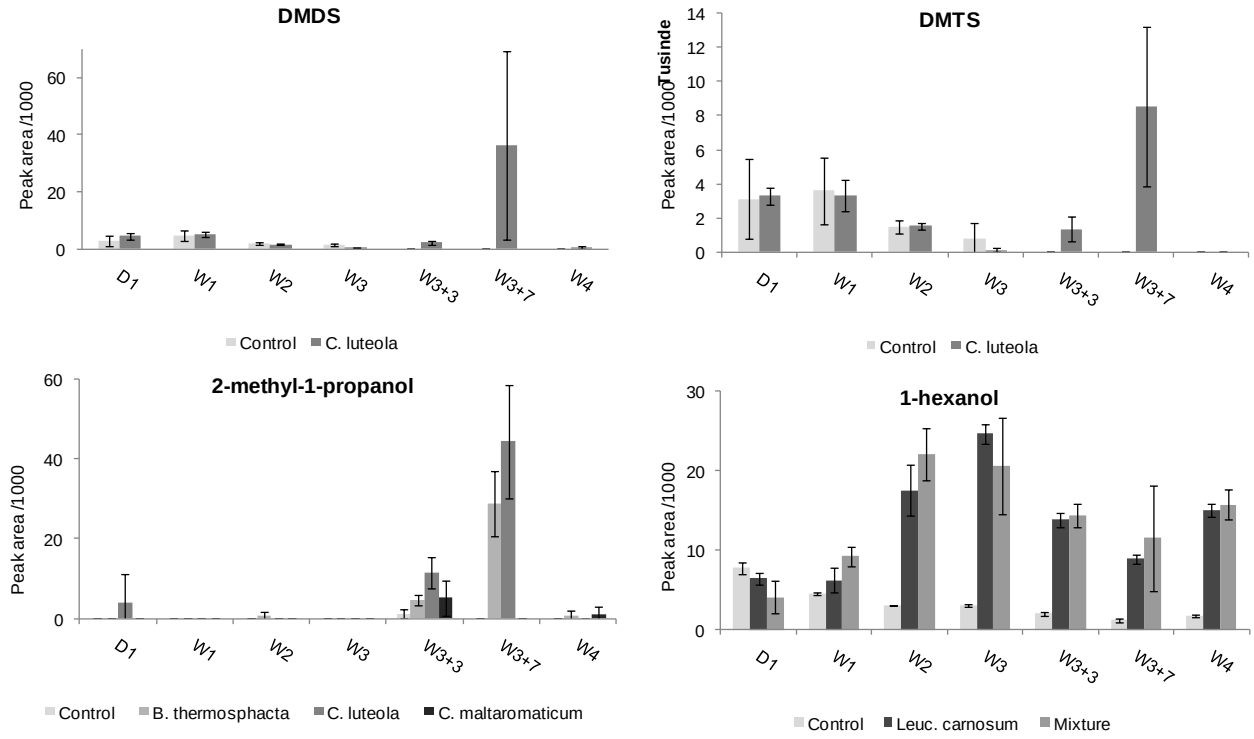


**Figure 2. The peak area and standard deviation: DMDS, DMTS, 2-methyl-1-propanol and 1-hexanol are shown for day 1 (D1), week 1 (W1), week 2 (W2), week 3 (W3), week 3+3 days with consumer simulated storage (CSS), week 3+7 days with CSS and week 4 (W4). Only the relevant experimental series are included.**

**Early detection of microbial metabolites.** When examining the effect of 'storage time *x* experimental series' it was noticed that none of the microbially produced aroma compounds diacetyl, acetoin, 2- and 3-methylbutanol and 2- and 3-methylbutanal changed significantly in the time span from day 1 to week 1 (results not shown). However, in the early stages after inoculation the bacteria could be in the lag-phase where their growth and metabolism is limited *(25)*. This indicates that an increase in the sensitivity of the applied method for aroma extraction and GC-MS analysis is needed in order to capture the initial changes in these aroma compounds. This could possibly be achieved by increasing the purge time or the purge flow during aroma extraction or by using single ion monitoring in the GC-MS analysis. PTR-MS, which is an on-line method for measurements of volatile compounds, has high sensitivity and could also be a potential method for early detection of aroma compounds in saveloy *(26)*. However, it is currently uncertain whether the early changes in the aroma composition can be detected and be linked with the eventual spoilage of saveloy.

**Table 2: Includes the sensory scores of each descriptor averaged over assessor and repetitions for each experimental series: Control (C), *B. thermosphacta* (Bt), *C. luteola* (Cl), *Leuc. carnosum* (Lc), *C. maltaromaticum* (Cm), Mixture (M). Letters a to d are used to indicate significant differences between experimental series (horizontal) and storage time (vertical) based on a Tukey HSD test.**

| *Sensory descriptor* | *Storage Time* | *Tukey HSD Test: Storage time* | *Sensory Scores* | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | ***C*** | ***Bt*** | ***Cl*** | ***Lc*** | ***Cm*** | ***M*** |
| **Saveloy Odor** | **Day1** | *a* | 5.9 | 5.9 | 5.8 | 6.3 | 6.6 | 6.0 |
| | **Week3** | *b* | 4.9 | 4.9 | 5.1 | 3.9 | 4.2 | 3.5 |
| | **Week3+3** | *c* | 5.0 | 2.3 | 2.0 | 3.5 | 2.2 | 2.8 |
| | **Week3+7** | *d* | 4.4 | 1.6 | 1.3 | 2.8 | 1.6 | 2.7 |
| | **Week4** | *c* | 4.7 | 4.6 | 2.2 | 2.6 | 2.0 | 2.3 |
| ***Tukey HSD Test: Series*** | | | *a* | *b* | *b* | *b* | *b* | *b* |
| **Spicy Odor** | **Day1** | *a* | 4.4 | 4.4 | 4.4 | 5.1 | 4.9 | 4.7 |
| | **Week3** | *b* | 3.8 | 3.8 | 4.0 | 3.1 | 3.3 | 3.1 |
| | **Week3+3** | *c* | 3.8 | 2.2 | 1.7 | 2.9 | 2.2 | 2.6 |
| | **Week3+7** | *d* | 3.5 | 1.5 | 1.1 | 2.4 | 1.4 | 2.1 |
| | **Week4** | *c* | 3.5 | 3.5 | 1.7 | 2.2 | 1.7 | 2.0 |
| ***Tukey HSD Test: Series*** | | | *a* | *bc* | *c* | *b* | *bc* | *bc* |
| **Acid-Like Odor** | **Day1** | *a* | 3.1 | 3.3 | 3.3 | 3.2 | 3.2 | 3.4 |
| | **Week3** | *ab* | 3.2 | 3.0 | 3.1 | 2.7 | 2.9 | 2.8 |
| | **Week3+3** | *bc* | 2.7 | 1.6 | 1.1 | 3.0 | 1.7 | 3.1 |
| | **Week3+7** | *bc* | 3.1 | 1.7 | 1.9 | 4.2 | 1.3 | 4.1 |
| | **Week4** | *c* | 2.7 | 2.8 | 1.6 | 3.2 | 1.3 | 3.9 |
| ***Tukey HSD Test: Series*** | | | *ab* | *bc* | *c* | *a* | *c* | *a* |
| **Butter-like Odor** | **Day1** | *d* | 0.8 | 0.5 | 0.8 | 0.4 | 0.9 | 0.8 |
| | **Week3** | *d* | 0.5 | 0.3 | 0.4 | 0.7 | 0.5 | 1.2 |
| | **Week3+3** | *b* | 0.6 | 4.5 | 1.5 | 0.9 | 5.1 | 1.5 |
| | **Week3+7** | *a* | 0.2 | 7.5 | 2.1 | 0.6 | 6.7 | 0.9 |
| | **Week4** | *c* | 0.5 | 0.7 | 1.4 | 1.1 | 5.5 | 1.1 |
| ***Tukey HSD Test: Series*** | | | *d* | *b* | *c* | *cd* | *a* | *cd* |
| **Sweet Odor** | **Day1** | *c* | 1.0 | 0.9 | 0.9 | 0.9 | 0.9 | 0.6 |
| | **Week3** | *c* | 0.5 | 0.5 | 0.4 | 1.0 | 0.9 | 1.4 |
| | **Week3+3** | *ab* | 0.7 | 3.9 | 2.8 | 1.3 | 3.4 | 1.5 |
| | **Week3+7** | *a* | 0.9 | 4.4 | 3.6 | 0.8 | 4.7 | 1.0 |
| | **Week4** | *b* | 0.6 | 0.9 | 2.3 | 1.3 | 4.1 | 1.6 |
| ***Tukey HSD Test: Series*** | | | *c* | *ab* | *b* | *c* | *a* | *c* |
| **Sour Odor** | **Day1** | *c* | 0.5 | 0.7 | 0.9 | 0.5 | 0.6 | 0.4 |
| | **Week3** | *c* | 0.5 | 0.6 | 0.2 | 1.6 | 1.1 | 2.3 |
| | **Week3+3** | *b* | 0.6 | 4.6 | 2.3 | 2.6 | 5.7 | 4.0 |
| | **Week3+7** | *a* | 0.6 | 5.9 | 5.3 | 3.8 | 6.5 | 4.1 |
| | **Week4** | *b* | 1.2 | 1.5 | 3.3 | 2.7 | 5.5 | 3.8 |
| ***Tukey HSD Test: Series*** | | | *c* | *b* | *b* | *b* | *a* | *ab* |
| **Old Odor** | **Day1** | *b* | 0.6 | 0.8 | 1.0 | 0.5 | 0.7 | 0.5 |
| | **Week3** | *b* | 0.3 | 0.6 | 0.2 | 1.3 | 1.1 | 1.9 |
| | **Week3+3** | *c* | 0.8 | 5.8 | 4.8 | 2.4 | 5.9 | 3.4 |
| | **Week3+7** | *c* | 1.2 | 5.6 | 8.7 | 3.4 | 5.8 | 3.3 |
| | **Week4** | *c* | 1.6 | 1.5 | 5.2 | 3.1 | 6.8 | 3.8 |
| ***Tukey HSD Test: Series*** | | | *c* | *b* | *a* | *b* | *a* | *b* |

**Sensory changes of the saveloy samples.** The results of the sensory assessments of are shown in Table 2. The sensory scores were averaged over assessor and repetitions for each combination of storage time and experimental series. All the fixed effects included in the ANOVA model had significant influence on each of the seven sensory descriptors. Significant changes in the sensory descriptors according to the Tukey HSD test are indicated in Table 2 using letters from *a* to *d*.

**Effect of storage time on the sensory evaluation.** Spicy odor, saveloy odor and acid-like odor are all positively associated with eating quality and generally showed similar behavior throughout the experiment. The highest scores for all three descriptors were found in the fresh saveloy samples from day 1. After three weeks storage in MAP the scores of these three descriptors were significantly lowered. When subjected to CSS in the fourth week of storage the scores of both saveloy odor, spicy odor and acid-like odor decreased even more and reached the overall lowest levels at week 3+7 days. A significant decrease in the intensity of these three descriptors was also observed when comparing the samples from week 3 and week 4 in MAP. Overall, these results suggests that storage time had a negative effect on both saveloy odor, spicy odor and acid-like odor and that this effect was enhanced by package opening and temperature loads.

None of the sensory descriptors butter-like odor, sweet odor, sour odor and old odor changed significantly during the first three weeks of storage in MAP but after the introduction of CSS in the fourth week of storage the scores of all four descriptors started to increase. The highest individual scores of all four descriptors were observed at week 3+7 days. A general increase in all four descriptors was also observed between week 3 and week 4 in MAP. This means that the spoilage processes starts affecting the sensory quality of the product during the fourth week of storage regardless of the storage conditions.

**Effect of experimental series on the sensory evaluation.** From Table 2 it was seen that inoculation with *B. thermosphacta*, *C. maltaromaticum* and *C. luteola* generally had a negative effect on the sensory descriptors during the four weeks storage period. The sensory scores of sweet-, sour- and old- odor were all significantly increased during the experiment when compared to the control samples. The scores in spicy odor and saveloy odor were furthermore significantly decreased during storage in samples inoculated with these bacteria. Table 2 also shows that butter-like odor almost exclusively described spoilage by *B. thermosphacta* and *C. maltaromaticum.* For samples inoculated with *C. luteola* old odor was the most characteristic sensory descriptor. From Table 2 it is seen that the overall highest scores in old odor was found for the *C. luteola* samples measured at week 3+7 days. When comparing the sensory scores from week 3+7 days and week 4 it is noticed that *B. thermosphacta*, which is a facultative anaerobic bacterium, did not induce sensory spoilage in the MA-packed saveloy samples from week 4. For the *C. maltaromaticum* and *C. luteola* samples the difference between week 3+7 days and week 4 was less significant. This suggests that these bacteria were able to cause spoilage both with and without the presence of oxygen.

For the samples inoculated with *Leuc. carnosum* a significant increase in both sour and old off-odors. However, the effect of inoculation with *Leuc. carnosum* on the sensory descriptors was generally not as pronounced as for samples inoculated with *B. thermosphacta*, *C. maltaromaticum* and *C. luteola*. This was noticed when investigating the 'storage time *x* experimental series' effect (data not shown) and when comparing the individual scores in Table 2. This could indicate that *Leuc. carnosum* does not cause as intense spoilage as the three other bacteria. Table 2 furthermore shows that the sensory scores of the *Leuc. carnosum* samples and the Mixture samples could not be separated statistically. This further supports the hypothesis that the Mixture samples were dominated by *Leuc. carnosum.*

**Microbial composition of the saveloy samples.** The purpose of the microbial analysis in this study was to examine the microbial growth in the 6 experimental series and secondly to validate the inoculation step in the experiment. In Table 3 the microbial counts are expressed as the logarithm of the total number of colony forming units (CFU) per grams of sample.

Though the control samples were not inoculated with spoilage bacteria some organisms were detected in these samples. This background flora could consist of sporulating bacteria deriving from the spices or contaminating bacteria from the slicing and packing process. However, as seen in Table 3 the background flora never exceeded $10^{5.0}$ CFU/g and is therefore not expected to contribute to spoilage of the saveloy samples in this experiment.

For the experimental series inoculated with potential spoilage bacteria there is generally good correlation between the microbial growth on BHI and the specific growth media (CFC, STA and ATP, Table 3). Samples inoculated with *B. thermosphacta* reached comparable counts on BHI and on the specific STA growth medium. *B. thermosphacta* was furthermore detected on ATP. For samples inoculated with *C. luteola* the BHI count corresponded well with the number of CFU of the pseudomonas specific CFC plates. At day 1 colonies were also observed on ATP for the *C. luteola* samples. However, these colonies were ascribed to the background flora as a similar number of colonies were observed for the control samples. Finally the number of *Leuc. carnosum* and *C. maltaromaticum* colonies detected on BHI was comparable to the number of colonies detected on the LAB specific growth medium ATP. Overall these results indicate that the inoculation step was successful.

**Table 3. An overview of the microbial counts of the six experimental series on the four growth substrates given in log CFU/g. BHI indicates the total microbial count, CFC counts Pseudomonas spp., STA counts B. *thermosphacta* and ATP counts lactic acid bacteria.**

| | *Time* | *BHI* | *CFC* | *STA* | *ATP* |
|---|---|---|---|---|---|
| Control | **Day1** | < 1 – 2.5 | < 1.0 | < 1.0 | 2.3 |
| | **Week3** | < 5.0 | - | - | - |
| | **Week3+3** | < 5.0 | - | - | - |
| | **Week3+7** | < 5.0 | < 1.0 | < 1 – 1.3 | < 5.0 |
| | **Week4** | < 5.0 | < 1.0 | < 1- 1.0 | < 5.0 |
| *B thermosphacta* | **Day1** | 2.5 | < 1.0 | 1.8 | 2.4 |
| | **Week3** | ≤5.0 | - | - | - |
| | **Week3+3** | 7.8 | - | - | - |
| | **Week3+7** | 8.0 | < 1.0 | 7.9 | 8.1 |
| | **Week4** | 5.5 | < 1.0 | 5.3 | 5.2 |
| C *luteola* | **Day1** | 1.8 | 1.6 | < 1.0 | 2.4 |
| | **Week3** | < 5.0 – 6.0 | - | - | - |
| | **Week3+3** | 8.6 | - | - | - |
| | **Week3+7** | 9.1 | 9.0 | < 1.0 | ≤ 5.0 |
| | **Week4** | < 6.0 – 8.0 | 7.7 | < 1.0 – 1.8 | < 5.0 |
| Leuc *carnosum* | **Day1** | 3.2 | < 1.0 | < 1.0 | 2.4 |
| | **Week3** | 8.2 | - | - | - |
| | **Week3+3** | 8.1 | - | - | - |
| | **Week3+7** | 8.0 | 2.4 | < 1.0 | 8.0 |
| | **Week4** | 8.3 | < 1.0 | ≤ 1.0 | 8.1 |
| C *maltaromaticum* | **Day1** | 2.4 | < 1.0 | < 1.0 | 2.3 |
| | **Week3** | 7.1 | - | - | - |
| | **Week3+3** | 8.3 | - | - | - |
| | **Week3+7** | 8.7 | < 1.0 | < 1.0 – 1.7 | 8.6 |
| | **Week 4** | 7.6 | < 1.0 | < 1.0 | 7.6 |
| Mixture | **Day1** | 3.1 | 2.1 | 1.7 | 3.1 |
| | **Week3** | 8.2 | - | - | - |
| | **Week3+3** | 8.3 | - | - | - |
| | **Week3+7** | < 6.0 – 8.0 | < 4.0 – 5.1 | < 4.0 – 4.9 | 7.9 |
| | **Week 4** | 8.3 | 5.8 | < 1 | 8.1 |

**Microbial growth and spoilage potential.** Due to the varying spoilage potential of different bacteria it is difficult to establish microbial spoilage using only the total microbial count. Nevertheless, growth to $10^7$ CFU/g is often used as an indicator that the risk of microbial spoilage is looming *(6, 11)*. *B. thermosphacta*, *C. maltaromaticum* and *C. luteola* were responsible for the most significant changes in sensory quality observed in the experiment. The microbial results shown in Table 3 further suggest a high risk of spoilage in samples inoculated with these bacteria during the fourth week of the experiment. The highest total microbial counts in samples inoculated with *B. thermosphacta*, *C. maltaromaticum* and *C. luteola* were found at week 3+7 days. As seen in Table 3 the *C. maltaromaticum* samples and *C. luteola* samples also reached microbial counts indicating a high spoilage risk in samples stored 4 weeks in MAP at 5 °C. However, the *B. thermosphacta* samples only reached a maximum of $10^{5.5}$ CFU/g after 4 weeks in MAP. This corresponds well with the aroma composition and the sensory evaluations of these samples which showed that *B. thermosphacta* did not spoil the MAP packed saveloy samples in this study.

After 3 weeks in MAP at 5 °C the total microbial count of the saveloy samples inoculated with *Leuc. carnosum* was $10^{8.2}$ CFU/g. This level of microbial growth was maintained throughout the experiment independent of the storage conditions. Even though the *Leuc. carnosum* samples reached high total microbial counts they did not cause as much sensory spoilage as the samples inoculated with fx. *C. maltaromaticum.* The *Leuc. carnosum* samples are therefore a good example that a high microbial count does not always equal severe spoilage.

The growth pattern of the samples inoculated with the mixture of all four potential spoilage bacteria was very similar to the growth pattern of the *Leuc. carnosum* samples. At week 3+7 days the spoilage flora of the Mixture samples was furthermore dominated by LAB with an ATP count of $10^{7.9}$ CFU/g whereas the *B. thermosphacta* and *C. luteola* counts were around $10^4$-$10^5$ CFU/g. Both the sensory results and the aroma composition indicated that the *Leuc. carnosum* was the dominating flora in the mixture of all four bacteria. The result of the microbial analysis is a final confirmation of that.

**Measurements of aroma precursors.** The precursors of the aroma compounds found in spoiled meats could be very interesting in relation to the understanding and modeling of spoilage of sliced meat products. In this experiment we have measured the content of FAA's and FFA's in the saveloy samples (Data not shown). However, there was no effect of inoculation with spoilage bacteria on the level of FFA's. The level of FAA's was generally lower in the inoculated samples than in the control samples. However, it was not possible to differentiate between the bacteria causing major sensory changes during the shelf-life period and those causing limited sensory changes. Based on these observations the aroma precursors were concluded to have no relation to spoilage of the saveloy samples this study.

**Modeling the relation between the sensory evaluation and the aroma composition with PCA.** In the present study we have found a significant effect of storage time and spoilage bacteria on the aroma composition and the sensory profile on the saveloy samples. An overview of the relation between the sensory profile and the aroma composition is given in Figure 3. This figure shows the bi-plot (principal component (PC)1 vs. PC2) from a four component PCA model including all the aroma compounds and the sensory descriptors. From Figure 3 it is seen that 2- and 3-methylbutanol (Ac3), 2- and 3-methylbutanal (A2), diacetyl (K3) and acetoin (K5) and 2-methyl-propanol (Ac2) are closely related to sweet-, butter-like-, sour- and old odor with negative scores on PC1 and positive scores on PC2. Samples inoculated with *B. thermosphacta*, *C. maltaromaticum* and *C. luteola* and measured at week 3+3 days and week 3+7 days plus the *C. maltaromaticum* samples measured after 4 weeks in MAP are also associated with these sensory descriptors. This corresponds well with the more progressed spoilage observed for these samples. The *Leuc. carnosum* samples and the mixture samples measured at week 3+3 days, week 3+7 days and week 4 are clustered together and are associated with the aroma compounds 1-hexanol (Ac7) and 2-heptanol (Ac6). These samples are clearly separated from the most spoiled samples on PC2. All the terpenes (T1-T19), the straight chain the aldehydes ethanal (A1), hexanal (A4), heptanal (A5) and octanal (A6) and the ketones Acetone (K1) 2-butanone (K2) and 2-heptanone (K3) are positively associated with saveloy odor and spicy odor with positive scores on PC1. The straight chain aldehydes and ketones are probably formed in thermal lipid oxidation processes during the cooking *(27)*. Together with the terpenes from the spices these lipid oxidation products make up the odor of fresh saveloy.

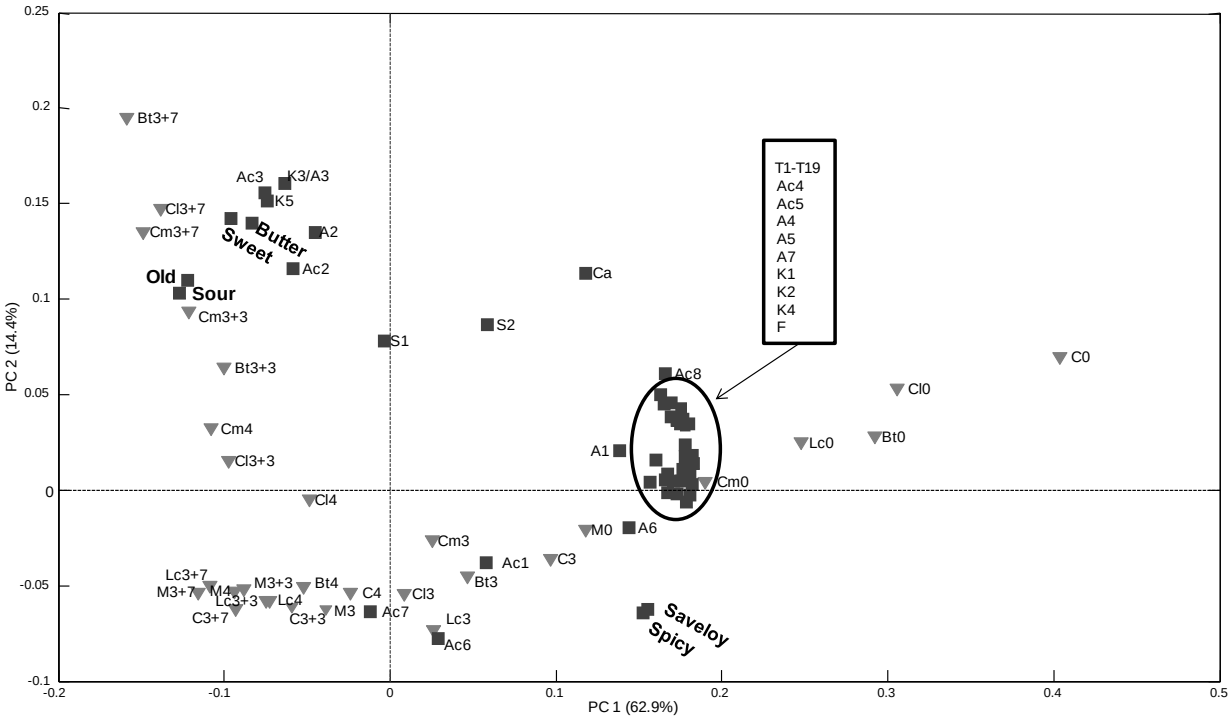


**Figure 3. The bi-plot (PC1 vs. PC2) from the PCA-model including all 42 aroma compounds and six sensory descriptors (squares) and the saveloy samples (triangles). The aroma compounds are denoted according to the compound code shown in Table 1. The samples are denoted according to the experimental series: control (c), B. *thermosphacta* (Bt), C *luteola* (Cl), Leuc. *carnosum* (Lc) and C. *maltaromaticum* (Cm) and the Mixture series (M) and the time of measurement: day 1 (0), week 3 (3), week 3+3days (3+3), week 3+7 days (3+7) and week 4 (4).**

**Modeling the relation between the sensory evaluation and the aroma composition with PLS.** PLS models were calculated for each of the seven sensory descriptors. The PLS-models included all the 42 aroma compounds as X-variables. An overview of the characteristics of the PLS-models is shown in Table 4. The PLS model of acid-like odor was excluded from the table as this descriptor was not modeled well. The table includes the validated root mean square error of calibration (RMSECV), the number of PC's included in the model, the coefficient of determination ($R^2$) for the predicted vs. measured plot and the percentage of Y-variance explained by the model. The table furthermore lists the ten aroma compounds which had the highest impact on the model based on their regression coefficient to the sensory descriptor. These compounds are sorted by the numeric value of the regression coefficient. From Table 4 it is seen that all the sensory descriptors resulted in PLS-models in which more than 70% of the sensory variation was captured using 2 or 3 PC's. The RMSECV of the PLS-models, shown in Table 4, was generally relatively high compared to the span of the sensory scores shown in Table 2. This means that the predictive ability of the PLS-models was relatively poor. A probable explanation of this is the large variation in the aroma composition of the 6 experimental series included in the PLS-models.

**Table 4: Characteristics of the PLS models of the sensory descriptors. The table includes the RMSECV, $R^2$ of the predicted vs. measured plot, The percentage of Y variation explained by the model and the ten most important aroma compounds based on their regression coefficient to the sensory descriptor.**

| Y-variable | RMSECV | $R^2$ | PC's | % Y-explained | Top ten aroma compounds |
|---|---|---|---|---|---|
| Saveloy odor | 1.02 | 0.69 | 2 | 80.1 | Ac3, K3, K5, Ac2, A2, A6, S1, Ca, Ac7, Ac1 |
| Spicy odor | 0.68 | 0.68 | 2 | 77.3 | Ac3, K3, K5, Ac2, A2, S1, Ca, A6, S2, Ac1 |
| Butter-like odor | 0.93 | 0.81 | 3 | 87.3 | A2, Ac3, K5, K3, S1, S2, Ca, Ac8, Ac7, A6 |
| Sweet odor | 0.88 | 0.63 | 2 | 74.0 | A2, Ac3, K3, K5, Ac2, Ca, Ac6, A6, S2, S1 |
| Sour odor | 1.24 | 0.63 | 2 | 73.1 | Ac3, A2, K5, K3, Ac2, Ca, T14, S1, T4, T11 |
| Old odor | 1.59 | 0.57 | 2 | 70.8 | Ac3, Ac2, K3, K5, S1, A2, S2, Ca, A6, T14 |

Table 4 shows that 2- and 3-methylbutanol (Ac3), diacetyl (K3) and acetoin (K5), were among the four most important aroma compounds in the PLS-models of all 6 sensory descriptors. 2- and 3-methylbutanal (A2) was also among the top ten aroma compounds in the PLS-models of all sensory descriptors and the most important compound to sweet odor and butter-like odor. 2-methylpropanol (Ac2) was found in the top five most important aroma compound in all the PLS models except that of butter-like odor. These results show that all sensory descriptors used in this study are closely related to the level of 2- and 3-methylbutanol, 2- and 3-methylbutanal, 2-methylpropanol, diacetyl and acetoin. Acetic acid (Ca), DMDS (S1), DMTS (S2) and octanal (A6) were also found frequently among the ten most important aroma compounds and could therefore also be important for the modeling of saveloy spoilage. No terpenes were among the most important aroma compounds in the PLS-models of spicy- or saveloy- odor. This indicates that it was the increase in microbial metabolites which limited the sensory shelf-life rather than the decrease in pleasant odors from the spices and the cooking process.

**Challenges for the practical application of a predictive model of sensory shelf-life of saveloy.** The development of sensory spoilage in cooked and sliced meat products is a complicated process which depends on several different factors. It is well established that the microbial interactions with the product are among the most important of these factors *(6, 11)*. This study shows that *B. thermosphacta*, *C. maltaromaticum* and *C. luteola* are important contributors to the microbial spoilage of cooked sliced meat products. Their volatile metabolites: 2- and 3-methylbutanol, 2- and 3-methylbutanal, diacetyl, acetoin and 2-methylpropanol were furthermore highlighted as important chemical markers for the sensory spoilage of saveloy. These results further confirm previous findings in industrially sliced saveloy samples where 2- and 3-methylbutanol, 2- and 3-methylbutanal, diacetyl and acetoin also were closely linked with sensory spoilage *(5)*.

The possibility of predicting sensory spoilage from measurements of selected aroma compounds would be of great value as a quality assurance tool in the meat industry. This tool could be used to estimate the expected shelf-life period of a given batch of saveloy shortly after packing. However, this requires that it is possible to measure the microbial production of aroma compounds in the meat products shortly after production. This was not the case in this study where no significant increase in the potential chemical markers was observed in the first week after production. It

remains uncertain whether an increase in the sensitivity of the applied GC-MS method or on-line MS methods will enable early detection of spoilage in cooked and sliced meat products based on aroma compounds.

It should be noted that the results of this study are primarily based on tests of the potential spoilage bacteria in monocultures. In industrially produced cooked and sliced meat products the microbial flora consists of several different bacteria and the composition of this microbial flora varies between meat processing facilities *(10)*. This implies that the competition and interactions between the different bacteria in the spoilage flora plays a major role in determining the rate and type of spoilage observed in a given product *(28)*. An example of this was seen in this study for the saveloy samples inoculated with a mixture of all four potential spoilage bacteria. In this mixture *Leuc. carnosum* became the dominating flora and, as noticed previously, this bacterium did not cause severe spoilage of the saveloy samples compared to *B. thermosphacta*, *C. maltaromaticum* and *C. luteola*. This result highlights the importance of addressing the composition of the spoilage flora in the development of a predictive spoilage model. In the present study different spoilage bacteria contributed to the aroma composition of the saveloy samples in 5 inoculated experimental series. This rather large variation in the aroma composition of the samples has probably reduced the predictive ability of the PLS-models. Future PLS-models for prediction of sensory spoilage based on aroma compounds should therefore be fitted to handle the variation in the microbial flora of a specific meat processing plant. This would probably increase the predictive ability of the PLS-models as the variation in the spoilage patterns would be low compared to this study.

Further analyses of the composition of the microbial flora in cooked and sliced meat products from different meat processing plants, with focus on the production of microbial metabolites during storage, would be interesting in order to assess the variation in the spoilage flora encountered in practice. This would moreover increase the knowledge of which microbial species that are particularly harmful in a meat processing environment.

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# Paper IV

## Quality changes during storage of cooked and sliced meat products measured with HS-GC-MS and PTR-MS

**In preparation for submission.**

**Quality changes during storage of cooked and sliced meat products measured with HS-GC-MS and PTR-MS**

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**Abstract**

The changes in the aroma composition of industrially produced saveloy were measured with proton transfer reaction-mass spectrometry (PTR-MS) and headspace gas chromatography-mass spectrometry (HS-GC-MS) during almost 6 weeks storage. Only limited changes in the aroma composition of the sliced saveloy were observed. However, there were good correspondence between the aroma composition measured with PTR-MS and HS-GC-MS. Furthermore, 4 other types of cooked and sliced meat products were measured with PTR-MS in the middle and at the end of their 4 weeks shelf-life period. These measurements showed an increase in m/z 61, 69, 71, 87 and 89. These masses were assigned to the microbially produced aroma compounds acetic acid, 2- and 3-methylbutanol, 2- and 3-methylbutanal, diacetyl and acetoin. The results of this study show that PTR-MS has a high potential for at-line measurements of the quality of cooked and sliced meat products.

## 1. Introduction

Odor is an important parameter for consumer acceptability and measurement of the aroma composition is therefore an important tool for evaluation of the eating quality of cooked and sliced meat products. The odor of fresh cooked and sliced meat products derives mainly from thermally induced lipid oxidation during cooking and from the spices added to the product (Ho et al., 1994; Mottram, 1998; Holm et al., 2012). However, during slicing and further processing the product is subjected to the in-house microbial flora (Samelis et al., 2000; Gounadaki et al., 2008). This flora is often dominated by lactic acid bacteria (LAB) typically in combination with *B. thermosphacta* and *Pseudomonas* spp. (Borch et al., 1996; Bjorkroth et al., 1998). During storage these bacteria metabolize the nutrients present on the surface of the product and start producing unpleasant aroma compounds. The aroma compounds contributing to the aroma of the fresh product furthermore starts to decrease. These changes in the aroma profile will have a negative impact on the sensory quality of the product and may in this way lead to spoilage (Dainty & Mackey, 1992; Borch et al., 1996; Leroy et al., 2009).

In a recent study the microbially produced aroma compounds: 2- and 3-methylbutanol, 2- and 3-methylbutanal, diacetyl and acetoin, measured by headspace gas chromatography-mass spectrometry (HS-GC-MS), have been suggested as chemical markers for the sensory shelf-life of sliced saveloy (Holm et al., 2012). Measurements of these chemical markers in the fresh meat product could provide an estimate of the expected shelf-life, which moreover could be used as an index of the current microbial quality of the product.

Proton transfer reaction-mass spectrometry (PTR-MS) could be a suitable method for rapid at- or on-line measurements of the chemical markers during industrial production of cooked and sliced meat products. The major advantages of PTR-MS are its ability to provide direct, fast and continuous measurements of the volatile organic compounds (VOC's) surrounding the product (Lindinger et al., 1998). Using the PTR-MS technique it is furthermore possible to detect VOC's present in pptv-levels. The PTR-MS instrument is equipped with a drift tube in which the VOC's are subjected to soft chemical ionization by protonated water. Although the PTR-MS relies on soft ionization aroma compounds such as aldehydes and alcohols are subjected to some degree of fragmentation during measurement (Buhr et al., 2002; Brown et al., 2010). The PTR-MS instrument furthermore does not include a GC-separation step and all VOC's obtained from the sample headspace therefore flow from the drift tube to the MS simultaneously (Lindinger et al., 1998). In combination with the fragmentation this presents a challenge to the data processing and identification of VOC's present in the headspace of complex food matrices. Nevertheless, PTR-MS has many interesting applications in food research (Biasioli et al., 2011). Of particular interest to this study Mayr et al. 2003 used PTR-MS to detect the emission of VOC's during spoilage of fresh poultry, pork and beef stored in atmospheric air or vacuum packing. This paper reported the formation of C4-esters and 2-butenal in the fresh meat stored in air whereas ethanol and acetate aldehyde were associated with vacuum packed beef and poultry (Mayr et al., 2003).

In the present study, changes in the aroma composition of industrially produced saveloy were monitored with PTR-MS and HS-GC-MS during a six weeks storage period. Furthermore, 4 other types of cooked and sliced meat products were purchased in a supermarket. The aroma composition of these products was measured with PTR-MS in the middle and at the end of their 4 weeks shelf-life period. The main objective of this study was to evaluate PTR-MS as a tool for detection of quality changes in cooked and sliced meat products with focus on the suggested chemical markers. A positive result would be an important step towards a practical application of the suggested chemical markers in the meat industry.

## 2. Materials and methods

### 2.1 Experimental setup

For the first part of the experiment sliced and modified atmosphere packed (MAP) saveloy samples were supplied by a meat processing facility. Saveloy is a gently seasoned sausage made from minced pork meat. During a six weeks storage period the saveloy samples were kept at 5 °C and the changes in aroma composition were measured with PTR-MS and HS-GC-MS. After 3, 4 and 5 weeks of storage series of saveloy packages were opened and kept at 5°C for yet another 4 or 6 days at 5 °C. This allowed oxygen to enter the packages and affect the microbial metabolism. Measurements of the aroma composition with PTR-MS were done at day 1, week 3, week 3+4 days (after package opening), week 3+6 days, week 4+6 days, week 5, week 5+4 days and week 5+6 days. HS-GC-MS measurements were done at day 1, week 4, week 4+4 days, week 4+6 days and week 5 in order support the identification of the PTR-MS mass fragments. A small sensory study was done at day 1, week 4, week 4+4 days and week 5 to reveal whether the changes in aroma composition affected the perception of the product.

For the second part of the experiment packages of four other types of cooked and sliced meat product: ‘jægerpølse’, ‘rullepølse’, sandwich ham and roasted pork loin were purchased in a supermarket. ‘Jægerpølse’ is a seasoned sausage made from minced pork which contained garlic and chives whereas the smoked ‘rullepølse is made from pork flank seasoned with chives and ramson. The aroma composition of these four products types was measured with PTR-MS in the middle of their four week shelf-life period and again near their expiration date after being subjected to atmospheric air during the final week of storage.

### 2.2 PTR-MS measurement

About 25 g of sample was coarsely chopped and placed in a 500 mL closed glass flask equipped with a purge top and conditioned in a water bath at 30 °C for 10 min. During the measurement a 150 mL/min flow of filtered air was let through the sample flask and then diluted with a filtered air flow of 300 mL/min. The inlet flow of the high sensitivity PTR-MS (Ionicon Analytik, Innsbruck, Austria) was ~84 mL/min and the remaining air flow did not enter the PTR-MS. The PTR-MS drift tube was operated at standard conditions with a temperature of 60 °C, a pressure of 2.14-2.20

mbar and voltage of 600 V. The E/N value was ~137 Td for all measurements. The MS scanned a m/z range from 21 to 200 with a dwell time of 200 ms. Ten cycles of each sample was recorded and the average values from the intermediate cycles of each m/z was subtracted background measurements made on an empty flask. 3 replications of the PTR-MS measurements were done using a new package for each measurement.

For identification purposes PTR-MS runs of authentic standards of: Hexanal (Merck KGaA, Darmstadt, Germany), acetoin (ChemService inc., West Chester, Pennsylvania, USA), 2-methylbutanal, diacetyl (Sigma-Aldrich, St. Louis, Missouri, USA) and α-pinene (Acros Organics, Geel, Belgium).

**2.3 Dynamic headspace sampling and HS-GC-MS measurement**

VOC's were extracted from the sample by dynamic headspace analyses coupled with HS-GC-MS. The method was described by Holm et al. 2012. However, the back purge flow was 20 mL/min in the present study. As in the PTR-MS measurements the samples were conditioned for 10 min at 30 °C. 3 replications were done of each sample. The dynamic headspace extraction and HS-GC-MS measurements were done using a fresh package of meat product for each measurement.

HS-GC-MS runs of authentic standards of the following compounds were done to aid the identification of aroma compounds: 2-methylbutanol and hexanal (Merck KGaA, Darmstadt, Germany), 3-methylbutanal and acetoin (ChemService inc., West Chester, Pennsylvania, USA), 1-octen-3-ol, acetic acid, diacetyl, 2-methylpropanol, 2-heptanol and 1-hexanol (Sigma-Aldrich, St. Louis, Missouri, USA), octanal, 2-heptanone, heptanal, α-pinene, limonene, 3-carene, dimethyl disulfide and dimethyl trisulfide (Acros Organics, Geel, Belgium) and 2-pentylfuran (Lancaster Synthesis, Windham New Hampshire, USA).

The retention times of the chromatographic peaks were furthermore standardized using the Kovats linear retention index (LRI) calculated from HS-GC-MS runs of a C5-C15 alkane standard (Air Liquide, Paris, France).

**2.4 Sensory test**

A small sensory test was included in the saveloy storage experiment. This test was performed with a panel consisting of ten people experienced in working with meat and meat spoilage. Four times during the six weeks storage experiment the sensory quality of the saveloy samples were compared to a reference sample stored at -1 °C. In previous experiments in our sensory laboratory sliced saveloy stored at -1 °C has been shown to be stable from a sensory perspective during a four weeks storage period (data not shown). The difference in odor and taste between the saveloy sample and the reference was evaluated on a scale from 0 to 5, where 0 was no deviation and 5 was strong deviation. The panel furthermore had the opportunity to comment on the observed

changes. The sensory test was done on day 1, week 4, week 4+4 and week 5 of the saveloy storage experiment.

### 2.5 Data processing

The HS-GC-MS data was processed using MSD Chemstation software (D.01.02.16, Agilent Technologies, Santa Clara, CA, USA). The chromatographic peaks were integrated based on target and qualifier ions which were characteristic to their mass spectra. Due to co-elution 2-methylbutanol and 3-methylbutanol were quantified together.

Identification of the chromatographic peaks was done using the NIST/EPA/NIH mass spectral library (V.1.7a, Agilent Technologies, Santa Clara, CA, USA) and HS-GC-MS runs of authentic standards. The LRI the of chromatographic peaks was moreover compared with the LRI of potential matching compounds using the C20M column from internet database www.flavornet.org (Acree & Arn, 2004). The maximum difference in LRI allowed for a potential match was 50 units. However, not all 3 methods were used for all compounds. The following denotation will be used: N (NIST mass spectral library), S (compound standards) and L (comparison of LRI).

Chemometric analysis was done using the PLS Toolbox (version 5.2.2, Eigenvector Research inc., Wenatchee, WA, USA). The PLS toolbox is running in the MATLAB environment (version 7.6.0.324, The Matworks inc., Natick, MA, USA).

Statistical analysis was performed in JMP v. 8.0.1 (SAS institute, Cary, North Carolina, USA). For the HS-GC-MS data and the PTR-MS data ANOVA models were built describing the relevant aroma variables using storage time as fixed effect. Based on the ANOVA models Tukey honest significant different (HSD) tests were done to locate significant differences between the levels of the relevant variables.

## 3. Results and Discussion

### 3.1 Changes in aroma composition of sliced saveloy measured with HS-GC-MS

A total of 46 aroma compounds were isolated from the sliced saveloy samples with HC-GC-MS during the storage experiment. 21 of these were terpenes but aldehydes, ketones, alcohols and sulfur containing compounds were other major compound classes extracted from the saveloy samples. The complete list of aroma compounds extracted from sliced saveloy and their peak areas is shown in Table 1. The table also includes the method used for identification, the LRI and the target ion used for quantification for each aroma compound. Significant differences in the peak area of the 46 aroma compounds between different samples are indicated in Table 1 using letters from *a* to *d* based on the Tukey HSD test.

From Table 1 it is seen that the odor of the fresh saveloy samples is roughly composed of aroma compounds formed by thermal degradation of lipids during cooking and aroma compounds from

the flavoring agents added to the product. Several of the straight chain aldehydes, ketones and alcohols found in the product headspace are well known lipid oxidation products and most of these compounds were found in the highest amount at day 1 (Ho et al., 1994; Mottram, 1998). Table 1 furthermore shows that the peak area of these lipid oxidation products, including hexanal and 2-butanone, decreased significantly with storage time. Similar observations were made for several of the terpenes and the sulfur containing compounds shown in Table 1. The terpenes originate from the spices added to the product whereas several of the sulfur-containing compounds could be derived from onion which was also added to the product (Järvenpää et al., 1998; Meynier et al., 1999). Overall a decrease is observed in the aroma compounds contributing to the fresh odor with storage time.

Previous studies have shown that the shelf-life of cooked and sliced meat products is determined mainly by microbial reactions (Samelis et al., 2000; Leroy et al., 2009; Holm et al., 2012). The increase in microbially produced aroma compounds with storage time has furthermore been shown to be closely related to a decrease in sensory acceptability of saveloy (Holm et al., 2012). However, from Table 1 it is seen that none of the aroma compounds extracted from the saveloy samples in this study increased significantly with storage time. Nevertheless, according to the Tukey HSD test in Table 1 there was an increase in acetoin release at week 4+4 days compared to the level at week 4. However, this increase in acetoin release was not significant compared to level in day 1 samples and the level of acetoin decreased again at week 4+6 days. Acetoin has been related to microbial spoilage of meat products and was also among the previously suggested chemical markers for shelf-life (Dainty & Hibbard, 1983; Holm et al., 2012). However, compared to the observations made in previous studies on sliced saveloy, acetoin did not appear to be a dominating part of the aroma composition in this study (Holm et al., 2012). Moreover, the HS-GC-MS measurements in this study generally suggest that the microbially produced aroma compounds only made a limited contribution to the aroma composition of the saveloy samples.

An overview of the distribution of the saveloy samples based on their aroma composition measured with HS-GC-MS is given in Figure 1. This figure shows the bi-plot from a PCA model including the saveloy samples and all 46 aroma compounds. This bi-plot shows principal component (PC) 1 vs. PC2 which accounts for roughly 67% of the variation in the aroma composition of the saveloy samples. PC1 accounts for the main variation in the dataset. On this PC the saveloy samples measured at day 1 and week 4 have positive scores whereas the samples from week 4+4 days, week 4+6 days and week 5 generally had negative scores. The majority of the aroma compounds included in the PCA model, including the terpenes, alcohols and aldehydes, were associated with the saveloy samples from day 1. This further supports that the decrease in aroma compounds contributing to the fresh odor with storage time was the main change in the aroma composition of the saveloy samples in this study. However, acetic acid (Ac1) and 2,2-dimethyl-propanoic acid (Ac2) are associated with the samples from week 4+4 days, week 4+6 days and week 5. Acetic acid is a known metabolite of LAB's, which has been related to spoilage of

meat products during storage (Dainty & Mackey, 1992; Laursen et al., 2009). However, according to the Tukey HSD test, this increase in acetic acid was not significant. Acetoin (K3) was moreover located right between the samples from day 1 and the samples from week 4+4 days and 4+6 days in the bi-plot in Figure 1. These observations suggest that the microbial contribution to the aroma composition is increasing at week 4+4 days and week 4+6 days.

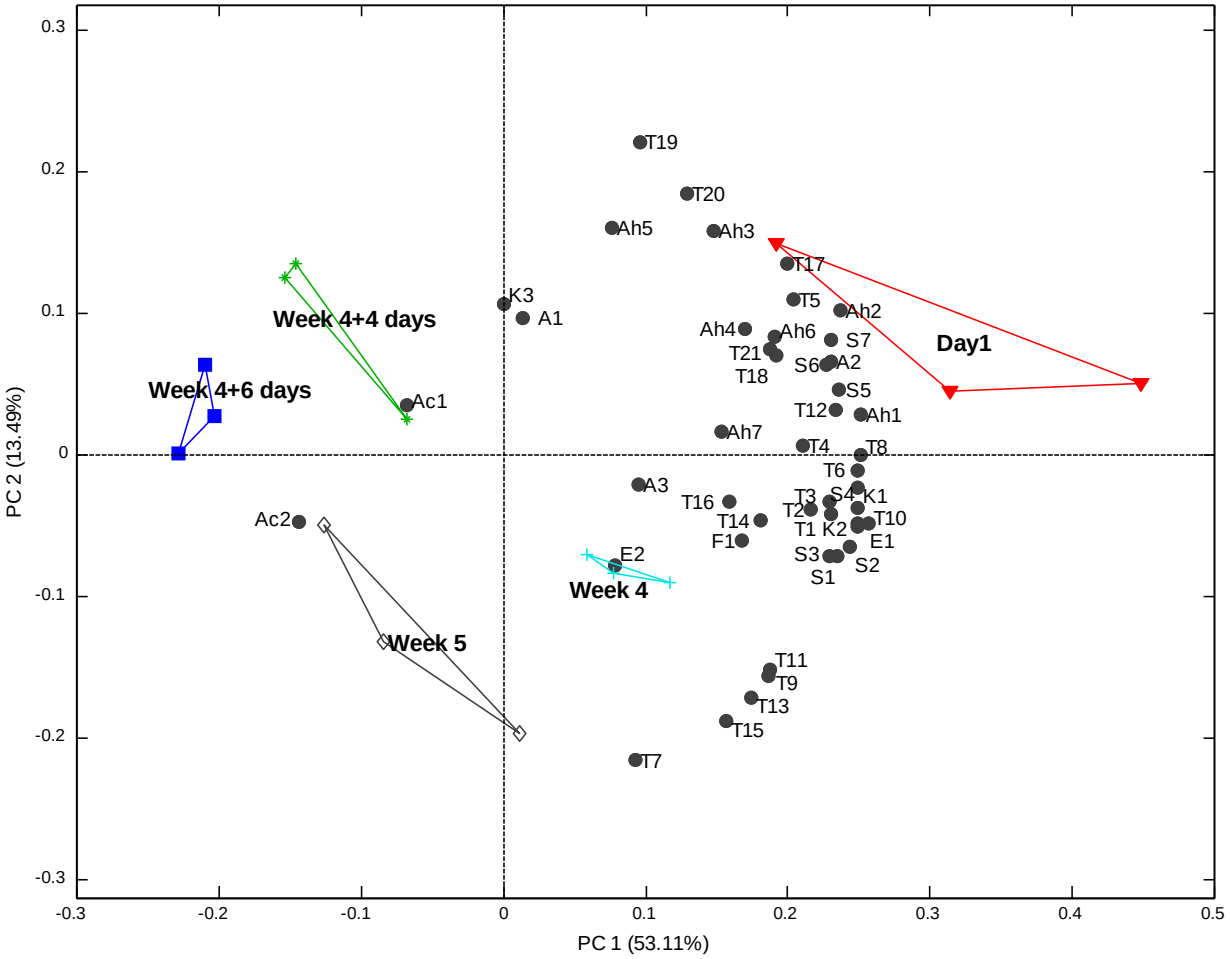


**Figure 1. Bi-plot (PC1 vs. PC2) from the PCA model including the 46 aroma compounds found with HS-GC-MS measurements of the saveloy samples at day 1, week 4, week 4+4 days, week 4+6 days and week 5. The aroma compounds are denoted by the compound code given in Table 1.**

### 3.2 Changes in aroma composition of sliced saveloy samples measured with PTR-MS

The results of the PTR-MS measurements made on the sliced saveloy samples during the storage experiment are shown in Table 2. The table shows the concentration of each mass obtained from the PTR-MS measurements that was above the background level. Significant differences between measurements from different days are indicated using letters *a* to *e* based on the Tukey HSD test. The list of aroma compounds contributing to specific masses was made based on fragmentation patterns reported in previous literature (Buhr et al., 2002; Maleknia et al., 2007; Lasekan & Otto,

2009; Brown et al., 2010; Feilberg et al., 2010). Authentic standards of acetoin, diacetyl, 2-methylbutanal, hexanal and α-pinene were also used to support the assignment of mass fragments to aroma compounds. As seen in Table 2, $C^{13}$-isotopes of the most abundant masses contribute to the neighboring fragments. The fragmentation of alcohols aldehydes, ketones and esters, which in general are important aroma components in foods, was studied by Buhr et al. 2002. Aldehydes and especially alcohols are subjected to fragmentation during PTR-MS measurement. Upon protonation aldehydes and alcohols undergo fragmentation by water elimination, in some cases followed by further reactions e.g. elimination of $H_2$. M/z 55 and 69 are mentioned as characteristic mass fragments for aldehydes whereas alcohols often contribute to m/z 43 and 57 (Buhr et al., 2002; Brown et al., 2010).

From Table 2 it is seen that several of the PTR-MS masses decreased significantly with storage time. The HS-GC-MS measurements of the aroma composition showed a decrease in the level of the terpenes, the sulfur compounds and the lipid oxidation products with storage time. This pattern was also observed in the PTR-MS measurements. A significant decrease in m/z 137 and 81 was observed with storage time. They are the major masses of monoterpenes which have a molecular mass of 136 g/mol. M/z 137 corresponds to the protonated parent molecule of monoterpenes and m/z 81 corresponds to the loss of a $C_4H_8$ fragment (Maleknia et al., 2007). M/z 95 and m/z 63, which are the protonated parent molecules of DMDS and dimethyl sulfide (DMS) respectively, also decreased significantly with storage time. The high fragmentation level of aldehydes and alcohols, which are the predominant lipid oxidation products, makes them difficult to relate to individual masses. Nevertheless, m/z 57, which can be attributed to several alcohols, decreased significantly with storage time. M/z 69, which is a characteristic aldehydes fragment (Buhr et al., 2002), decreased until week 3+6 days and then started to increase again. The microbially produced aroma compounds 2- and 3-methylbutanal also contribute to m/z 69 which could explain this increase observed in the end of the storage experiment. Moreover, no significant change was observed for m/z 55, which is another characteristic aldehyde fragment (Buhr et al., 2002). However, the $H_3O^+(H_2O)_2$-cluster also contributes to this mass and could interfere with the measurements. The PTR-MS measurements furthermore showed that ethanol was the most abundant compound in the headspace of sliced saveloy. The protonated parent molecule (m/z 47) was found in levels between 1921 ppb and 7087 ppb and the ethanol fragments at m/z 29 and m/z 45 plus the ethanol plus water cluster at m/z 65 was also found in high amounts. Ethanol was also found to be an abundant peak in the HS-GC-MS measurements.

An overview of the development in aroma composition measured with PTR-MS is given in Figure 2. This figure shows the bi-plot (PC1 vs. PC2) from a PCA model which includes the concentration of all the PTR-MS masses found in the saveloy samples at the different days of measurements. PC1 and PC2, which are shown in the bi-plot, account for 61% of the variation in the data. The variation between the five samples measured on the same day is outlined in the figure which enables an evaluation of the variation in the aroma composition of the saveloy samples stored at the same

conditions. The samples from day 1, week 3 and week 5, which were kept in closed packages until measurement, were generally characterized by positive score on PC1 and low or negative scores on PC 2. This was most pronounced for the samples from day 1 which had the highest scores on PC1. The samples from week 3 and week 5 had lower scores on PC1 and were located closer to the saveloy samples which were opened for 4 or 6 days prior to measurement. Several mass fragments were associated with the samples from day 1 including m/z 137 and 81 from the terpenes and m/z 63 and 95 from the sulfur compounds DMS and DMDS. Samples from week 3+4 days, week 3+6 days and week 4+6 days had similar aroma composition, and were positively associated with m/z 28, 31, 45, 46 and 109 and negatively associated with the m/z ratios associated with the closed packages. From the PCA model shown in Figure 2 it is seen that the samples from week 5+4 days and week 5+6 days were subject to some variation in the aroma composition. For both week 5+4 days and week 5+6 days one of the five replications was separated from the remaining samples with high scores on PC2. These two extreme samples were related to a group of mass fragments which included m/z 71 and 87. As seen in Table 2, the protonated molecular ion of diacetyl and 2- and 3-methylbutanal plus a fragment of acetoin all contribute to m/z 87. A fragment of 3-methylbutanol moreover contributes to m/z 71. As mentioned these aroma compounds have previously been identified as chemical markers for the sensory shelf-life of saveloy (Holm et al., 2012). The increase in m/z 71 and 87 therefore indicates that the microbial spoilage processes starts to have an impact on the aroma composition of the saveloy samples at week 5+4 weeks and week 5+6 days. The expected shelf-life of the saveloy samples was 4 weeks according to the declaration label and the observed changes in aroma composition beyond this point are therefore mainly of theoretical interest.

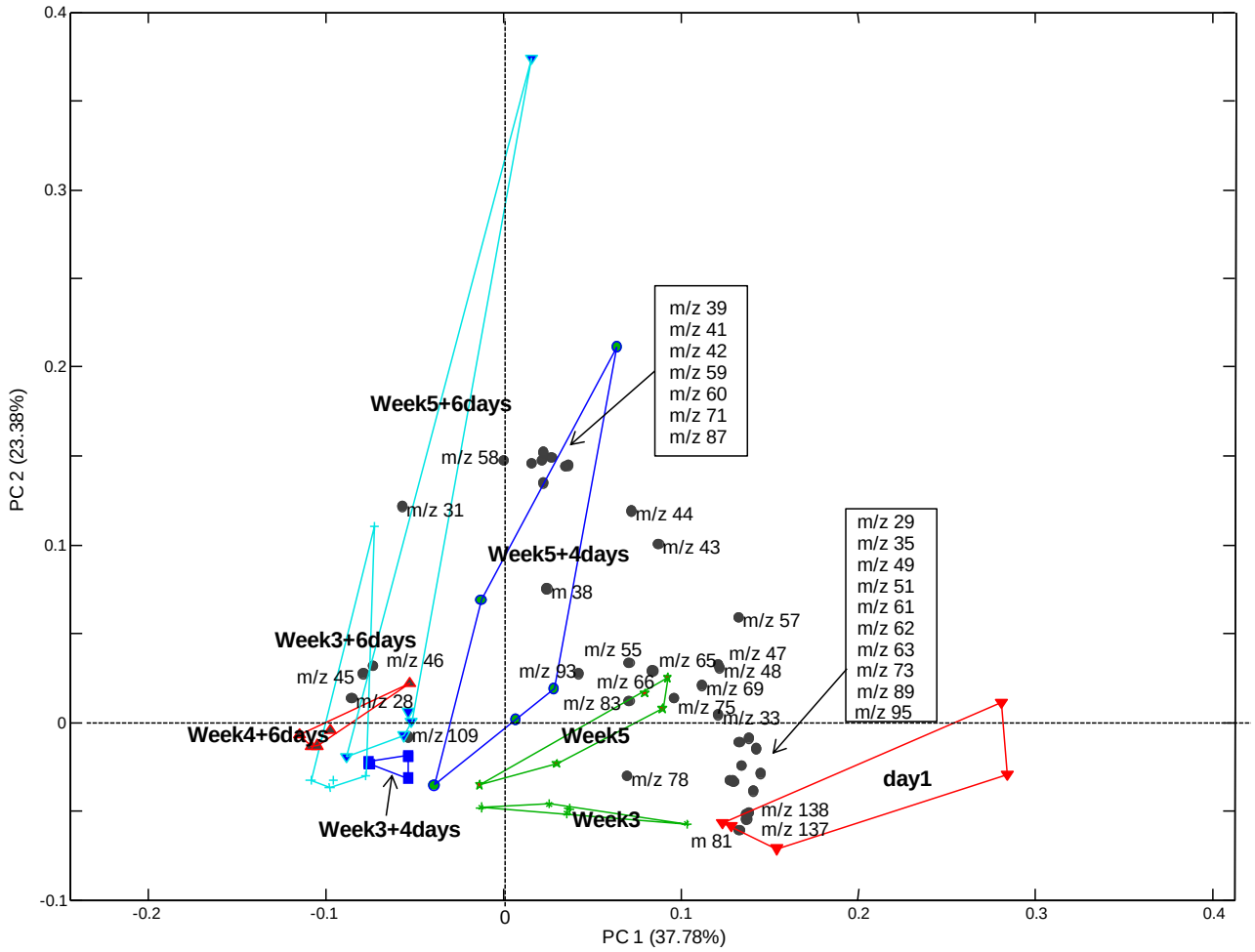


**Figure 2. Bi-plot (PC1 vs. PC2) from the PCA model including the PTR-MS mass fragments and the saveloy samples measured at day 1, week 3, week 3+4 days, week 3+6 days, week 4+6 days, week 5, week 5+4 days and week 5+6 days.**

### 3.3 Sensory evaluation of the quality changes of the sliced saveloy samples

Though the sensory test was not made using a conventional trained sensory panel the results give a good indication of the quality changes in the product perceived by the consumers. The results of the sensory test are seen in Figure 3 where the perceived quality changes in odor and taste relative to a reference sample are shown on a scale from 1 to 5. From Figure 3 it is seen that there was a tendency towards an increased difference between sample and reference with storage time. However, the standard deviation on these evaluations was relatively high. The difference between the samples and the references was highest at week 4+4 days. At this point seven of the ten assessors noted that the sample had a less spicy odor compared to the reference. This corresponds well with the observed decrease the concentration of the terpernes observed in HS-GC-MS and PTR-MS measurements. Furthermore, two of the assessors noted the detection of a slightly sour odor at week 4+4 days. This could indicate that the souring processes had been initiated by the LAB present in the product. This was to some extend confirmed by the HS-GC-MS

measurements where acetic acid was associated with saveloy samples measured at week 4+4 days and week 4+6 days in the PCA model.

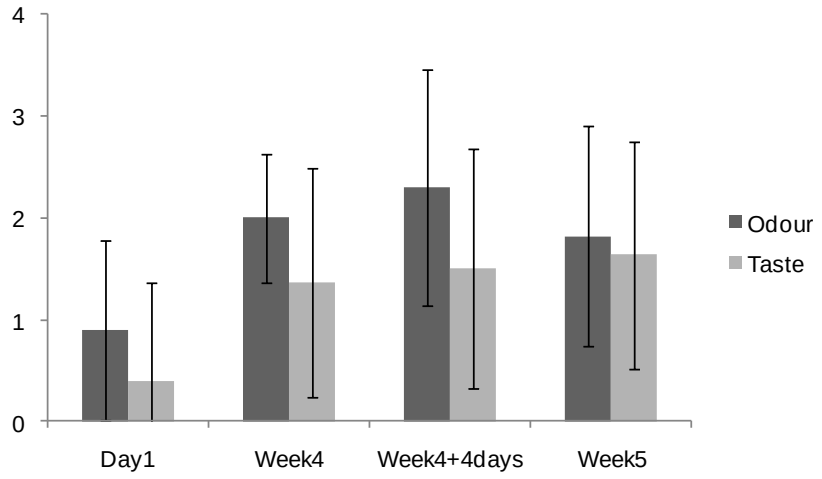


**Figure 3. The results of the sensory test on sliced saveloy samples made on day1, week 4, week 4+4 days and week 5 evaluating the deviation from a control sample. A 10 person sensory panel evaluated the saveloy samples in relation to a reference stored at -1°C. The difference in odor and taste was assessed on a scale from 0 to 5 where 0 was no difference and 5 was large difference.**

### 3.4 PTR-MS measurements of 4 types of cooked and sliced meat product

The PTR-MS measurements made on the packages of cooked and sliced: 'jægerpølse', 'rullepølse', sandwich ham and roasted pork loin were included in the experiment in order to study the rate and extent of spoilage in other types of cooked and sliced meat products from different manufactures. The saveloy samples used in the storage experiment were obtained directly from the production facility and afterwards kept at 5 °C until measurement. In contrast the samples of 'jægerpølse', 'rullepølse', sandwich ham and roasted pork loin were purchased in local super markets. Compared to the saveloy samples there is therefore an increased risk that these products have been subjected to some degree of temperature fluctuations during storage and distribution. The aroma composition of the 4 types of cooked and sliced meat product was measured with PTR-MS in the middle of their shelf-life period and again close to their expiration date after being subjected to package opening for 1 week. This was longer than suggested by the manufactures which recommended between 2 and 3 days storage in opened packages. However, the prolonged storage time in open packages was used to induce microbial changes in the products. In the final PTR-MS measurement made near the expiration date the samples of especially roasted pork loin and 'jægerpølse' appeared spoiled, and clear sour and butter-like off odors were noted when preparing these samples for measurement.

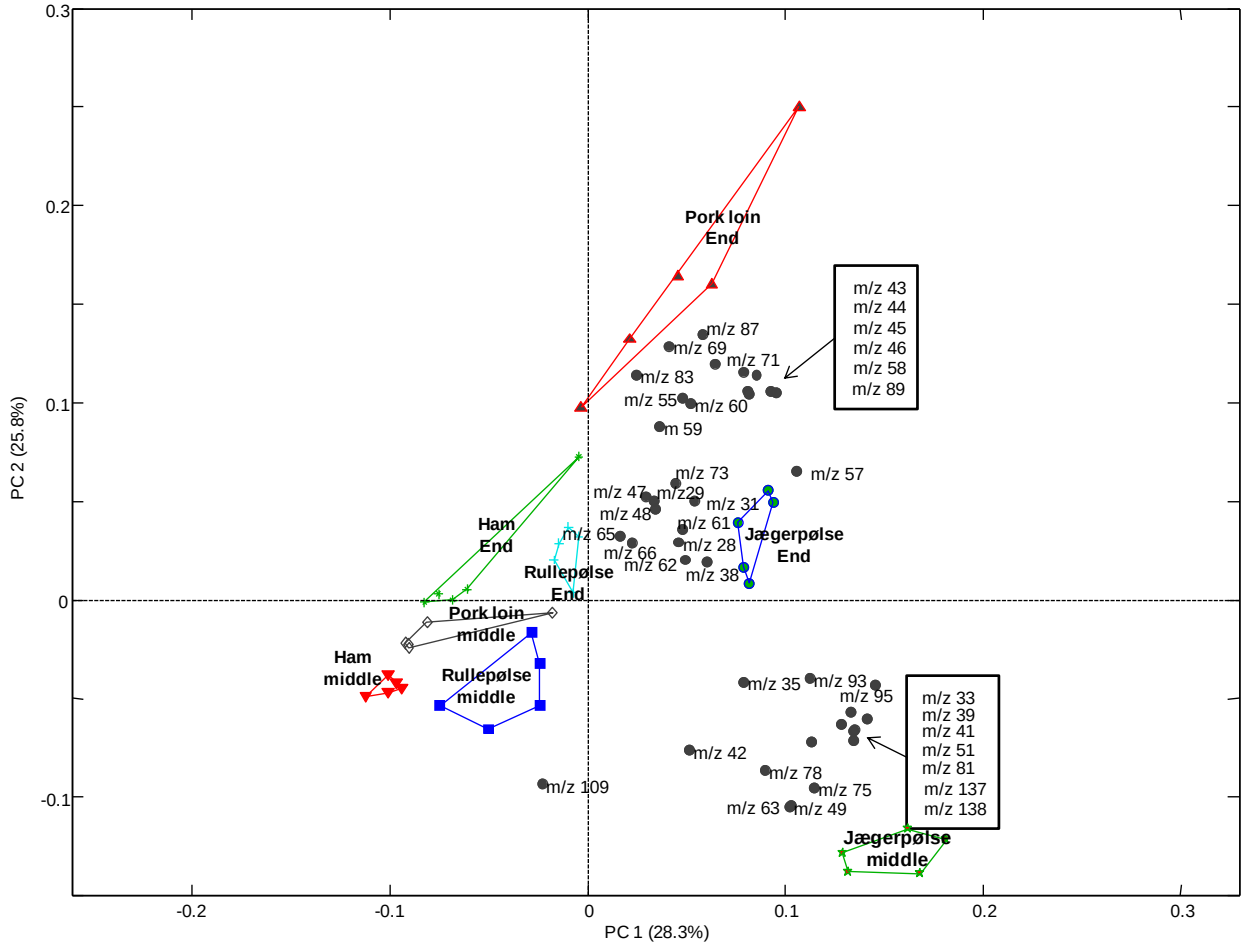


**Figure 4. Biplot (PC 1 vs. PC 2) from the PCA model including the PTR-MS mass fragments and samples of 4 types of cooked and sliced meat product measured in the middle and the end of their 4 week shelf-life period.**

The changes in aroma composition determined by PTR-MS were accounted for in a PCA model from which the bi-plot (PC1 vs. PC2) is shown in Figure 4. In this bi-plot the 'jægerpølse' samples measured in the middle of the shelf-life period were separated from the remaining samples on both PC1 and PC2. This is partly due to a high content of the masses corresponding to the monoterpenes (m/z 81, 137 and 138). This suggests that 'jægerpølse' contained the highest level of spices of the 4 cooked meat products tested in this study. PC 2 in the PCA model in Figure 4 shows the difference between the two days of PTR-MS measurement. The samples measured in the middle of the shelf-life period have negative scores on PC2 whereas the samples measured at the end of the shelf-life have positive scores on this PC. Figure 4 furthermore shows that the pork loin samples measured at the end of the shelf-life period had the highest scores on PC2. These samples were closely related with several masses including m/z 69, 71, 87 and 89. From Table 2 it is seen that fragments of 2- and 3-methylbutanal contribute to m/z 69 whereas the molecular ion of acetoin has m/z 89. As previously highlighted 2- and 3-methylbutanol, diacetyl and acetoin

contribute to m/z 71 and 87. These aroma compounds were all among the previously suggested chemical markers of spoilage of sliced saveloy (Holm et al., 2012). The PCA-model therefore indicates that the pork loin samples were the most spoiled of the four tested product types.

The change in m/z 69, 71 and 87 are shown in Figure 5 along with m/z 61, which is the protonated molecular mass of acetic acid. From Figure 5 it is seen that the samples of pork loin measured at the end of the shelf-life period contained the highest amounts of m/z 69, 71 and 87 corresponding to 2 and 3-methylbutanal, 2- and 3-methylbutanol, diacetyl and acetoin. This confirms the observations made from the PCA-model. The 'jægerpølse' samples measured at the end of the shelf-life period also contained relatively high amounts of m/z 69, 71 and 87 though not as high as for the pork loin samples. However, the 'jægerpølse' samples contained increased amounts of m/z 61 (acetic acid) at the end of the shelf-life period compared to the pork loin samples. In figure 5 it is seen that the increase in m/z 69, 71 and 87 was relatively low for samples of sliced 'rullepølse' and sliced sandwich ham. However, a significant increase in m/z 61 was observed for the rullepølse samples in the end of their shelf-life period.

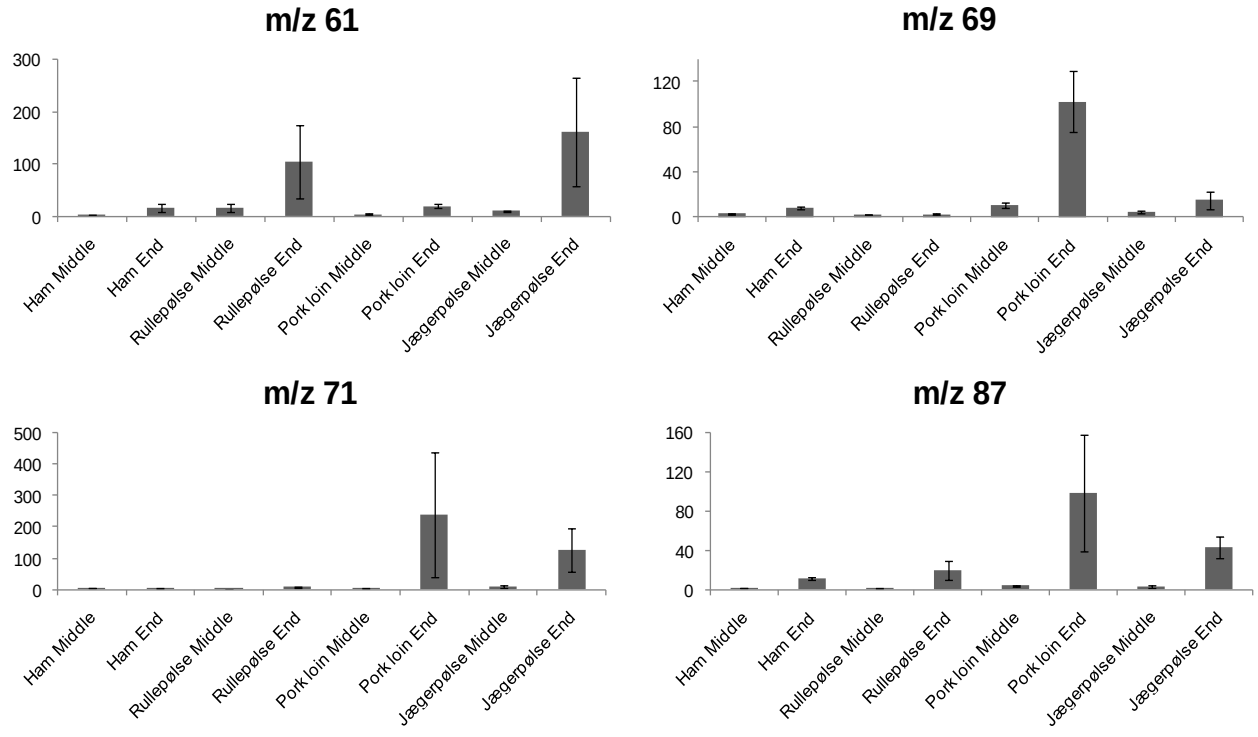


**Figure 5. The development in m/z 61, 69, 71 and 87 between the middle and the end of the 4 week shelf-life period for cooked and sliced ham, rullepølse, pork loin and jægerpølse. m/z 61 corresponds to acetic acid, m/z 69 corresponds to 2- and 3-methylbutanal, m/z 71 corresponds to 2- and 3-methylbutanol whereas both diacetyl, acetoin and 2-and 3- methylbutanol contributes to m/z 87.**

The variation in the concentration of m/z 61, 69, 71 and 87 suggests that there were major differences in the microbial composition and activity of these 4 types of meat products at the end of the shelf-life period. These differences are probably caused by variation in the composition and growth rate of the specific spoilage flora of the products. The specific spoilage flora consists of the small fraction of bacteria present in the microbial flora of the processing facility which are able to grow and dominate the product under the given environmental conditions (Mataragas et al., 2007; Nychas et al., 2008). Variation in factors such as the pH, water activity, availability of nutrients and the type/concentration of preservation agents used could also contribute to explain the differences in microbial activity between the 4 products (Samelis et al., 2000; Nychas et al., 2008; Holm et al., 2012).

### 3.5 Evaluation of PTR-MS for detection of quality changes in cooked and sliced meat products

The objective of this study was to investigate PTR-MS as a tool for detection of quality changes in cooked and sliced meat products. The changes in aroma composition were measured with PTR-MS in 5 types of cooked and sliced meat products during refrigerated storage. The saveloy samples were studied in most detail. However, only limited changes in these samples were observed during the first 5 weeks of the storage experiment where the aroma composition was measured with both PTR-MS and HS-GC-MS. The main change in the saveloy samples in this period was a decrease in aroma compounds contributing to the fresh odor of the product. As seen in Table 1 and Table 2 this change was observed both when measured with PTR-MS and HS-GC-MS. In general there was good correspondence between the two methods for measurement of the aroma composition.

The PTR-MS measurements of the 4 types of cooked and sliced meat product showed an increase m/z 69, 71, 87 and 89 between the middle and the end of their 4 week shelf-life period. This observation was most significant in the samples of cooked pork loin and 'jægerpølse'. M/z 71 and 87 were also observed in increased levels in one of five saveloy samples measured at week 5+4 days and 5+6 days. As seen in Table 2, these m/z-ratios correspond to mass fragments or the protonated molecular mass of 2- and 3-methylbutanal, 2- and 3-methylbutanol, acetoin and diacetyl. However, it should be considered that other aroma compounds also could contribute to these m/z-ratios. When considering previous studies made on similar meat products it is considered very probable that the significant increases in the concentration of m/z 61, 69, 71 and 87 is related to microbial spoilage (Stanley et al., 1981; Leroy et al., 2009; Holm et al., 2012).

The degree of fragmentation of the potential chemical markers for shelf-life, when subjected to chemical ionization, could pose a problem when using PTR-MS. Major fragments of 3-methylbutanal and 3-methylbutanol have been found at m/z 39, 41, 43 and 45 (Buhr et al., 2002; Lasekan & Otto, 2009). At these low masses the fragments of several other alcohols and aldehydes also contribute to the total signal and therefore interfere with the aroma compounds of interest (Buhr et al., 2002; Brown et al., 2010). The fragmentation therefore results in a loss of sensitivity

when using PTR-MS to measure the aroma composition of complex food matrices. The degree of fragmentation can be reduced by lowering the voltage of the drift tube but this will instead result in formation of water clusters and lower the concentration of $H_3O^+$-ions available for proton transfer reaction (Brown et al., 2010).

Nevertheless, the results of this study show that PTR-MS is capable of detecting changes in the aroma composition of cooked and sliced meat products during storage. M/z 69, 71, 87 and 89 were found to correspond largely to the previously identified chemical markers for shelf-life. Based on these findings PTR-MS is a very promising technique for measurement of microbially induced quality changes in cooked and sliced meat products. The ability of the PTR-MS to provide direct, rapid and continuous measurements makes it suitable for at-line measurements directly at the production facility. PTR-MS measurements made in the fresh packages of cooked and sliced meat products could potentially be developed into a quality assurance tool which could provide an estimate of the expected expiry date. This study is an important step towards the development of this tool which should be based on a thoroughly validated shelf-life model fitted to account for the variation in the output of the single production facility.

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**Table 1. An overview of the aroma compounds extracted from the saveloy samples with HS-GC-MS. The table includes peak area/1000 of the 46 aroma compounds for each of the 5 days of measurement, the linear retention index (LRI) and the target ion used for quantification. The aroma compounds were identified using the NIST mass spectral data base (N), comparisons of the observed LIR with the LRI in internet database flavornet.com (L) or HS-GC-MS runs of authentic standard compounds (S). Letters a to d are used to indicate significant differences in the peak area of the aroma compounds between measure points based on a Tukey HSD test.**

| Nr. | Code | Compound name | LRI | Id-quality | T-ion | D1 | W4 | W4+4 | W4+6 | W5 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | A1 | propanal | 765 | N | 58 | 8228 | 7122 | 15210 | 2241 | 3903 |
| 2 | K1 | acetone | 802 | N | 43 | $28487^{a}$ | $23234^{b}$ | $13659^{c}$ | $10342^{c}$ | $19635^{b}$ |
| 3 | E1 | ethyl acetate | 865 | N-L | 43 | $92925^{a}$ | $73430^{b}$ | $38859^{c}$ | $26292^{c}$ | $63582^{b}$ |
| 4 | K2 | 2-butanone | 884 | N | 43 | $4766^{a}$ | $4022^{ab}$ | $2316^{cd}$ | $1550^{d}$ | $3289^{bc}$ |
| 5 | S1 | 1-(methylthio)-propane | 903 | N | 61 | $1527^{a}$ | $1262^{ab}$ | $599^{c}$ | $356^{c}$ | $1149^{b}$ |
| 6 | Ah1 | ethanol | 941 | N-L-S | 45 | $500180^{a}$ | $400045^{b}$ | $351536^{bc}$ | $324850^{c}$ | $378199^{bc}$ |
| 7 | S2 | 1-(methylthio)-(Z)-1-Propene | 999 | N | 88 | $4216^{a}$ | $3596^{ab}$ | $1947^{c}$ | $1317^{c}$ | $3110^{b}$ |
| 8 | T1 | α-pinene | 1004 | N-L-S | 93 | $20004^{a}$ | $16306^{ab}$ | $8398^{b}$ | $6398^{ab}$ | $9926^{ab}$ |
| 9 | T2 | α -phujene | 1009 | N-L | 93 | 5163 | 4824 | 2552 | 2016 | 2495 |
| 10 | T3 | camphene | 1040 | L-N | 93 | $1245^{a}$ | $737^{ab}$ | $456^{ab}$ | $252^{b}$ | $643^{ab}$ |
| 11 | S3 | dimethyl disulfide | 1050 | N-L-S | 94 | $33286^{a}$ | $23372^{ab}$ | $2445^{b}$ | $1117^{b}$ | $18855^{ab}$ |
| 12 | A2 | hexanal | 1068 | N-L-S | 56 | $941^{a}$ | $793^{ab}$ | $786^{ab}$ | $642^{b}$ | $718^{b}$ |
| 13 | T4 | β-pinene | 1078 | N-L | 93 | 12274 | 9846 | 7206 | 5247 | 6296 |
| 14 | T5 | sabinene | 1094 | N-L | 93 | $16687^{a}$ | $8480^{ab}$ | $8453^{ab}$ | $5611^{b}$ | $3721^{b}$ |
| 15 | T6 | 3-carene | 1116 | N-L-S | 93 | $2294^{a}$ | $1758^{ab}$ | $1276^{b}$ | $1004^{b}$ | $1320^{b}$ |
| 16 | T7 | α-phellandrene | 1133 | N-L | 93 | $1170^{ab}$ | $1474^{ab}$ | $727^{b}$ | $727^{b}$ | $1821^{a}$ |
| 17 | T8 | β-myrcene | 1140 | N-L | 93 | $3185^{a}$ | $2293^{ab}$ | $1704^{b}$ | $1391^{b}$ | $1715^{b}$ |
| 18 | T9 | (+)-4-carene | 1148 | N | 121 | 2610 | 2755 | 1539 | 1485 | 2400 |
| 19 | Ah2 | 1-ethoxy-2-propanol | 1164 | N | 45 | $947^{a}$ | $512^{b}$ | $464^{b}$ | $419^{b}$ | $438^{b}$ |
| 20 | T10 | limonene | 1166 | N-L-S | 68 | $4896^{a}$ | $3945^{ab}$ | $2807^{ab}$ | $2447^{b}$ | $3279^{ab}$ |
| 21 | T11 | β-phellandrene | 1174 | N-L | 93 | 5900 | 6216 | 4139 | 3835 | 5519 |
| 22 | T12 | eucalyptol | 1182 | N-L | 43 | $1101^{a}$ | $850^{ab}$ | $659^{b}$ | $606^{b}$ | $680^{b}$ |
| 23 | S4 | methyl propyl disulfide | 1199 | N | 122 | $8715^{a}$ | $5878^{ab}$ | $971^{bc}$ | $429^{c}$ | $2939^{bc}$ |
| 24 | Ah3 | 2- and 3-methylbutanol | 1200 | N-L-S | 55 | $146^{a}$ | $65^{ab}$ | $104^{ab}$ | $46^{ab}$ | $21^{b}$ |
| 25 | F1 | 2-pentylfuran | 1203 | N-L-S | 81 | 158 | 126 | 34 | 101 | 112 |
| 26 | T13 | γ-terpinene | 1212 | N-L | 93 | 5873 | 6742 | 4355 | 3734 | 5832 |
| 27 | Ah4 | 3-methyl-3-buten-1-ol | 1234 | N | 41 | $106^{a}$ | $92^{ab}$ | $87^{ab}$ | $57^{b}$ | $73^{ab}$ |
| 28 | T14 | ρ-cymene | 1238 | N-L | 119 | 10841 | 8443 | 4271 | 4939 | 6724 |
| 29 | Ah5 | 1-pentanol | 1242 | N-L | 55 | 201 | 89 | 134 | 142 | 53 |
| 30 | T15 | δ-terpinene | 1248 | N-L | 93 | 1353 | 1589 | 1039 | 879 | 1465 |
| 31 | K3 | acetoin | 1265 | N-L-S | 45 | $647^{ab}$ | $480^{b}$ | $1201^{a}$ | $418^{b}$ | $434^{b}$ |
| 32 | Ah6 | 1-hexanol | 1331 | N-L-S | 56 | $292^{a}$ | $251^{ab}$ | $239^{ab}$ | $217^{b}$ | $217^{b}$ |
| 33 | S5 | dimethyl trisulfide | 1341 | N-L-S | 126 | $20931^{a}$ | $4037^{b}$ | $586^{b}$ | $258^{b}$ | $1514^{b}$ |
| 34 | S6 | dipropyl disulfide | 1343 | N | 43 | $4450^{a}$ | $2466^{b}$ | $461^{c}$ | $174^{c}$ | $537^{c}$ |
| 35 | A3 | nonanal | 1368 | N-L | 57 | 115 | 87 | 87 | 93 | 96 |
| 36 | S7 | 1,2-dithiolane | 1400 | N | 148 | $312^{a}$ | $123^{b}$ | $13^{c}$ | $0^{c}$ | $10^{c}$ |
| 37 | T16 | p-a-dimethyl-styrene | 1407 | N-L | 132 | 725 | 559 | 174 | 282 | 336 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 38 | E2 | ethyl octanoate | 1409 | N-L | 88 | 177 | 193 | 170 | 164 | 173 |
| 39 | Ah7 | 1-octen-3-ol | 1427 | N-L-S | 57 | 134 | 97 | 70 | 92 | 84 |
| 40 | Ac1 | acetic acid | 1429 | N-L-S | 43 | 103 | 653 | 1248 | 238 | 431 |
| 41 | T17 | cis-beta-terpinol | 1452 | N | 71 | 318[a] | 0[b] | 33[b] | 7[b] | 0[b] |
| 42 | T18 | camphor | 1488 | N-L | 95 | 81 | 63 | 68 | 57 | 58 |
| 43 | T19 | linalool | >1488 | N | 71 | 546[a] | 287[bc] | 463[ab] | 369[ab] | 184[c] |
| 44 | Ac2 | 2,2-dimethyl-propanoic acid | >1488 | N | 57 | 0 | 7 | 0 | 445 | 280 |
| 45 | T20 | terpinen-4-ol | >1488 | N | 71 | 960[a] | 679[ab] | 808[ab] | 692[ab] | 461[b] |
| 46 | T21 | safrole | >1488 | N | 162 | 788[a] | 600[ab] | 573[ab] | 508[b] | 554[ab] |

**Table 2: The concentration (ppb) of mass fragments found in the saveloy samples measured with PTR-MS at day 1, week 3, week 3+4 days, week 3+6 days, week 4+6, week 5, week 5+4 days and week 5+6 days. Letters a to e are used to indicate significant differences in the concentration of PTR-MS mass fragments between measure points based on a Tukey HSD test. Possible assignments of the mass fragments are provided based on compound standards and the existing literature.**

| M/z | D1 | W3 | W3+4 | W3+6 | W4+6 | W5 | W5+4 | W5+6 | Identification |
|---|---|---|---|---|---|---|---|---|---|
| **28** | $1^{e}$ | $1^{e}$ | $4^{bc}$ | $4^{cd}$ | $7^{a}$ | $4^{b}$ | $1^{e}$ | $3^{d}$ | |
| **29** | $182^{a}$ | $147^{ab}$ | $133^{bc}$ | $69^{d}$ | $90^{cd}$ | $127^{bc}$ | $107^{bcd}$ | $124^{bc}$ | ethanol[IV] , acetoin[V] |
| **31** | $33^{c}$ | $30^{c}$ | $91^{abc}$ | $92^{abc}$ | $164^{a}$ | $111^{abc}$ | $72^{bc}$ | $135^{ab}$ | methanal[II] |
| **33** | $714^{a}$ | $673^{ab}$ | $637^{ab}$ | $419^{d}$ | $509^{c}$ | $654^{ab}$ | $622^{b}$ | $607^{b}$ | methanol[II,IV] |
| **35** | $26^{a}$ | $4^{b}$ | $2^{b}$ | $1^{b}$ | $1^{b}$ | $2^{b}$ | $2^{b}$ | $1^{b}$ | hydrogensulfide[VI] |
| **38** | 2 | 3 | 3 | 4 | 6 | 10 | 11 | 10 | |
| **39** | 17 | 16 | 16 | 23 | 19 | 33 | 53 | 53 | various compounds[IV, V] |
| **41** | 67 | 48 | 38 | 75 | 25 | 35 | 143 | 135 | various compounds[III] |
| **42** | 7 | 6 | 6 | 6 | 7 | 7 | 10 | 10 | |
| **43** | 188 | 117 | 75 | 136 | 56 | 142 | 137 | 116 | various compounds[III] |
| **44** | 5 | 3 | 2 | 4 | 2 | 5 | 4 | 4 | |
| **45** | $373^{c}$ | $298^{c}$ | $766^{b}$ | $669^{b}$ | $1187^{a}$ | $832^{b}$ | $387^{c}$ | $686^{b}$ | ethanol[III], ethanal |
| **46** | $10^{d}$ | $8^{d}$ | $20^{bc}$ | $18^{c}$ | $33^{a}$ | $24^{b}$ | $11^{d}$ | $19^{c}$ | ethanol $C^{13}$-isotope |
| **47** | $7087^{a}$ | $3433^{ab}$ | $3184^{ab}$ | $1921^{b}$ | $2591^{b}$ | $4645^{ab}$ | $4771^{ab}$ | $4413^{ab}$ | ethanol[III] |
| **48** | $189^{a}$ | $84^{ab}$ | $78^{ab}$ | $47^{b}$ | $68^{b}$ | $120^{ab}$ | $119^{ab}$ | $105^{ab}$ | ethanol $C^{13}$-isotope |
| **49** | $596^{a}$ | $181^{b}$ | $28^{c}$ | $13^{c}$ | $17^{bc}$ | $94^{c}$ | $25^{c}$ | $16^{c}$ | methanthiol[VI] |
| **51** | $41^{a}$ | $11^{b}$ | $4^{b}$ | $3^{b}$ | $7^{b}$ | $14^{b}$ | $11^{b}$ | $5^{b}$ | |
| **55** | 49 | 9 | 10 | 10 | 35 | 58 | 64 | 9 | butanal[III], hexanal[I,III], $H_3O^+(H_2O)_2$-cluster |
| **57** | $12^{a}$ | $8^{b}$ | $6^{b}$ | $7^{b}$ | $6^{b}$ | $10^{ab}$ | $7^{b}$ | $7^{b}$ | 2-methylpropanol, 1-pentanol, 1-butanol, 1-hexanol, 1-octanol[III,IV] |
| **58** | 1 | 1 | 1 | 3 | 2 | 2 | 2 | 6 | |
| **59** | 332 | 221 | 157 | 198 | 91 | 213 | 634 | 570 | acetone[VI], propanal[III], diacetyl[I,V] |
| **60** | 12 | 8 | 5 | 7 | 4 | 9 | 22 | 20 | |
| **61** | $248^{a}$ | $153^{bc}$ | $71^{de}$ | $114^{cd}$ | $35^{e}$ | $186^{b}$ | $65^{de}$ | $56^{de}$ | acetic acid[V], ethyl acetate[III] |
| **62** | $7^{a}$ | $4^{bc}$ | $2^{cd}$ | $3^{cd}$ | $1^{e}$ | $5^{ab}$ | $2^{cd}$ | $2^{cd}$ | |
| **63** | $10^{a}$ | $5^{b}$ | $2^{b}$ | $2^{b}$ | $3^{b}$ | $9^{a}$ | $2^{b}$ | $1^{b}$ | dimethyl sulfide[VI] |
| **65** | 287 | 30 | 27 | 17 | 118 | 389 | 207 | 16 | ethanol*$H_3O^{+}$[IV] |
| **66** | 7 | 1 | 1 | 0 | 3 | 10 | 5 | 0 | |
| **69** | $7^{a}$ | $5^{b}$ | $4^{b}$ | $3^{c}$ | $5^{b}$ | $4^{b}$ | $7^{a}$ | $5^{b}$ | 2- and 3-methylbutanal[I,V], 1-octen-3-ol[III], pentanal, octanal, nonanal[III] |
| **71** | 4 | 3 | 2 | 3 | 2 | 3 | 4 | 9 | 3-methylbutanol[III], 1-pentanol[IV] |
| **73** | $16^{a}$ | $11^{b}$ | $6^{de}$ | $3^{e}$ | $7^{cd}$ | $11^{b}$ | $10^{bc}$ | $7^{cd}$ | 2-butanone[III, VI] |
| **75** | $12^{b}$ | $7^{b}$ | $4^{b}$ | $5^{b}$ | $4^{b}$ | $21^{a}$ | $13^{ab}$ | $4^{b}$ | propanoic acid[VI] |
| **78** | $18^{bc}$ | $32^{b}$ | $5^{c}$ | $5^{c}$ | $3^{c}$ | $60^{a}$ | $7^{c}$ | $3^{c}$ | |
| **81** | $95^{a}$ | $65^{b}$ | $34^{cd}$ | $33^{d}$ | $38^{cd}$ | $56^{bc}$ | $37^{cd}$ | $32^{d}$ | monoterpenes[I,II] |
| **83** | $6^{a}$ | $4^{bc}$ | $6^{ab}$ | $3^{c}$ | $4^{c}$ | $4^{bc}$ | $6^{a}$ | $4^{bc}$ | hexanal[I, III] |
| **87** | 3 | 2 | 1 | 2 | 3 | 3 | 6 | 8 | diacetyl[I,V,VI], 2- and 3-methylbutanal[I,V], acetoin[I] |
| **89** | $67^{a}$ | $40^{ab}$ | $15^{bc}$ | $7^{c}$ | $8^{c}$ | $55^{a}$ | $19^{bc}$ | $15^{bc}$ | ethyl acetate[III], acetoin[I] |
| **93** | 11 | 7 | 6 | 4 | 7 | 65 | 55 | 5 | toluene |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **95** | 7[a] | 5[b] | 3[d] | 3[d] | 3[d] | 5[bc] | 3[cd] | 3[d] | dimethyl disulfide[VI], monoterpenes[II] |
| **109** | 1[c] | 1[c] | 2[b] | 3[a] | 2[ab] | 2[ab] | 1[cd] | 1[d] | 4-methylphenol[VI] |
| **137** | 74[a] | 55[b] | 27[c] | 26[c] | 27[c] | 49[b] | 30[c] | 26[c] | monoterpenes[I,II] |
| **138** | 8[a] | 5[b] | 3[c] | 3[c] | 3[c] | 5[b] | 4[c] | 3[c] | monoterpenes[I,] |

[I]Compound standard, [II]Maleknia et al. 2007, [III] Buhr et al. 2002, [IV]Brown et al. 2010, [V]Lasekan et al. 2009, [VI]Feilberg et al. 2010.

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