Quality control of raw cows' milk by headspace analysis

- a new approach to mastitis diagnosis -

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All truths are easy to understand once they are discovered;
the point is to discover them
Galileo Galilei

Abstract

The objectives of the work described in this thesis were twofold. The first aim was to test the suitability of headspace analysis for quality control of raw milk in general. The second aim was to further develop the headspace analysis for the identification of mastitis causing pathogens in raw milk. Fresh raw milk without quality defects was shown to always contain the same 7 volatile components. Treatments like heating and homogenization of raw milk drastically changed this basic pattern. Using the headspace analysis, variation in the composition of the regular diet could not be detected, and *Pseudomonas* only when present in high numbers. On the other hand, the headspace analysis can be used for quantifying the extent of lipolysis, the amount of chloroform, and the detection of feeding specific vegetable byproducts. The evaluated headspace method is thus able to detect several quality defects simultaneously and therefore is a valuable supplementary method for raw milk quality control. Additionally, it was investigated if headspace analysis could be used as a faster method for identification of mastitis causing pathogens, based on the analysis of volatile bacterial metabolites. This method, supported by an artificial neural network, was found to reliably identify the most important groups of mastitis causing pathogens. To study the origin of these metabolites, milk was inoculated with isolated mastitis pathogens. Most metabolites found in inoculated milk samples corresponded with their occurrence in mastitis milk. Finally, the influence of incubation on the formation of these metabolites was studied. Incubation was shown to be a necessary step for the detection of volatile metabolites. It was found that after 8 hours of incubation, all important metabolites were formed.

Contents

1

Introduction

1.1 Milk

Milk is the nutritional fluid secreted by the mammary gland of mammals. It is the main source of nutrition for newborns. The composition of milk varies by species, but it always contains significant amounts of carbohydrate, fat, and protein. Milk is an important source of calcium.

In the Western world, cows' milk is nowadays produced on a large scale. It is the most commonly consumed type of milk. Most cows have been bred for high milk production as well as high protein and fat content of the milk. The largest producers of milk today are India and the USA, although if the 25 countries of the European Union are combined, the EU is the largest producer of cows' milk.

The composition of cows' milk is very complex, containing carbohydrates (approx. 4.6%), fat (approx. 4.3%), and proteins (approx. 3.3%). Almost all carbohydrates in milk are in the form of the disaccharide lactose. Milk fat is a very complex mixture of triglycerides having a wide variety of chain lengths (2 to 22 C-atoms), saturation, and branching of the fatty acids. Milk protein can be divided in two fractions: the caseins (approx. 80%) and the whey proteins (approx. 20%). In addition to these major components, milk also contains many components in a lower concentration, like minerals, organic acids, peptides, and vitamins (Walstra et al., 2008).

1.2 Quality control of raw milk

Raw milk quality is very important for the quality of milk and dairy products made of it. Therefore, quality of raw milk is under strict control. Every milk delivery is inspected with regard to certain quality parameters. Other parameters, for quality defects which are less common, are examined only periodically.

In The Netherlands, the first step in quality control of raw milk is the farmer. The farmer has to check the cow for abnormal milk before starting to milk the cow. The second step is performed by the truck driver. Before the truck driver collects the milk, it has to be inspected. The milk should be free from undesirable odours and should not be flocculated or discoloured. Milk temperature and age should also be inspected. The third step in the Dutch quality control system is the milk control station. For every milk delivery, the milk composition (fat, protein, and lactose), urea content, and presence of bacterial growth inhibitors (e.g. antibiotics) are determined. Most of these analyses are based on Fourier transform infrared (FTIR) spectroscopy. FTIR is

a fast and simple method, but is only able to detect the relative abundant components in raw milk. Periodically (every 2 weeks to twice a year), bacterial growth, somatic cell count (SCC), butyric acid bacteria, free fatty acid content, freezing point, as well as visible contamination are examined.
Other quality aspects than the ones mentioned above are not regularly monitored. Changes in raw milk quality can, for example, be caused by cow diet, chemical contamination, and enzymatic deterioration but are not routinely checked. These quality defects may be detectable using a chemical analysis (Azzara and Campbell, 1992). Volatile components in milk (e.g., aldehydes, ketones, and alcohols) may also be good indicators of milk quality (Azzara and Campbell, 1992; Vallejo-Cordoba and Nakai, 1994a, 1994b; Kim and Morr, 1996; McSweeney et al., 1997; Marsili, 1999, 2000; Valero et al., 2001). Because of speed and simplicity, these components can probably best be analyzed using headspace sampling techniques.

1.3 Analytical procedure

As described in the previous paragraph, volatile components are thought to be valuable additional indicators of milk quality. Because of speed and simplicity, headspace analysis can be used for the detection of these components. Headspace analysis of milk and milk products can be performed using different headspace sampling techniques. These techniques either sample the volatiles already available in the headspace (static headspace), actively extract volatiles from the headspace (e.g. solid-phase microextraction (SPME)), or purges the sample with a gas to force the volatiles out of the sample (purge and trap).
After taking a sample of the volatiles with one of these techniques, the volatiles need to be identified and quantified. For this purpose, the headspace sampling techniques are usually coupled to gas chromatography/mass spectrometry (GC/MS) (Fabre et al., 2002). The GC separates volatiles using interactions of the volatiles with the stationary phase coated on the wall of a capillary. These interactions cause different components to elute at different times, called retention times.
The MS identifies the volatiles. Usually, in the MS volatiles interact with electrons to form ionized fragments. The pattern of fragments formed is characteristic for the component that entered the MS. In addition, the amount of fragments detected is correlated with the quantity of the component.
The data produced by the GC/MS can be analyzed using deconvolution for identification of the different volatile components from the sample. After

quantification of the volatile components, different statistical techniques can be used for data analysis (Horimoto et al., 1997).
Based on literature and own experiments, solid-phase microextraction combined with GC/MS was selected as the most appropriate detection technique for volatile components. In chapter 2, comprehensive background information on the different steps of the analytical procedure, including the data analysis, can be found.

1.4 Mastitis

In the course of the research project, mastitis was selected as the main subject to be studied using headspace analysis. Mastitis is an inflammation of the mammary gland. It is a reaction of the udder tissue to bacterial, chemical, thermal, or mechanical injury. Although mastitis can have different origins, it is most often associated with bacterial infections.
Mastitis is the most costly disease of dairy cows. In The Netherlands, the average costs of a clinical mastitis case are estimated between €168 and €277 depending mainly on stage of lactation (Hogeveen, 2005).
A range of more than 140 microorganisms may cause mastitis in cows. However, a limited group of 5 bacteria (*Staphylococcus aureus*, coagulase-negative staphylococci, *Streptococcus uberis*, *Streptococcus dysgalactiae*, and *Escherichia coli*) is responsible for approximately 80% of the Dutch mastitis cases in which bacteria could be cultured from the milk (Barkema et al., 1998; Sampimon et al., 2008).
Mastitis pathogens can be classified as either contagious or environmental. Contagious pathogens transmit from cow to cow, often at the time of milking trough contact with contaminated milking equipment. The most common contagious pathogen is *Staph. aureus*. Environmental pathogens are bacteria which normally occur in the environment. On the farm, these pathogens may be present on the farm in manure, bedding material, or soil. The most common environmental pathogen is *E. coli*.
Mastitis can further be classified as either clinical or subclinical mastitis. Clinical mastitis is characterized by abnormalities of the udder (e.g. swelling and redness), and/or changes in milk appearance and composition. Subclinical mastitis is based on the SCC and bacteriological culturing: a quarter is infected while udder nor milk are clinically abnormal. In practice, the diagnosis of subclinical mastitis is based on SCC. Individual udder quarters with a SCC above a certain threshold (e.g. 200,000/mL) are considered to have subclinical mastitis (Schukken et al., 2003).

Milk from cows with clinical or subclinical mastitis is often analyzed using bacteriological culturing to detect the pathogens causing the mastitis. Determination of mastitis causing pathogens is of great interest, both for choice of treatment of the cow as well as for possible measures that have to be taken on the farm to prevent the spread of mastitis. Bacteriological culturing, however, has the disadvantage of being time-consuming. Because the bacteria have to grow before they can be identified based on phenotypical characteristics, it takes a few days before results are available.
A second disadvantage of bacteriological culturing is the existence of false-negative results. False-negative samples give no result in bacteriological culturing, even though there is a bacterial cause of the mastitis. This may for example be due to samples containing too few pathogens to be detected or pathogens which are already dead before sampling (Sears et al., 1990; Zorah et al., 1993). Finally, failure to detect pathogens in samples taken from quarters with clinical mastitis can be caused by contamination of the sample (Zorah et al., 1993).
Faster and more accurate methods of pathogen identification are thus advantageous, because farmers are earlier able to choose an optimal treatment at the cow and the herd level.

1.5 Outline of the thesis

The objective of the work described in this thesis was twofold. The first aim was to test the suitability of the headspace analysis for quality control of raw milk. The second aim was to further develop the headspace analysis for the identification of mastitis causing pathogens in raw milk.
Chapter 2 contains comprehensive background information on the applied analytical techniques selected, including the data analysis.
Cow diet, microbiological and chemical contamination, as well as enzymatic deterioration may change the volatile composition of raw milk. As headspace analysis can detect a whole range of volatile components at the same time, it may be able to detect a wide range of quality defects with a single analysis. In chapter 3, quality defects of raw milk are described that can be studied simultaneously by a headspace analysis.
Subsequently, mastitis was studied in more detail because this gave the most promising results. The focus of this study was on the possibility to identify the mastitis causing pathogens. A pilot study, as proof-of-principle of mastitis pathogen identification using headspace analysis, is described in chapter 4.

The volatiles described in chapter 4 may be bacterial metabolites as well as metabolites formed by enzymes from the cow with mastitis, or from an interaction between these two. During mastitis, leakage of enzymes from the blood to the milk may occur. Also, enzymes present in the somatic cells may influence the milk composition. Chapter 5 describes the formation of volatile metabolites by pathogens inoculated in milk, compared to milk of mastitis cows.
In the pilot study described in chapter 4, the samples were incubated overnight before analysis. To keep the analysis as simple and fast as possible, this incubation step should be minimized or preferably even be removed at all from the procedure. Chapter 6 describes the effect of incubation time on the formation of volatile metabolites in mastitis samples.
Finally, chapter 7 contains a general discussion of the results presented in the thesis and is finished with the main conclusions.

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2

Analytical procedure

2.1 Introduction

The analytical procedure used for the research described in this thesis consists of several steps. First, the volatiles are extracted from the headspace using a headspace sampling technique. Second, the extracted volatiles are separated using gas chromatography (GC) and identified using mass spectrometry (MS). The identification of the volatiles, based on the results of the GC/MS analysis, is done with the automatic mass spectral deconvolution and identification system (AMDIS) software. After quantification of the volatiles, multivariate statistics are applied for sample classification. The different steps of the analytical procedure are discussed in this chapter.

2.2 Headspace sampling techniques

Headspace analysis of milk and milk products can be performed using static headspace, solid-phase microextraction (SPME), and purge and trap (P&T) (Contarini et al., 1997; Fabre et al., 2002; Vallejo-Cordoba and Nakai, 1994a).

2.2.1 Static headspace

Static headspace is the most rapid of these techniques. It only removes the available headspace, with no pre-concentration, which makes this technique also the least sensitive (Fabre et al., 2002; Peres et al., 2002). In both references it is concluded that static headspace sampling is not a suitable technique due to the high limit of detection (LOD). However, this may be caused by the volume of injected headspace being too large to be transferred to the column in a short period of time. To have good peak shape, refocusing of the sample is needed. Thus, concentrating the volatiles on a cryogenic trap can help in refocussing the volatiles, which may also lower the LOD of the static headspace method (Kolb, 1999; Pillonel et al., 2002).

2.2.2 Solid-phase microextraction

SPME is a technique based on a fiber, coated with a sorbent, which extracts volatiles from the headspace of a sample. After an equilibration time, the fiber is removed, and the volatiles desorbed from the fiber by heating (Arthur and Pawliszyn, 1990). Depending on the components to be extracted from the headspace, a fiber with a specific sorbent can be chosen. This method can be automated, but takes more time than static headspace. Also, the SPME fiber tends to deteriorate over time, so drift can cause problems with reproducibility.

However, using this technique, a high number of volatiles in milk can be determined (Marsili, 1999b, 2000).

2.2.3 Purge and trap

With P&T, the sample is purged with a carrier gas to transfer the volatiles from the sample to a trap with sorbent material (Pillonel et al., 2002). P&T is a more complicated technique, which in general will extract a higher amount of volatiles from the sample, resulting in a lower LOD. P&T is the most often used technique for headspace sampling of milk (Park and Goins, 1992; Vallejo-Cordoba and Nakai, 1993, 1994a, 1994b; Kim and Morr, 1996; Contarini et al., 1997).

2.2.4 Comparing the headspace techniques

Results of SPME and P&T analysis of milk have been compared (Contarini et al., 1997; Contarini and Povolo, 2002). The reported number of components extracted using P&T were higher than for SPME. The sensitivity of the two methods differed depending on the type of component. For fat oxidation products, Marsili (1999a) reported that SPME and P&T had the same sensitivity, and that the accuracy is highest for SPME.

The difference between all three headspace sampling techniques was studied by analyzing 10 volatile components at room temperature, in milk protein solutions (Fabre et al., 2002). The concentrations varied between 0.16 and 1 mg/L. With static headspace, only 3 out of 10 components could be detected. With P&T, 7 components were detected, and only with SPME all components added to the protein solution could be detected. However, they did not report on the LOD of the three methods.

Based on the cited literature, it was expected that static headspace detects less components compared to SPME and P&T. Also, static headspace is expected to have relatively high detection limits.

The difference between SPME and P&T will be influenced by the absorbing materials used for these techniques. P&T is mostly performed with Tenax for absorption of volatile metabolites. For SPME, a much wider range of absorbing materials is available. When SPME and P&T parameters and absorbing materials are optimized, they will probably extract the same number of volatile components, while P&T is expected to have a lower LOD. In this thesis, SPME and P&T will be compared (chapter 3) and the best performing method will be selected for the research described in this thesis.

2.3 GC/MS analysis

After extraction of the volatile components from the sample, these components are separated using GC, followed by fragmentation in the MS. The combination of the retention time with the mass spectrum is used for identification of the components.

2.3.1 Gas chromatography

After injection, the volatile components are separated on a GC column. For separation of these components, both polar and non-polar columns can be applied. The drawback of polar columns is the low heat tolerance, which can cause practical problems. For this reason, a non-polar column with a thick film (e.g. 0.5 to 1 μm) is most common. For the research described in this thesis, however, a non-polar column with a normal film thickness (0.25 μm) was used.
Theoretically, a normal film thickness in combination with a lower temperature (-30°C) will give the same separation as the more common combination of a thick film and a higher GC start temperature (around 30 to 40°C). With non-polar columns, separation is mainly based on the volatility of the components. This separation is influenced by the equilibrium between the gas phase and the stationary phase, which is affected by both the temperature of the column and the relative volumes of the gas and stationary phase (Christie, 1989; Scott, 2003a, 2003b). Thus, to have sufficient retention of very volatile components in the liquid phase of the column, either the relative volume of stationary phase can be increased (thicker film) or the temperature can be decreased.
A normal film thickness has both advantages and disadvantages. The main advantage is that temperature programming can be quicker, which in turn reduces the time needed for the analysis as well as increasing the sharpness of the peaks. A disadvantage of a normal film thickness is the reduced sample capacity. However, with headspace sampling, a relatively low amount of sample material is introduced onto the GC column compared to the sample capacity of the column.

2.3.2 Mass spectrometry

For identification of volatile components, mass spectrometry based on electron ionization (EI) and chemical ionization (CI) can be applied to ionize the sample molecules. After ionization, the ions are separated based on their mass to charge ratio (m/z). As charge (z) is often 1, the resulting spectrum usually shows the masses and abundances of the ions formed.

For EI, accelerated electrons, often with an energy of 70eV, interact with the sample molecules. The sample molecules will then form radical-cations. These cations disintegrate into fragments. These fragments are characteristic of a certain component, and can be found in databases, like the NIST database. The results of these measurements are very reproducible between different mass spectrometers based on EI.
For CI, sample molecules are mixed with a large surplus of reagent molecules. Again, accelerated electrons collide with the molecules. However, because there is a large surplus of reagent molecules, these will be ionized to radical-cations. These reagent molecules will then collide with the sample molecules, so the sample molecules are ionized. After this ionization, the sample ions will not have a large surplus of energy, so fragmentation will almost not occur. The data from these experiments is thus the m/z value of the sample molecules, which often is not characteristic for one component. Also, the results of these measurements are not very reproducible between different mass spectrometers based on CI. CI can be applied if the molecules of interest are unstable, causing too much fragmentation with EI (Herbert and Johnstone, 2002).
EI is the method selected for headspace analysis of milk, as it produces mass spectra which can be compared to e.g. databases to help identify the components.

2.3.3 Other detectors

Besides MS, other detectors can also be applied after separation of the volatile components by GC. One of the most used detectors is the flame ionization detector (FID). The FID is a general detector for hydrocarbons, and is often utilized for quantitative detection of volatile components. Examples of other detectors are the electron capture detector (ECD; for halocarbons) and the pulsed flame photometric detector (PFPD; for specific detection of sulfur and phosphorus containing components). The more specific detectors, like ECD or PFPD, are in general more sensitive than wide range detector, like FID or MS. The disadvantage of detectors other than MS is that signals produced cannot be used for identification (Atar, 1991; Skoog, 2007). Therefore, MS was used.

2.3.4 Electronic nose

With GC/MS, the volatiles are separated before being identified. However, there are also systems without separation of the volatile components which are based on a detector receiving a mixture of all components. Some of those are called “electronic noses” by their manufacturer. According to the usually

accepted definition (Gardner and Bartlett, 1999), an electronic nose is a device composed of an array of gas sensors, with non-specific responses, having pattern recognition ability from the multivariate data analysis of the whole set of responses. However, not all analytical equipment sold as electronic nose complies with this definition, such as a stand-alone MS.
Two often employed "electronic noses" are a stand-alone MS and a system with a set of sensors. Both are based on obtaining a pattern of signals, which can normally not be used for identification of individual components. Groups of components can sometimes be assigned to certain signals. Generally, multivariate statistics is used to compare the spectra of different types of samples (Marsili, 2000; Ampuero et al., 2002; Korel and Balaban, 2002).
A system based on sensors has the advantage of being relatively cheap. However, the identification of the volatile components is important, for the research described in this thesis. The identification is important to explain differences between samples and to give information on the processes from which the components arise.
To reach the objectives of this thesis, GC/MS was selected because of its ability to quantify and identify the different volatile components. However, when developing a method for practice based on the work described in this thesis, an "electronic nose" may be a good and affordable alternative analytical device to perform the analysis.

2.4 Data analysis

To get the maximum amount of information from GC/MS data in a proper way, sound data analysis techniques must be applied. This applies to both the peak identification and the subsequent analysis of the obtained peak pattern.

2.4.1 GC/MS peak identification

The MS records the signals for all ions separately, which leads to a separate chromatogram for every m/z value. These chromatograms are called the ion traces. Usually, the different ion traces are combined to one chromatogram, containing the summed signals of all ions, called the total ion count (TIC). Usually, GC/MS software plots this TIC chromatogram, which consists of peaks against a background. The mass spectrum obtained at a certain peak is used to identify the peak. The mass spectrum recorded at the top of a peak is, however, not the pure mass spectrum of the component. Both the background signal as well as coeluting components can cause contamination of the mass spectrum.

The most common method for extracting "pure" spectra for a component is to subtract spectra in a selected background region of the chromatogram from spectra at the component maximum. This, however, is only appropriate when background signal levels are relatively constant. Moreover, highly complex chromatograms may have no identifiable "background" region. To obtain high quality, “pure”, mass spectra, AMDIS (Automated Mass spectral Deconvolution & Identification System; NIST, Gaithersburg, MD, USA) was used for the research described in this thesis. AMDIS is based on four steps to identify components. AMDIS starts by analyzing the background and calculating a noise level for later processing. It then analyzes the data for an increase in specific ion traces. If there is a maxima for other ion traces at the same time, is assumes there is a peak and shapes a model peak. In the next step it calculates a “clean” spectra for each peak. The last step is the identification of the component via a library search on the “clean” spectra. The theory of AMDIS has been more extensively described elsewhere (Stein, 1999).

2.4.2 GC/MS peak quantification

After peak identification, the detected volatile components can be quantified in all samples. Some quality changes in milk may be detected based on the presence or absence of certain specific components. These changes are easy to detect. However, many quality defects cause changes in the concentration of a range of volatile components (Marsili and Miller, 1998; Bendall, 2001). If several changes occur at the same time, it may be difficult to detect these changes. Thus, multivariate statistics may be valuable for the process of discriminating between patterns of volatile components. Multivariate statistics may also help in the automation of the detection of milk quality defects (Vallejo-Cordoba and Nakai, 1994a; Horimoto et al., 1997).

2.4.3 Artificial neural networks

For the research described in this thesis, data classification based on multivariate statistics was used. A lot of different techniques exist to classify data. A frequently applied method is artificial neural networks (ANN). ANN are computerized models based on biological neural networks. Like biological networks, ANN are build of data processing elements called neurons. ANN were chosen for the research described in this thesis, because ANN are robust data processing models which don’t put much restraints (e.g. of normality) on the data. They are also able to model non-linear relationships between input parameters and output categories.

A lot of different types of ANN exist. The best known ANN are multi-layer feedforward networks. Another type of ANN specifically designed for classification purposes are probabilistic neural networks (PNN). PNN were selected because of the fast training times, the sound statistical background, as well as the type of output, which is the probability of all categories.

Probabilistic neural networks

PNN, unlike other ANN, are based on well-established statistical principles derived from Bayes decision theory and non-parametric kernel based estimators of probability density functions. The most important advantages of PNN are the very short training times and the probability per category as output (Specht, 1990; Beltrán et al., 2006).

PNN consist of simple data processing elements called neurons. Figure 2.1 gives a schematic representation of a PNN network.

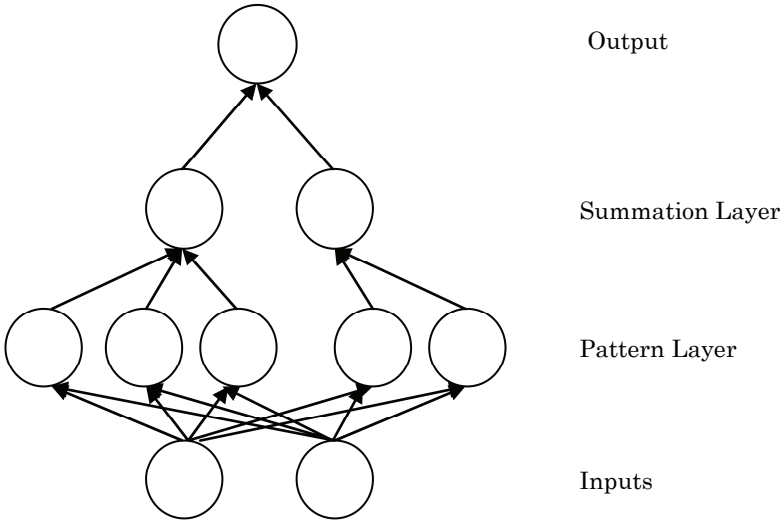

Figure 2.1. Schematic representation of a probabilistic neural network.

The number of input neurons is equal to the number of input variables. The data of a test case proceed from the input layer to the pattern layer. The pattern layer has one neuron for every training case. In the pattern layer, the distance between the test case and all training cases is calculated. The pattern layer calculates (non-linear) weighted distances for comparison of the test case to all known training cases. The calculated value for the distance is then

passed on to the summation layer. The summation layer contains one neuron for every category. All training cases belonging to one category send the value for the distance to their respective summation neuron. This neuron calculates a weighted distance from the test case to all training cases of the specific category. Finally, all neurons from the summation layer send their output to the output neuron. This output neuron calculates the probability that the test case belongs to any one of the categories. Finally, it selects the category with the smallest average distance/highest probability (Specht, 1990).

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3

Quality control of raw cows' milk by headspace analysis

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Quality control of raw cows' milk by headspace analysis
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Abstract

Headspace analysis of volatile components can be used for monitoring of food quality. This study investigated whether this type of analysis can also be applied for raw cows' milk. The detection of different quality defects caused by cow feed, microbiological and chemical contamination, as well as enzymatic deterioration was studied. Fresh raw milk without quality defects was shown to always contain the same 7 volatile components. It was also shown that treatments like heating and homogenization of raw milk may drastically change this basic pattern resulting in as much as a tenfold increase in the number of volatile components. The growth of *Pseudomonas* could not be detected in an early stage using headspace analysis. Cow feed was shown to have an effect on the volatile composition if specific vegetable byproducts were fed to the cow. Chloroform contamination was quantified using the proposed headspace method. Also, the extent of lipolysis could be quantified by measuring the free fatty acids. For quantification of both chloroform and lipolysis, the sensitivity and reproducibility of the method is sufficient for quality control purposes. The proposed headspace method was thus able to detect several quality defects with a single analysis and may therefore be a valuable supplementary method for raw milk quality control.

3.1 Introduction

Chemical analyses are an important tool to monitor the quality of food products. One of the fastest and easiest chemical analyses performed with this goal is based on headspace analysis of volatile components. Analysing volatile components has been proven to be useful for quality monitoring of a wide range of food products, such as oils (Aprea et al., 2006), vegetables (Barra et al., 2007), fruits (Beaulieu and Lea, 2006), ready to eat products (Limbo et al., 2005), wine (Carrillo and Tena, 2006), juices (Ros Chumillas et al., 2007), cheese (Andersen et al., 2006), and milk (Valero et al., 1999; Marsili, 2000; Contarini and Povolo, 2002). Headspace analysis of milk and milk products has been performed using static headspace, solid-phase microextraction (SPME), and purge and trap (P&T). To identify the individual volatile components, the headspace sampling techniques were usually coupled to gas chromatography/mass spectrometry (GC/MS) (Vallejo-Cordoba and Nakai, 1994a; Contarini et al., 1997; Fabre et al., 2002).

Although quality control of milk based on headspace analysis focused on (heated) milk products (Vallejo-Cordoba and Nakai, 1994a; McSweeney et al., 1997; Marsili, 2002), also changes in raw milk quality may be detectable by analysing the volatile composition. Cows' diet, microbiological and chemical contamination, as well as enzymatic deterioration may change the volatile composition of raw milk (Shipe et al., 1978; Azzara and Campbell, 1992). As headspace analysis can detect a whole range of volatile components at the same time, it may be able to detect a wide range of quality defects with a single analysis. Headspace analysis may thus be a valuable supplementary method for raw milk quality control.

Differences within the normal cows' diet as well as feeding of specific vegetable byproducts may alter the volatile composition of raw milk. The differences in volatile composition between cows which received either fresh grass or silage have been compared before (Bendall, 2001). This showed almost no effect of the ration on the volatile components detected in milk. However, the difference in volatile composition of milk from cows receiving either a starch-rich (e.g. maize) or crude fiber-rich (e.g. grass) diet has not been studied before. These differences in diet have an influence on the overall composition of the milk, by influencing the fermentation in the rumen (Ekern et al., 2003). Also, the transfer of volatile components from specific vegetable byproducts to the milk may be studied using a headspace analysis. The transfer of terpenes from cows' feed to the milk has been studied before (Fernandez et al., 2003). The transfer of a wider variety of flavour components to the milk, causing specific off-

flavours has also been described before (Shipe et al., 1978). Whether this transfer of volatile components from the feed to the milk can be detected using a headspace analysis has, however, not been studied.
Microbiological contamination has a major influence on raw milk quality. The major group of bacteria causing problems in cold stored raw milk are psychotrophic bacteria. *Pseudomonas* species are the most important group of psychotrophic bacteria causing spoilage of raw milk. In literature, the production of volatile components by *Pseudomonas* has only been described for pasteurized milk (Reddy et al., 1968; Cormier et al., 1991). A headspace analysis may thus be a useful method to detect *Pseudomonas* also in raw milk, as a marker for microbiological contamination and growth.
Chemical contamination of raw milk is mainly due to the cleaning and disinfection of the milking equipment. The main volatile component associated with this problem is chloroform (Resch and Guthy, 1999). Static headspace combined with GC/ECD has been used before to quantify chloroform (Miller and Uhler, 1988; Resch and Guthy, 1999). It is, however, not known whether our headspace analysis is sensitive enough to quantify chloroform in raw milk correctly.
One of the main enzymatic reactions causing quality deterioration in raw milk is lipolysis. Lipolysis is the breakdown of triglycerides to free fatty acids (FFA). This can cause a rancid off-flavour in milk (Santos et al., 2003). A method by the Bureau of Dairy Industries (BDI) is the current method to determine the extent of lipolysis (Deeth, 2006). This BDI method is laborious, expensive, and does not always correspond very well with the off-flavour in milk. A headspace analysis may, however, correspond better with the off-flavour and is easier and cheaper (Evers, 2003; Gonzalez-Cordova and Vallejo-Cordoba, 2003). A headspace method may thus be a suitable alternative to the BDI method.
The aim of this study was to show which quality defects of raw milk can be studied simultaneously with a simple, fast, and robust headspace analysis.

3.2 Materials and methods

3.2.1 Milk samples

Fresh raw milk

Fresh raw cows' milk samples from individual milk trucks were frozen at -20°C (n=10; 250 mL sample per truck). Loss of volatile components is not specifically

prevented in this experiment, as sampling is performed as currently usual for raw cows' milk.
Also, 10 mL fresh raw cows' milk samples were analysed from 10 different individual Dutch farms (bulk tank milk sample) as well as from 46 individual cows of two experimental farms of Wageningen University, "De Ossekampen" and "Zegveld".

Feeding experiment: grass/maize
Ten cows were selected at the experimental farm of Wageningen University, "De Ossekampen". The cows were randomly distributed over 5 groups of 2 cows in a latin square design. Every group of cows received *ad lib*. a diet containing 55% silage and 45% concentrate, based on dry matter (DM). Grass silage (GS), maize silage (MS), starch-rich concentrate (SC), and crude fiber-rich concentrate (CC) were fed in different ratios. Cows were fed one of five diets:
55% GS, 22.5% SC, and 22.5% CC
27.5% GS, 27.5% MS, 45% SC
27.5% GS, 27.5% MS, 22.5% SC, 22.5% CC
27.5% GS, 27.5% MS, 45% CC
55% MS, 22.5% SC, and 22.5% CC
Each diet was given for 3 weeks. After 3 weeks, a 10 mL milk sample was taken.

Feeding experiment: specific vegetable byproducts
The effect of feeding onions, green cabbage, orange peel, and spent beer brewers barley to cows was investigated. These products were chosen based on their usage as cow feed in The Netherlands, as well as their possible off-flavour effect. Eight cows were selected at the experimental farm of Wageningen University, "De Ossekampen". The cows were randomly distributed over 4 groups. Next to a normal diet (mixture of maize and grass silage), every cow received a total of 0.5 kg DM/day of one vegetable byproduct for 5 days. The vegetable byproducts were mixed with the normal diet. Every day, for 8 days, a 50 mL milk samples was taken twice a day.

Microbiological contamination: Pseudomonas
One L fresh raw cows' milk was obtained from the experimental farm of Wageningen University, "De Ossekampen" and divided in aliquots of 250 mL in sterile 250 mL bottles. The samples were spiked with *Pseudomonas fragi* (supplied by the Laboratory of Food Microbiology of Wageningen University) at

approx. 10^5 colony forming units (CFU)/mL in triplicate. The bottles were stored at 7°C. Samples were taken every day for counting of the number of *Pseudomonas* according to Hayes et al. (2002). Also, 10 mL samples were taken daily for headspace analysis.

Chemical contamination: chloroform
Fifty mL fresh raw cows' milk was obtained from the experimental farm of Wageningen University, "De Ossekampen". The sample was divided over 15 vials containing 2 mL of milk. For determination of linearity, 2 samples were analysed without spiking and 8 samples were spiked in duplicate with chloroform at a level of 2.5, 5, 12.5, or 25 µg/L. For determination of reproducibility, one sample was spiked in five-fold at a level of 12.5 µg/L.

Enzymatic deterioration: lipolysis
Two L fresh raw cows' milk was obtained from the experimental farm of Wageningen University, "De Ossekampen". Within 1 h after obtaining the milk, 250 mL milk was heated to 40°C and subsequently mixed with a blender for 1 min. The milk sample was cooled with cold running water. Various amounts (0 to 5 mL) of the milk mixed with a blender were added to 200mL fresh raw milk. After keeping the samples for 72 h at 4°C, 0.2 mL hydrogen peroxide was added to stop lipase activity. Samples were analysed for extend of lipolysis with the BDI method according to Driessen et al. (1977).

All samples were kept frozen at -20ºC for a maximum of 1 month before analysis. A vial with 10 mL demineralized water was used as blank for SPME analysis and a glass flask with 25 mL demineralized water was used as blank for P&T analysis.

3.2.2 SPME analysis
Ten mL milk samples were heated in 20 mL vials sealed with silicone/Teflon septa and magnetic caps for 1 min at 60°C. Volatiles were extracted from the headspace for 5 min with a 75 µm PDMS-carboxen SPME fiber (Supelco, Bellefonte, PA, USA) using the combiPAL autosampler (CTC Analytics AG, Zwingen, Switzerland). The volatiles were thermally desorbed from the fiber by heating for 3 min at 250ºC. The fiber was subsequently cleaned for 10 min at 275ºC. GC/MS analysis was performed on a Finnigan Trace GC gas chromatograph (ThermoFinnigan, San Jose, CA, USA) coupled to a Finnigan DSQ mass spectrometer (ThermoFinnigan, San Jose, CA, USA). Volatiles were

separated on a BPX-5 column of 30 m length, 0.15 mm i.d., and 0.25 µm film thickness (SGE, Austin, TX, USA). Oven temperature was held at -30°C for 3 min, raised to 190°C at 20°C/min, followed by 1 min holding. Helium was used as the carrier gas at a flow rate of 0.6 mL/min.
The MS interface and the ion source were kept at 250°C. Acquisition was performed in electron impact mode (70 eV) with 2 scans/s; the mass range used was m/z 33-250. For the determination of the extent of lipolysis, the acquisition was performed in SIM mode, quantifying the ions with m/z 60 and 73.

3.2.3 Purge and trap analysis

Twenty-five mL milk samples were heated at 40°C in a closed glass flask (70 mL). The flask was placed in a water bath of 37°C and a flow of purified nitrogen (50 mL/min) was passed through the sample for 30 min. The sample was constantly mixed by a magnetic stirrer. Volatiles were adsorbed on a glass tube (length 100 mm, 3.0 mm internal diameter) filled with 90 mg of Tenax TA (20/30 mesh, Alltech Nederland BV, Zwijndrecht, The Netherlands) using P&T. A cold trap with ethanol of -10°C was used to prevent water vapour from entering the Tenax tube. Volatiles were desorbed onto the column using a thermal desorption unit for 10 min at 250°C (Chrompack TCT injector 16200, Chrompack, Middelburg, The Netherlands). GC/MS analysis was performed on a Varian 3400 gas chromatograph (Varian, Bergen op Zoom, The Netherlands) coupled with a Finnigan MAT95 mass spectrometer (Thermo Electron, Bremen, Germany). Volatiles were separated on a Agilent J&W DB-5 column of 60 m length, 0.25 mm i.d., and 0.25 µm film thickness (VWR International B.V., Amsterdam, The Netherlands). The oven temperature was held at 40°C for 4 min, raised to 200°C at 6°C/min, followed by 4 min holding. Helium was used as carrier gas at a constant pressure of 125 kPa. The mass spectrometer was operated in electron impact mode (70 eV) with 1 scan/s; the mass range used was m/z 33-300.

3.2.4 Data analysis

The results of the measurements were analysed using the Automated Mass Spectral Deconvolution and Identification System (AMDIS) software (NIST, Gaithersburg, MD, USA). Identification of milk volatiles was based on matching deconvoluted mass spectra from AMDIS with spectra from the NIST/EPA/NIH mass spectral database. Whenever possible, the retention times were matched to standards, spiked to milk. Analytical standards were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands), with the

exception of FFA which were obtained from Supelco (Zwijndrecht, The Netherlands), and ethanol, chloroform, and acetone which were obtained from Merck (Darmstadt, Germany). Subsequent peak integration was performed using the XCalibur software package.
SPSS for Windows version 12.0 (SPSS Inc., Chicago, IL, USA) was used for comparisons between groups (ANOVA). SPSS was also used to determine the mean, correlation coefficient, and relative standard deviation (RSD) of the results. $P<0.05$ was considered statistically significant.

3.3 Results and discussion

3.3.1 Fresh raw milk

Using SPME-GC/MS, 7 volatile components were found in raw milk (Figure 3.1). This pattern of 7 volatile components was the same for all 20 fresh raw milk samples. No other volatiles were found in any of the samples. Also samples from individual farms as well as individual cows were analysed using SPME-GC/MS. In all these samples, the same 7 components were detected. Next to the 7 components, some of the individual cow's milk samples also contained a very small amount of FFA (just above the detection limit).
The number of volatiles detected was much lower compared to numbers in literature references reporting headspace analyses of milk and milk products. This can probably be explained by two factors. First, pasteurized and sterilized milk samples were often analysed (Contarini et al., 1997; Valero et al., 1999, 2001; Contarini and Povolo, 2002). In the sterilized milk samples, the highest number of volatile components was found; up to 77 (Valero et al., 1999, 2001). During sterilization of milk, volatile components are formed (Azzara and Campbell, 1992; McSweeney et al., 1997).
Recently, the volatile components in raw milk have been described twice (Vazquez-Landaverde et al., 2005, 2006). In both cases they reported approximately 20 components in raw milk, which were mainly aldehydes and ketones. However, they used homogenized raw milk. After homogenization without pasteurization, natural milk lipase will cause lipolysis of the raw milk, causing the formation of FFA. Subsequently, these FFA will be broken down to the aldehydes and ketones reported (Deeth and Fitz-Gerald, 1994). When we induced lipolysis in milk samples, we could find all the 20 components reported by Vazquez-Landaverde et al. (2005), see table 3.1. The concentration of the aldehydes and ketones was about 100 times lower than the concentration of

FFA. Thus, these papers do not describe the volatile components found in *fresh* raw milk.

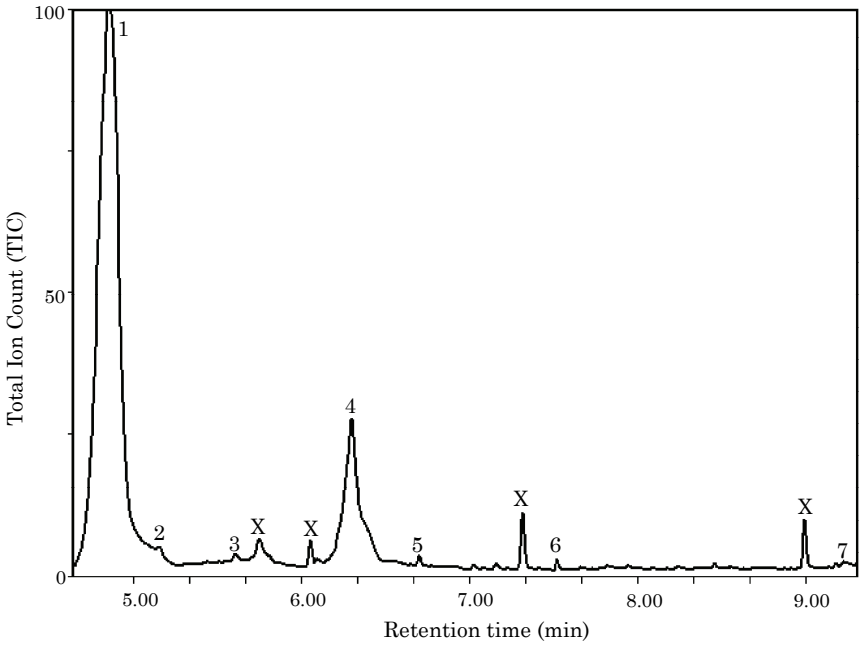

Figure 3.1. GC/MS chromatogram after SPME extraction of fresh raw milk. Identification of peaks: (1) acetone, (2) dimethyl sulfide, (3) carbon disulfide, (4) 2-butanone, (5) chloroform, (6) pentanal, and (7) hexanal. Peaks marked with X are blank peaks (mainly cyclosiloxanes, which are known artifacts from the SPME fiber).

Table 3.1. Number of components detected in raw, homogenized, and sterilized milk, ordered by group of components. Number of components reported in literature between brackets.

Type of component	Raw	Homogenized [1]	Sterilized [2]
Aldehydes	2	8 (8)	7 (6)
Ketones	2	6 (7)	8 (9)
Alcohols		1 (1)	5 (9)
Free fatty acids		4 (?)	3 (0)
Esters		6 (1)	2 (1)
Sulphurous	2	0 (1)	3 (2)
Alkanes			5 (7)
Other	1	1 (0)	4 (5)

[1] Data: Vazquez-Landaverde et al., 2005

[2] Data: Valero et al., 2001

As shown by Moio et al. (1993), vacuum distillation is able to extract a larger quantity and a wider range of volatile components. These methods aim at components which are slightly less volatile than the components detected using headspace sampling. However, for our aim (a fast chemical analysis) the relatively slow method of vacuum distillation is not practical.
The only reference of headspace analysis of *fresh* raw milk is from 1966 (Bassette et al., 1966). This paper only describes the concentration of 4 volatile components in fresh raw milk, which could be identified. It also reports a few more peaks in the chromatogram which could not be identified. This is thus the first time, as far as the authors know, that all components that can be detected using a simple headspace analysis of fresh raw milk were identified and reported.

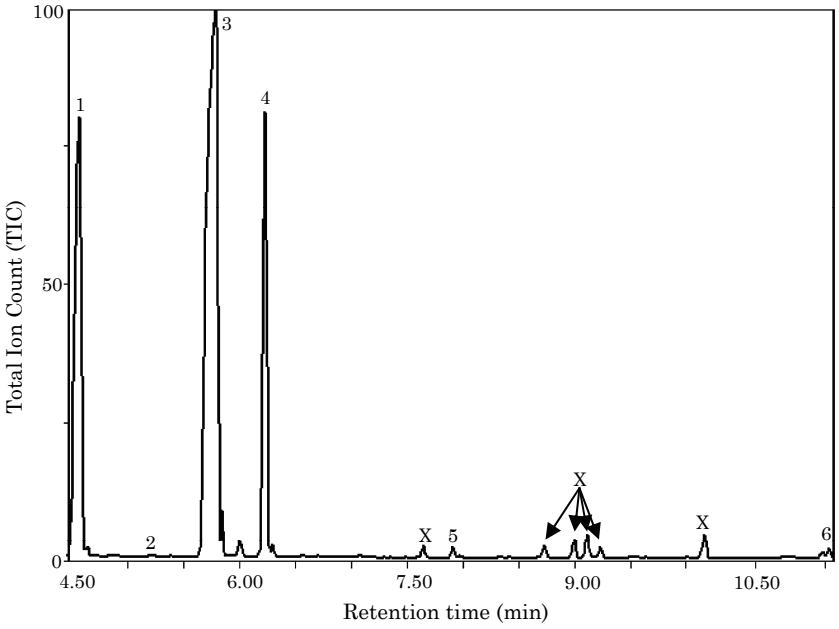

Figure 3.2. GC/MS chromatogram after purge and trap extraction of fresh raw milk. Identification of peaks: (1) acetone, (2) dimethyl sulfide, (3) 2-butanone, (4) chloroform, (5) pentanal, and (6) hexanal. Peaks marked with X are blank peaks (mainly alcohols from the cold trap).

To remove all doubts about the low number of volatiles found in fresh raw milk, we also performed P&T-GC/MS analyses which should have a higher sensitivity compared to the SPME-GC/MS method. Using P&T, just 6

components could be detected in fresh raw milk (Figure 3.2). Compared to SPME, only carbon disulfide was not detected using P&T. The 6 peaks, except dimethyl sulfide, had a much higher signal-to-noise ratio compared to SPME, demonstrating the higher sensitivity of P&T. This higher sensitivity did not increase the number of detected peaks. The relative insensitivity of P&T towards the most volatile sulphurous components was previously reported by Contarini et al. (2002).

3.3.2 Feeding experiment: grass/maize

All 50 milk samples were analysed for their volatile components. All 7 components already reported in section 3.1 were quantified. Next to these 7 components, no other volatile components were detected. An ANOVA test showed that there were no significant differences between the groups for any of the volatiles. This shows that differences in the starch/crude fiber content of the diet do not change the volatile composition of the milk, even though it has an influence on the fermentation in the rumen and the non-volatile composition of the milk (Ekern et al., 2003).

3.3.3 Feeding experiment: specific vegetable byproducts

Headspace analyses of the samples from cows fed one of the four vegetable byproducts were performed as well as analyses of 1 gram of the vegetable byproducts involved. The samples from the cows fed orange peel contained 7 different terpenes, which could also be found in the headspace of the orange peel itself. The samples from the cows fed onion contained only one additional component, next to the 7 components found regularly. This was a sulphurous component, not detected in the onion itself. No direct transfer of any of the volatiles found in onion could be detected. The milk did, however, have a sulphurous off-flavour. We did not determine if this off-flavour was caused by the sulphurous component detected in the milk sample.

A higher number of sulphurous components may be present in the milk sample than found by SPME-GC/MS, due to the fact that the human nose is much more sensitive to sulphurous components compared to the GC/MS (Senger-Emonnot et al., 2006). Moreover, extraction techniques like Solvent Assisted Flavour Evaporation (SAFE) will probably be more sensitive to sulphurous components compared to SPME (Havemosea et al., 2006). SAFE is, however, not a simple and fast method for quality control.

The samples from the cows fed green cabbage or spent beer brewers barley did not contain any additional volatile component to the 7 already regularly found.

3.3.4 Microbiological contamination: Pseudomonas

The number of *Pseudomonas* in spiked and unspiked milk samples is shown in Figure 3.3. After 3 days, the spiked milk sample was obviously spoiled, for the control milk sample this took 5 days. In both cases, spoilage was probably due to *Pseudomonas* growth, as also in the control milk sample, there was considerable growth of *Pseudomonas* and both spoiled milk samples had a fruity off-flavour, which can be attributed to the growth of *Pseudomonas* (Cormier et al., 1991; Hayes et al., 2002).

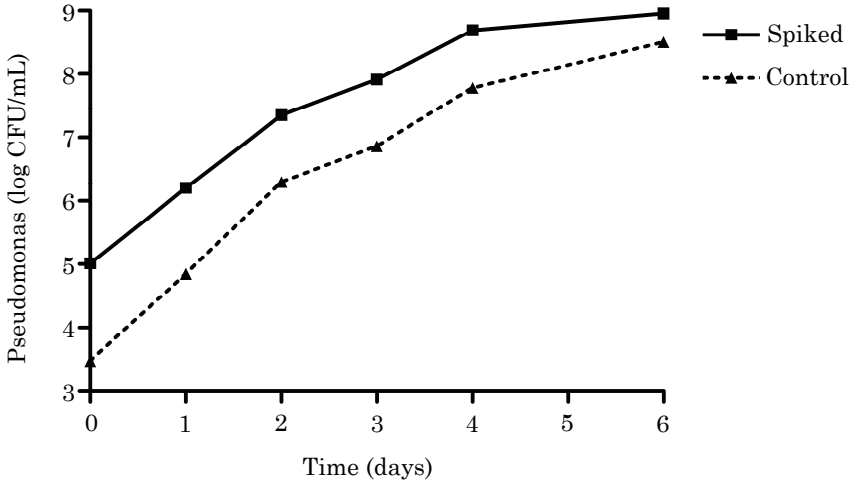

Figure 3.3. Number of *Pseudomonas* detected in raw milk (control) and raw milk spiked with 10^5/ml of *Pseudomonas*.

With GC/MS analysis of the spiked milk samples, no differences were detected in the volatile composition in the first days, when the milk was not yet spoiled. Only when the milk was obviously spoiled, the GC/MS pattern changed, and the previously reported ethyl-esters, ethyl butanoate and ethyl hexanoate, of FFA were detected (Reddy et al., 1968; Cormier et al., 1991). These ethyl-esters are most likely responsible for the fruity off-flavour noticed in the spoiled milk samples (Cormier et al., 1991; Hayes et al., 2002). On day 5, the same pattern of volatiles could be detected in the control milk sample.

It was expected that the detection of *Pseudomonas* metabolites would already occur at an earlier stage. However, for the production of the ethyl ester, the presence of ethanol is necessary. In the chromatogram, no ethanol could be detected. Also, *Pseudomonas* normally does not produce ethanol, although under special conditions it can produce small amounts of it (Pereira and

Morgan, 1958). Ethanol may thus be a limiting factor for the production of the ethyl esters. Cormier et al. (1991), who detected a range of metabolites which could be contributed to *Pseudomonas*, did add some ethanol to the sample before incubation to increase the formation of the flavour metabolites.
However, as the goal of our method was to have a fast and simple analysis directly on non-incubated raw milk, the detection of relatively low amounts of *Pseudomonas* was not feasible. Only when the milk was obviously spoiled, the metabolites could be detected.

3.3.5 Chemical contamination: chloroform

As already reported in section 3.3.1, chloroform was detected in all raw milk samples. When spiked samples were analysed, the peak area of chloroform was found to be linear with the amount of chloroform added ($R^2 = 0.99$). The method was also reproducible, as the RSD was approximately 7.5%.
Although there is no European MRL for chloroform in food products, the German government uses a limit of 100 µg/kg (SHmV, 2006). The quantification limit for the used SPME method was approximately 0.1 µg/kg, based on a theoretical calculation of a signal-to-noise ratio of 10. The quantification limit is thus much lower compared to the German legal limit.
Currently, chloroform is analysed using GC/ECD (Resch and Guthy, 1999). This method can however only detect halogens. The used headspace method with GC/MS, can identify a wider range of components, and may thus be used to detect a wider range of quality defects with a single analysis. The used headspace method may thus be a suitable alternative for the quantification of chloroform.

3.3.6 Enzymatic deterioration: lipolysis

Seven samples were analysed using both the BDI method and the SPME-GC/MS method. The BDI value of the samples ranged from 0.4 for fresh milk to 0.9 mmol FFA/100g fat (a value of 1.0 is the farmer penalty limit for the FFA content in The Netherlands). With the SPME-GC/MS analysis, a range of FFA with 4 to 12 C-atoms could be quantified separately. Butyric acid had the highest peak of the different FFA, which can be expected based on its volatility and relative abundance. After the analyses, the correlation between the two methods was determined (Figure 3.4). The correlation coefficient of the two methods was 0.98 ($P<0.05$). The SPME-GC/MS method had an acceptable RSD of 9.3%.

As the sensitivity and repeatability of the method are sufficient for quality control purposes, the SPME-GC/MS method is thus a good candidate to replace the current BDI method.

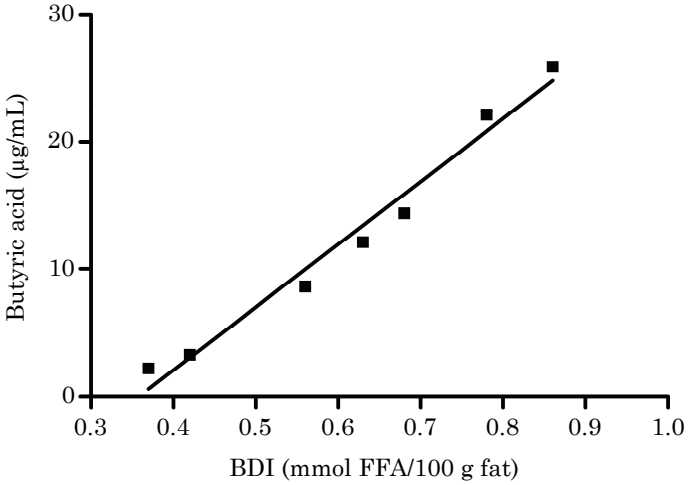

Figure 3.4. Butyric acid as determined with SPME-GC/MS versus the BDI value of one milk sample in which lipolysis was induced at different levels.

3.4 Conclusions

The suitability of headspace analysis for quality control of raw milk was tested for a range of quality defects. Fresh raw milk without quality defects was shown to always contain the same 7 volatile components. Treatments like heating and homogenization of raw milk drastically changed this basic pattern resulting in as much as a tenfold increase in the number of volatile components. The growth of *Pseudomonas*, or variation in the composition of the regular diet could not be detected using headspace analysis. On the other hand, the headspace analysis could be used for quantifying the extent of lipolysis, the amount of chloroform, as well as the detection of feeding specific vegetable byproducts. The proposed headspace method was thus able to detect several quality defects with a single analysis and may therefore be a valuable supplementary method for raw milk quality control.

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4

Detection of mastitis pathogens by analysis of volatile bacterial metabolites

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Detection of Mastitis Pathogens by Analysis of Volatile Bacterial Metabolites
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Abstract

The possibility to detect mastitis pathogens based on their volatile metabolites was studied. Milk samples from cows with clinical mastitis, caused by *Staphylococcus aureus*, coagulase-negative staphylococci (CNS), *Streptococcus uberis*, *Streptococcus dysgalactiae*, and *Escherichia coli* were collected. Also, samples from cows without clinical mastitis and with low somatic cell count were collected for comparison. All mastitis samples were examined with classical microbiological methods, followed by headspace analysis for their volatile metabolites. Milk from culture negative samples contained far less metabolites compared to cows with clinical mastitis. Due to variability between samples within a group, comparisons between pathogens were not sufficient for identification of the pathogens in the samples by univariate statistics. Therefore, an artificial neural network was trained to classify the pathogen in the milk samples based on the bacterial metabolites. The trained network differentiated milk from uninfected and infected quarters very well. When comparing pathogens, *Staph. aureus* produced a very different pattern of volatile metabolites compared to the other samples. Samples with coagulase-negative staphylococci and *E. coli* had enough dissimilarities with the other pathogens, making it possible to separate these two pathogens from each other and the other samples. The two *streptococcus* species did not show significant differences between each other but could be identified as a different group from the other pathogens. Five groups can thus be identified based on the volatile bacterial metabolites: *Staph. aureus*, CNS, *streptococcus* (*Strep. uberis* and *Strep. dysgalactiae* as one group), *E. coli*, and uninfected quarters.

4.1 Introduction

The most costly disease of dairy cows is mastitis. Mastitis is most often caused by bacteria. Although many bacteria can cause mastitis, a limited group of 5 bacterial species (*Staphylococcus aureus*, coagulase-negative staphylococci (CNS), *Streptococcus uberis*, *Streptococcus dysgalactiae*, and *Escherichia coli*) is responsible for approximately 80% of the Dutch mastitis cases in which bacteria could be cultured from the milk (Barkema et al., 1998). However, in over 30% of clinical mastitis cases, no pathogen is detected (Bradley et al., 2007).

Determination of mastitis causing pathogens is of great interest, both for choice of treatment of the cow as well as for possible measures that have to be taken on the farm to prevent the spread of mastitis. Currently, determination of the pathogen is generally done by bacteriological culturing (NMC, 2004). This method, however, has the important disadvantage that it is time-consuming. Because the bacteria have to grow before they can be identified based on phenotypical characteristics, it takes a few days before results are available. Other diagnostic methods like polymerase chain reaction (PCR) on milk are, although promising, very labor intensive and thus expensive. Also, it is difficult to perform PCR directly on milk, as it is a complex matrix (Yamagishi et al., 2007).

A disadvantage of bacteriological culturing is the existence of false-negative results, in which samples contain too few pathogens to be detected (Sears et al., 1990). The pathogens may also already be dead before sampling. Finally, failure to detect pathogens in samples taken from quarters with clinical mastitis can be caused by contamination of the sample (Zorah et al., 1993). Thus, faster and more accurate methods of pathogen identification could be advantageous, because farmers are earlier able to choose an optimal treatment.

Fast automatic on-line detection of mastitis has been described using the variables milk temperature, milk electrical conductivity, and milk production (Nielen et al., 1994). Similarly, Heald et al. (2000) showed that a classification could be made between 3 types of mastitis (contagious, environmental, or "other" pathogen) using a wide variety of already available variables from milk and herd screening (DHIA program), like somatic cell count (SCC), days in lactation, and (average) milk production. However, none of these methods were able to identify individual mastitis causing pathogens.

In microbiology, screening of volatile bacterial metabolites for detection and/or identification purposes is well known. The identification is based on the fact that all microorganisms have their own group of enzymes, producing their own

range of volatile metabolites (Gardner et al., 1998; Marilley et al., 2004; Turner and Magan, 2004). Eriksson et al. (2005) used this principle to detect mastitis using an electronic nose. They were able to discriminate between uninfected and infected quarters based on the bacterial metabolites, but they could not differentiate between pathogens. This may be due to the fact that electronic noses only detect groups of metabolites, but are unable to identify individual metabolites.
For identification of volatile metabolites, other headspace based chemical analytical methods can be used. An often used headspace extraction method is solid-phase microextraction (SPME). SPME is a technique using a fiber, coated with a sorbent, which extracts volatiles from the headspace of a sample (Arthur and Pawliszyn, 1990). To identify the individual volatile components, SPME is usually coupled to gas chromatography/mass spectrometry (GC/MS) (Marsili, 1999).
In our study, clinical mastitis samples were examined with classical microbiological methods and by headspace analysis for their volatile metabolites, comparing the results of both methods.

4.2 Materials and methods

4.2.1 Milk samples

Fifty milk samples from cows with clinical mastitis were selected from the bacteriological diagnostic lab of the GD Animal Health Service (Deventer, The Netherlands). Samples had first been screened for the presence of bacteria. If one of the 5 pathogens of interest (*Staph. aureus*, CNS, *Strep. uberis*, *Strep. dysgalactiae*, or *E. coli*) was cultured, the remainder of the sample (approx. 5 mL) was frozen at -20°C for later use. Ten samples per pathogen were collected. Ten milk samples of cows, without clinical mastitis and with low SCC (below 75,000), were used as controls. The samples were supplied by "De Ossekampen", the university farm of Wageningen University and Research Centre. These samples were also kept frozen at -20°C for later use.
Bacteriological culturing was carried out according to NMC protocols (NMC, 2004). All plates were incubated at 37ºC and examined after 24 and 48 h. Because these were milk samples from routine mastitis diagnosis, the milk samples were incubated overnight at 37ºC, to be able to use them again in case of "no growth". Thus, all milk samples were incubated for 14 hours prior to analysis of their volatile metabolites. Only milk samples from which one

bacterial species was cultured before incubation of the sample, were included in the study.

4.2.2 Analysis of volatile metabolites

Five mL milk samples were preheated in 20 mL vials sealed with silicon/Teflon septa and magnetic caps for 1 min at 60°C. Volatile metabolites were extracted from the headspace for 5 min with a 75 µm PDMS-carboxen SPME fiber (Supelco, Bellefonte, PA, USA) using the combiPAL autosampler (CTC Analytics AG, Switzerland). The volatile metabolites were thermally desorbed from the fiber by heating it in a Best PTV injector with an empty liner for 5 min at 250ºC. The fiber was subsequently cleaned for 10 min at 290ºC. A vial with 5 mL demineralized water was used as blank.

GC separation of the volatile components was performed on a Finnigan Trace GC gas chromatograph (ThermoFinnigan, San Jose, CA, USA) coupled to a Finnigan DSQ mass spectrometer (ThermoFinnigan, San Jose, CA, USA). Volatiles were separated on an apolar BPX-5 column of 30 m length, 0.15 mm i.d., and 0.25 µm film thickness (SGE, Austin, TX, USA). Oven temperature was held at -30°C for 3 min, raised to 230°C at 20°C/min, followed by 1 min holding. Helium was used as the carrier gas at a flow rate of 0.6 mL/min. The MS interface and the ion source were kept at 250°C. Acquisition was performed in electron impact mode (70 eV) with 2 scans/s; the mass range used was m/z 33-250. The resulting chromatograms were analyzed using the AMDIS software (NIST, Gaithersburg, MD, USA); data was deconvoluted to obtain pure mass spectra, for improved peak identification. Identification of volatile metabolites was based on matching mass spectra and retention time with pure standards, if possible. Otherwise, spectra were compared to the NIST/EPA/NIH mass spectral database and the kovats index was compared to data from literature (Acree and Arn, 2004). Peak integration was subsequently performed using the XCalibur software package (ThermoFinnigan, San Jose, CA, USA). Peak area was corrected for the blank sample. The peak area, which is in arbitrary units, was used for subsequent statistical analysis.

4.2.3 Statistical analysis

SPSS for Windows version 12.0 (SPSS Inc., Chicago, IL, USA) was used for comparisons between groups. Because data were not normally distributed, first the Kruskal-Wallis test was performed to test for differences between groups. If significant differences between groups were observed, the Nemenyi test (Zar, 1999) was used for subsequent pairwise multiple comparisons.

NeuralTools (Palisade, Ithaca, NY, USA) was used to develop artificial neural networks (ANN). Probabilistic neural networks (PNN) were the type of ANN used for this study. The conjugate gradient descent method was used for training the PNN. Training of the neural networks was carried out using cross validation, with 70% of the samples used for training and 30% for validation. Samples were distributed randomly between the training and validation group. To validate the model, both leave-one-out as well as 10-fold cross-validation were used.

4.3 Results and discussion

4.3.1 Analysis of volatile metabolites

After microbiological analysis of the milk samples, their volatiles were analyzed using GC/MS. Table 4.1 contains an overview of the results of the GC/MS analysis, grouped by pathogen. The samples from uninfected quarters contained a lower number and lower amount of volatile components, compared to the mastitis milk samples.

The formation of 2-butanone, 2- and 3-methylbutanal, acetoin, and isopentanol by *Staph. aureus* has been described by Zechman et al. (1986). Furthermore, the formation of 2- and 3-methylbutanal, as well as the (esters of) branched fatty acids by *Staph. aureus* has been described Ritter and Hanni (1960). Of the volatile metabolites found in samples with *E. coli*, acetaldehyde, 2,3-butadione, ethyl acetate, and ethyl butyrate have been described before (Morales et al., 2004). No literature references were found for the volatile metabolites formed by CNS and the two *streptococcus* species.

The univariate statistical analysis of the differences between uninfected and infected quarters showed a clear distinction between the two groups. The subsequent statistical analysis of differences between pathogens did, however, not give a clear distinction between the different pathogens (Table 4.1). *Staph. aureus* could be differentiated from other pathogens based on the (esters of) the branched fatty acids, such as 2-methylbutyrate. Also, *Staph. aureus* and *E. coli* formed a higher amount of ethyl acetate and acetic acid. *Staph. aureus* and CNS produced similar amounts of the branched aldehydes, which were higher than for the other groups of pathogens. The two *streptococcus* species did, however, not differ in anyway from each other.

Table 4.1. Quantity of volatile metabolites in the milk samples. Numbers are mean area values (arbitrary units) of the different volatiles of the samples which did contain the components of interest.

Metabolite [1]	Mastitis Pathogen					Control [3]
	Staph. aureus	CNS [2]	*Strep. uberis*	*Strep. dysgalactiae*	*E. coli*	
Acetaldehyde	$7.4\times10^{6\ a}$	$6.4\times10^{4\ b}$	$2.8\times10^{5\ b}$	$1.4\times10^{5\ ab}$	$2.7\times10^{5\ ab}$	$1.2\times10^{5\ b}$
Ethanol	$1.1\times10^{8\ a}$	$1.6\times10^{5\ b}$	$1.6\times10^{6\ b}$	$5.8\times10^{6\ b}$	$1.3\times10^{8\ a}$	$0^{\ b}$
2,3-Butadione	$1.1\times10^{6\ a}$	$5.0\times10^{4\ ab}$	$5.6\times10^{5\ ab}$	$1.4\times10^{5\ ab}$	$1.5\times10^{4\ b}$	$0^{\ b}$
2-Butanone	$2.1\times10^{6\ a}$	$3.2\times10^{5\ b}$	$1.4\times10^{6\ ab}$	$7.0\times10^{5\ ab}$	$5.0\times10^{5\ ab}$	$3.0\times10^{6\ a}$
Eth. acetate	$1.1\times10^{6\ a}$	$0^{\ b}$	$5.0\times10^{4\ b}$	$2.0\times10^{4\ b}$	$6.9\times10^{5\ a}$	$0^{\ b}$
3-Meth.butanal	$2.8\times10^{7\ a}$	$2.7\times10^{6\ a}$	$8.8\times10^{4\ b}$	$4.5\times10^{5\ b}$	$4.6\times10^{4\ b}$	$6.4\times10^{3\ b}$
2-Meth.butanal	$3.7\times10^{5\ a}$	$5.9\times10^{4\ a}$	$2.7\times10^{3\ b}$	$2.7\times10^{3\ b}$	$0^{\ b}$	$0^{\ b}$
2-Pentanone	$8.1\times10^{4\ a}$	$8.3\times10^{3\ b}$	$3.6\times10^{4\ b}$	$7.1\times10^{4\ ab}$	$9.6\times10^{5\ ab}$	$0^{\ b}$
Acetic acid	$6.0\times10^{6\ a}$	$5.1\times10^{5\ b}$	$2.7\times10^{6\ ab}$	$3.4\times10^{6\ ab}$	$9.8\times10^{6\ a}$	$3.7\times10^{6\ ab}$
Isopentanol	$7.3\times10^{6\ a}$	$1.1\times10^{6\ ab}$	$0^{\ b}$	$1.3\times10^{5\ b}$	$3.9\times10^{6\ b}$	$0^{\ b}$
Acetoin	$8.2\times10^{5\ a}$	$3.2\times10^{3\ b}$	$3.6\times10^{3\ b}$	$0^{\ b}$	0 b	$0^{\ b}$
Eth. butyrate	$7.2\times10^{5\ a}$	$0^{\ b}$	$7.2\times10^{4\ b}$	$1.0\times10^{5\ b}$	$3.3\times10^{5\ ab}$	$0^{\ b}$
Butyric acid	$2.9\times10^{6\ ab}$	$2.0\times10^{5\ c}$	$7.8\times10^{5\ c}$	$1.0\times10^{6\ abc}$	$1.0\times10^{6\ abc}$	$4.2\times10^{6\ a}$
Eth. 2-meth.but.	$2.2\times10^{4\ a}$	$3.6\times10^{2\ b}$	$0^{\ b}$	$0^{\ b}$	$0^{\ b}$	$0^{\ b}$
Eth. 3-meth.but.	$9.6\times10^{4\ a}$	$4.6\times10^{2\ b}$	$3.2\times10^{3\ b}$	$2.8\times10^{3\ b}$	$0^{\ b}$	$0^{\ b}$
3-Meth.but.	1.4×10^{2}	0	0	7.2×10^{2}	0	0
2-Meth.but.	6.0×10^{4}	0	0	0	0	0
2-Heptanone	$1.7\times10^{5\ a}$	$1.9\times10^{4\ b}$	$1.0\times10^{5\ ab}$	$1.3\times10^{5\ ab}$	$3.2\times10^{5\ ab}$	$2.0\times10^{4\ b}$
Butyl but.	$1.2\times10^{4\ a}$	$0^{\ b}$	$0^{\ b}$	$0^{\ b}$	$0^{\ b}$	$0^{\ b}$
Eth. hexanoate	$3.7\times10^{5\ a}$	$7.6\times10^{2\ b}$	$6.4\times10^{4\ b}$	$9.2\times10^{4\ ab}$	$2.2\times10^{5\ a}$	$0^{\ b}$

[a, b, c] Means within a row with different superscripts differ ($P < 0.05$)

[1] Eth. = Ethyl; Meth. = Methyl; But. = Butyrate

[2] CNS = coagulase-negative staphylococci

[3] Milk from cows without clinical mastitis and with low somatic cell count

Five groups can thus be identified based on the statistical analysis of the volatile bacterial metabolites: *Staph. aureus*, CNS, *streptococcus* (both *streptococcus* species as one group), *E. coli*, and culture-negative samples. However, identification based on univariate statistical analysis alone was difficult, because in every group, some samples did have different results compared to the other samples in their group, e.g. a specific metabolite was not detected, or the concentration of a specific metabolite was different. Thus, a clear identification of the mastitis pathogens in individual samples based on

this univariate statistical analysis alone were complicated, time-consuming, and not fully reliable. Therefore multivariate statistics were used.

4.3.2 Multivariate statistics

A lot of automatic identification techniques depend on multivariate statistics for identification. Among the wide variety of techniques, artificial neural networks (ANN) are often used. ANN are non-linear models which can be trained to quantify as well as classify samples based on a large number of input variables. Multilayer feedforward networks trained by back-propagation (MLF) are the best known and most commonly applied artificial neural networks for classification purposes (González-Arjona et al., 2006).

Another kind of ANN are probabilistic neural networks (PNN). PNN are specifically developed for classification purposes. Unlike other ANN, like MLF, it is based on well-established statistical principles derived from Bayes decision theory and non-parametric kernel based estimators of probability density functions. The most important advantages of PNN are the very short training times and the probability per category as output (Specht, 1990; Beltrán et al., 2006). A PNN consists of simple data processing elements called neurons. Figure 2.1 gives a schematic representation of a PNN. The number of input neurons is equal to the number of input variables (in our case, the number of volatile components). The data of a test case proceeds from the input layer to the pattern layer. The pattern layer has one neuron for every training case. In the pattern layer, the distance between the test case and all training cases is calculated. The calculated value for the distance is then passed on to the summation layer. The summation layer contains one neuron for every category. All training cases belonging to one category send the value for the distance to their respective summation neuron. This neuron calculates a weighted distance from the test case to the training cases. Finally, all neurons from the summation layer send their output to the output neuron. This output neuron calculates the probability that the test case belongs to any one of the categories. Finally, it selects the category with the smallest average distance (Specht, 1990).

4.3.3 Training the PNN to classify milk samples

A PNN was first trained to categorize milk as either from uninfected or infected quarters. The correct classification rate for this categorization was 100%.

The PNN was then trained for categorizing all 5 pathogens in infected quarters. The correct classification rate for this PNN was 66% (Table 4.2).

Table 4.2. Cross-validation results of training a PNN to differentiate between 5 mastitis pathogens.

	Predicted by PNN [1]					
Microbiological identification	*Staph. aureus*	CNS [2]	*Strep. uberis*	*Strep. dysgalactiae*	*E. coli*	Correct (%)
Staph. aureus	3	0	0	0	0	100
CNS	0	3	0	0	0	100
Strep. uberis	0	0	1	1	1	33
Strep. dysgalactiae	0	0	2	1	0	33
E. coli	0	0	0	1	2	66
Total correct						66

[1] The number of samples classified in the respective group by the PNN
[2] CNS = coagulase-negative staphylococci

The prediction results showed that the main error was with the two *streptococcus* species, which could not be well classified by the PNN. The bad classification of the two types of *streptococcus* was already expected based on table 4.1; all volatile metabolites are formed in approximately equal amounts in both *streptococcus* species. Because of this, for subsequent PNN training, the two different *streptococcus* species were treated as one group.
The PNN was subsequently trained to classify the samples in 4 groups. Table 4.3 contains the classification results for the validation group.

Table 4.3. Cross-validation results of training a PNN to differentiate between 4 mastitis groups.

	Predicted by PNN [1]				
Microbiological identification	*Staph. aureus*	CNS [2]	*Streptococcus*	*E. coli*	Correct (%)
Staph. aureus	3	0	0	0	100
CNS	0	2	1	0	66
Streptococcus	0	0	6	0	100
E. coli	0	0	0	3	100
Total correct					93

[1] The number of samples classified in the respective group by the PNN
[2] CNS = coagulase-negative staphylococci

The correct classification rate was 93%, with all samples classified with more than 95% probability. The sample which was not correctly classified was a CNS sample. The wrongly classified CNS sample differed from the other 9 CNS samples by its relatively low amount of 2- and 3-methylbutanal.
To confirm the validity of PNN for the relatively small data-set, the model was checked using leave-one-out as well as 10-fold cross-validation. With leave-one-out cross-validation, also a correct classification rate of 93% was obtained. Using 10-fold cross-validation, a correct classification rate of 90% was reached. This confirms that PNN is a valid data analysis technique for this data.
There is a debate whether mastitis cows from which no pathogens are cultured, or with Gram-negative pathogens (e.g. *E. coli*) in their milk, should be treated using antibiotics (Neeser et al., 2006). Fast classification of samples between no pathogen, *E. coli*, and other pathogens is thus very useful for a treatment decision. Therefore we trained a new PNN to classify samples in one of these three groups. The correct classification rate was 94% (Table 4.4), with all samples classified with more than 95% probability.

Table 4.4. Cross-validation results of training a PNN to differentiate between Gram-positive, Gram-negative, and control samples.

	Predicted by PNN [1]			
Microbiological identification	Gram-positive	Gram-negative	Control [2]	Correct (%)
Gram-positive	11	0	1	91
Gram-negative	0	3	0	100
Control	0	0	3	100
Total correct				94

[1] The number of samples classified in the respective group by the PNN
[2] Milk from cows without clinical mastitis and with low somatic cell count

4.3.4 Application in practice

The method was shown to be able to differentiate pathogens based on volatile metabolites. This has two advantages, cost of analysis and analytical time. The cost of a headspace analysis, when performed on a large scale, may be up to 5 times lower compared to classical microbiological culturing. The reduction in analytical time will depend on the necessity of incubation of the samples prior to analysis of their volatile metabolites. In that respect, it must be emphasized that in this study all samples were incubated overnight prior to analysis of their volatile metabolites. During this incubation step, bacteria may have

further grown and produced more volatile metabolites. This was however a necessary step to obtain the samples for this research. Before application of this method in practice, the necessity of the incubation step, and if needed the minimum incubation time, should be determined, to achieve maximum reduction in the time needed for analysis. The results of this study were obtained with milk samples with unambiguous bacteriological results (only one pathogen was detected). Thus, before application in practice, the method needs to be tested with a wider range of samples from different origins. These results, however, show that mastitis detection and identification using volatile bacterial metabolites looks very promising. Detection of these metabolites can be done using a simple and fast analytical method. This method may be used for detection of mastitic udder quarters as well as identification of the pathogen causing mastitis.

4.4 Conclusions

Volatile metabolites found in milk from uninfected quarters differed significantly from metabolites found in milk from infected quarters. Additionally, different pathogens were found to differ in the formation of volatile metabolites. Especially S*taph. aureus*, CNS, and *E. coli* differed from the other pathogens. The two *streptococcus* species did not show significant differences between each other but were different from the other pathogens.

A PNN was trained to classify the milk samples based on the amount of volatile metabolites. The trained PNN was able to differentiate between samples from uninfected and infected quarters, with a correct classification of 100%. Another trained PNN was able to differentiate between *Staph. aureus*, CNS, *Streptococci*, and *E. coli* with a correct classification of 93%. If only a classification between uninfected quarters, *E. coli,* and non-*E. coli* was needed, the correct classification rate was 94%. These results show that mastitis pathogen classification using volatile bacterial metabolites looks very promising.

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5

The origin of the volatile metabolites found in mastitis milk

This chapter is submitted as:
The origin of the volatile metabolites found in mastitis milk
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Abstract

The possibility to detect mastitis pathogens based on their volatile metabolites was previously studied. Because the origin of the metabolites is unknown, the formation of volatile metabolites by 5 mastitis pathogens inoculated in milk of healthy cows was studied. The volatile metabolites from inoculated samples were compared to those of mastitis milk samples from which the inoculated pathogens were isolated. Most metabolites formed in the inoculated samples were similar to the metabolites formed in mastitis samples, both in presence and in amount. Prediction by a neural network showed that the similarity between the inoculated samples and mastitis samples was sufficient for correct prediction of the pathogen in the inoculated sample. The main difference between the inoculated samples and the mastitis samples was the absence of ethyl esters of free fatty acids in inoculated samples. This was explained by disturbance of the milk-blood barrier, allowing the transfer of esterase from the cows' blood to the milk in cows with mastitis.

5.1 Introduction

The most costly disease of dairy cows is mastitis. Mastitis is most often caused by bacteria. Although many bacteria may cause mastitis, a limited group of 5 bacteria (*Staphylococcus aureus*, coagulase-negative staphylococci (CNS), *Streptococcus uberis*, *Streptococcus dysgalactiae*, and *Escherichia coli*) is responsible for approximately 80% of the Dutch mastitis cases from which bacteria can be cultured (Barkema et al., 1998; Sampimon et al., 2008). Determination of mastitis causing pathogens is of great interest, both for choice of treatment as well as for herd level management measures to prevent the spread of mastitis. Currently, determination of mastitis pathogens is generally done by bacteriological culturing (NMC, 2004). Previous research (Hettinga et al., 2008b) showed that results of a method for mastitis pathogen identification based on the analysis of volatile metabolites in milk samples were comparable with bacteriological culturing.

Specific volatile metabolites detected in mastitis milk samples are formed by the pathogen itself. For example, the formation of 2- and 3-methylbutanal, as well as branched free fatty acids by *Staph. aureus* have been described by Ritter and Hanni (1960). In mastitis samples with *Staph. aureus*, other, not previously described metabolites, like ethyl esters of free fatty acids, were also detected. Whether these components are formed by *Staph. aureus* or are the result of other processes occurring during mastitis is not known.

Detected metabolites may, for example, also be formed by enzymes which travel from the blood trough the blood-milk barrier to the milk, or by enzymes associated with endothelial cells of the udder tissue. Additionally, a combination of enzymes from several sources may be involved in the formation of volatile metabolites. For example, somatic cells are known to contain proteases which break proteins down to peptides (Le Rouw et al., 1995). Subsequently, bacterial enzymes may break the peptides down to volatile metabolites like branched aldehydes (Zechman et al., 1986).

To determine whether the volatile metabolites originate from the pathogens or from other sources, inoculation of milk samples from healthy cows can be used. In cows with healthy udders, the somatic cell count (SCC) is low and the blood-milk barrier is intact. This minimizes the presence of enzymes from the blood and from the somatic cells in milk. By inoculating pathogens to these samples, the effect of the pathogen itself can be studied, which gives more insight in the mechanisms of formation of volatile metabolites in mastitis milk.

Additionally, if the pattern of volatile metabolites found in inoculated samples is similar to that of mastitis samples, inoculation can be used as a model for

mastitis samples. This can be a simple and cost-effective way to obtain the large number of samples needed for validation of the previously developed diagnostic method with a wider range of pathogens in a higher number of samples.
The goal of this study was to compare the volatile metabolites from mastitis samples with those from inoculated samples containing the same pathogen to get insight in the mechanism of volatile metabolite formation and to evaluate whether inoculated samples can be used as a model for mastitis samples in studying volatile metabolites.

5.2 Materials and methods

5.2.1 Milk samples

Mastitis samples

The mastitis samples in this study were the same as described in our previous publication (Hettinga et al., 2008b). Fifty milk samples from cows with mastitis were selected from the bacteriological diagnostic lab of GD Animal Health Service (Deventer, The Netherlands). Samples were first screened for the presence of bacteria. If one of the 5 pathogens of interest (*Staph. aureus*, CNS, *Strep. uberis*, *Strep. dysgalactiae*, or *E. coli*) was cultured, the remainder of the milk sample (approx. 5 mL) was frozen at -20°C for later analysis of the volatile metabolites. Also, the pathogen cultured from the mastitis samples was frozen at -80°C for the inoculation procedure.
Bacteriological culturing was carried out according to NMC protocols (NMC, 2004). All plates were incubated at 37°C and examined after 24 and 48 h. Because these were milk samples from routine mastitis diagnosis, the milk samples were incubated overnight at 37°C, to be able to use them again in case of "no growth". Only milk samples from which one bacterial species was cultured (before incubation) were included in the study.

Inoculated samples

Fifteen milk samples from 3 cows (5 samples/cow), without clinical signs of mastitis and with low SCC (<75,000/ml), were supplied by the "De Ossekampen", the university farm of Wageningen University and Research Centre (Wageningen, The Netherlands). After disinfection of the teat and forestriping, 10 mL foremilk samples were taken into sterile 12 mL tubes. Directly after taking these samples, they were transported to the laboratory

and inoculated, in the same tube, with one of the five pathogens cultured from the mastitis samples (3 samples per pathogen). Inoculation was performed using BHI broth (CM0225, Oxoid, Cambridge, UK) containing 10^9 CFU/mL, diluted to a final inoculation level of 10^5 CFU/mL in the milk sample.
The inoculated samples were incubated at 37°C for 8 h. After incubation, the samples were cooled on ice to approx. 0°C. Five mL of the sample was subsequently transferred to a headspace vial. The headspace vial was frozen at -20°C for later analysis of the volatile metabolites.

5.2.2 Analysis of volatile metabolites

After thawing, 5 mL milk samples were preheated for 1 min at 60°C in 20 mL vials sealed with silicon/Teflon septa and magnetic caps. Volatile metabolites were extracted from the headspace for 5 min with a 75 µm PDMS-carboxen SPME fiber (Supelco, Bellefonte, PA, USA) using the combiPAL autosampler (CTC Analytics AG, Zwingen, Switzerland).
The volatile metabolites were thermally desorbed from the fiber by heating it for 5 min at 250ºC in a Best PTV injector with an empty liner. The fiber was subsequently cleaned for 10 min at 290ºC. A vial with 5 mL demineralized water was used as blank.
GC separation of the volatile metabolites was performed on a Finnigan Trace GC gas chromatograph (Thermo-Finnigan, San Jose, CA, USA) coupled to a Finnigan DSQ mass spectrometer (Thermo-Finnigan, San Jose, CA, USA). Volatiles were separated on a BPX-5 column of 30 m length, 0.15 mm i.d., and 0.25 µm film thickness (SGE, Austin, TX, USA). Oven temperature was held at -30°C for 3 min, raised to 230°C at 20°C/min, followed by 1 min holding. Helium was used as the carrier gas at a flow rate of 0.6 mL/min. The MS interface and the ion source were kept at 250°C. Acquisition was performed in electron impact mode (70 eV) with 2 scans/s; the mass range used was m/z 33-250.
The resulting chromatograms were analyzed using the AMDIS software (NIST, Gaithersburg, MD, USA). To improve peak identification, pure mass spectra were obtained by deconvoluting the data. Identification of volatile metabolites was based on matching mass spectra and retention time with pure standards, if obtainable. Otherwise, spectra were compared to the NIST/EPA/NIH mass spectral database, and the kovats index was compared to data from literature (Acree and Arn, 2004). Peak integration was subsequently performed using the XCalibur software package (Thermo-Finnigan, San Jose, CA, USA). Peak area was corrected for the blank sample.

5.2.3 Statistical analysis

SPSS for Windows version 12.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. An ANOVA was performed for comparison of inoculated and mastitis samples, with a Welch test in the case of unequal variances.
The obtained data were used in the prediction module of NeuralTools (Palisade, Ithaca, NY, USA) using the previously developed probabilistic neural network (PNN) to identify the mastitis causing pathogen (Hettinga et al., 2008b).

5.3 Results

5.3.1 Analysis of volatile metabolites

The results of the analysis of the mastitis samples have been described elsewhere (Hettinga et al., 2008b). The results of the inoculated samples were per pathogen compared with the mastitis samples.
Table 5.1 contains an overview of the results of the comparison between the mastitis samples and the inoculated samples.

5.3.2 Comparison of volatile metabolites per pathogen

Staphylococcus aureus
When comparing the data of the inoculated samples with the mastitis samples, especially the group of ethyl esters of the free fatty acids stands out. Four of the ethyl esters were not found at all in the inoculated samples, only ethyl acetate was detected in one of the samples. Another difference between the inoculated and mastitis samples is that the samples inoculated with *Staph. aureus* contained a higher amount of dimethyl sulfide. Finally, hexanoic acid was found in the inoculated samples but not in the mastitis samples.

CNS
Just like *Staph. aureus*, samples inoculated with CNS contained hexanoic acid, whereas the mastitis samples did not. Additionally, the concentration of dimethyl sulfide and 2-butanone were significantly higher for the inoculated samples compared to the mastitis samples. The inoculated samples did not contain isopentanol, acetoin, and ethyl esters of branched fatty acids whereas some of the mastitis samples did contain these components. These differences were, however, not significant.

Table 5.1. Amount (arbitrary units of area) of volatile metabolites detected in mastitis samples and inoculated samples containing *Staph. aureus*, coagulase-negative staphylococci (CNS), *Strep. uberis*, *Strep. dysgalactiae*, and *E. coli* (n=10 for mastitis samples, n=3 for inoculated samples).

	Staph. aureus		CNS		*Strep. uberis*		*Strep. dysgalactiae*		*E. coli*	
Metabolite [1]	Mastitis	Inoculated	Mastitis	Inoculated	Mastitis	Inoculated	Mastitis	Inoculated	Mastitis	Inoculated
Acetaldehyde	7×10^{6}	2×10^{7}	6×10^{4}	1×10^{5}	3×10^{5}	1×10^{5}	1×10^{5}	1×10^{5}	3×10^{5}	2×10^{5}
Ethanol	1×10^{8}	3×10^{7}	1×10^{5}	4×10^{4}	1×10^{6}	0	6×10^{6}	0	1×10^{8}	4×10^{7}
Dimethyl sulfide	9×10^{4}	1×10^{6} [a]	3×10^{4}	6×10^{5} [a]	2×10^{5}	9×10^{5} [a]	2×10^{5}	8×10^{5} [a]	1×10^{5}	2×10^{7} [a]
2,3-Butadione	1×10^{6}	3×10^{6}	5×10^{4}	3×10^{3}	6×10^{5}	1×10^{4}	1×10^{5}	4×10^{3}	2×10^{4}	4×10^{5} [a]
2-Butanone	2×10^{6}	3×10^{6}	3×10^{5}	3×10^{6} [a]	1×10^{6}	3×10^{6}	7×10^{5}	5×10^{6} [a]	5×10^{5}	4×10^{6}
Eth. Acetate	1×10^{6}	4×10^{4} [a]	0	0	5×10^{4}	0 [a]	2×10^{4}	0 [a]	7×10^{5}	4×10^{5}
3-Meth.butanal	3×10^{7}	8×10^{7}	3×10^{6}	2×10^{6}	9×10^{4}	8×10^{5}	5×10^{5}	5×10^{5}	5×10^{4}	3×10^{5}
2-Meth.butanal	4×10^{5}	2×10^{6}	6×10^{4}	6×10^{4}	3×10^{3}	0	3×10^{3}	0	0	0
2-Pentanone	8×10^{4}	1×10^{5}	8×10^{3}	4×10^{4}	5×10^{4}	1×10^{5}	7×10^{4}	7×10^{4}	1×10^{6}	6×10^{4}
Acetic acid	6×10^{6}	4×10^{7}	5×10^{5}	2×10^{6}	3×10^{6}	2×10^{6}	3×10^{6}	3×10^{6}	1×10^{7}	9×10^{6}
Isopentanol	7×10^{6}	2×10^{6}	1×10^{6}	0	0	0	1×10^{5}	0	4×10^{6}	0
Acetoin	8×10^{5}	5×10^{5}	3×10^{3}	0	4×10^{3}	0	0	0	0	0
Eth. Butyrate	7×10^{5}	0 [a]	0	0	7×10^{4}	0 [a]	1×10^{5}	0 [a]	3×10^{5}	2×10^{4}
Butyric acid	3×10^{6}	4×10^{5}	2×10^{5}	4×10^{5}	8×10^{5}	3×10^{5}	1×10^{6}	4×10^{5}	1×10^{6}	2×10^{6}
Eth. 2-meth.but.	2×10^{4}	0 [a]	4×10^{2}	0	0	0	0	0	0	0
Eth. 3-meth.but.	1×10^{5}	0 [a]	5×10^{2}	0	3×10^{3}	0	3×10^{3}	0	0	0
3-Meth.but.	1×10^{6}	4×10^{4}	0	0	0	0	7×10^{2}	0	0	0
2-Meth.but.	6×10^{6}	1×10^{5}	0	0	0	0	0	0	0	0
2-Heptanone	2×10^{5}	4×10^{4}	2×10^{4}	4×10^{4}	1×10^{5}	3×10^{4}	1×10^{5}	4×10^{4}	3×10^{5}	5×10^{4}
Hexanoic acid	0	3×10^{5} [a]	0	2×10^{5} [a]	0	1×10^{5} [a]	0	2×10^{5} [a]	0	3×10^{6} [a]
Eth. Hexanoate	4×10^{5}	0 [a]	8×10^{2}	0	6×10^{4}	0	9×10^{4}	0	2×10^{5}	0 [a]

[a] significant difference between mastitis samples and inoculated sample ($p<0.05$)

[1] Eth. = Ethyl; Meth. = Methyl; But. = Butyrate

Streptococcus uberis
The absence of the ethyl esters and the presence of hexanoic acid in the inoculated samples compared to the mastitis samples was similar to the other pathogens. Additionally, 2-methylbutanal and acetoin were absent in the inoculated samples. Furthermore, the inoculated samples contained a higher concentration of dimethyl sulfide compared to the mastitis samples.

Streptococcus dysgalactiae
In the inoculated samples, the ethyl esters, 2-methylbutanal, isopentanol, and 3-methylbutanoic acid were all absent. Hexanoic acid was present in all inoculated samples, but not in the mastitis samples. Like the other inoculated samples, samples inoculated with *Strep. dysgalactiae* contained a higher concentration of dimethyl sulfide compared to mastitis samples.

E. coli
The samples inoculated with *E. coli*, like the inoculated *Staph. aureus* samples, had very limited formation of ethyl esters of free fatty acids. As for other pathogens, the presence of hexanoic acid in inoculated samples was also seen in the samples inoculated with *E. coli*. In addition to these two differences, a 100-fold increase in dimethyl sulfide was seen for the inoculated samples. This increase was much higher than for the other pathogens.

5.3.3 Prediction by PNN
The results of the inoculated samples were used in the prediction module of the previously developed PNN. Of the 15 inoculated samples, 14 were correctly classified (93%). Only 1 of the *E. coli* samples was incorrectly classified.

5.4 Discussion
Comparing the mastitis samples to the inoculated samples, the majority of the volatile metabolites were present in both types of samples in similar amounts. On average, 80% of the metabolites did not show a significant difference between the two types of samples. The prediction of the PNN showed that the similarity between the inoculated samples and mastitis samples was sufficient for correct prediction of the pathogen in the inoculated sample. The correct classification rate for the inoculated samples (93% correct) is identical to the correct classification rate for the mastitis samples (Hettinga et al., 2008b).

Looking at the 20% of metabolites which were different between the inoculated samples and mastitis samples, some differences are seen for all 5 pathogens. The main difference is that in inoculated samples, ethyl esters of free fatty acids are absent, or significantly lower. This may be explained by the activity of the esterase enzyme. Esterases are enzymes which are produced by the cow and occur in the blood (McSweeney and Sousa, 2000). In case of mastitis, this enzyme can probably transfer from the blood to the milk due to disturbance of the blood-milk barrier (Raulo et al., 2002). These enzymes would then not be present in the milk of healthy cows, thus explaining the absence of the ethyl esters in inoculated samples. Also, Marquardt et al. (1965) showed an association between the severity of mastitis and the level of esterases in the milk. These esterases were associated with the leukocytes in the blood that entered the milk during mastitis.

Hexanoic acid was detected in all inoculated mastitis samples, but not in any of the 50 mastitis samples. However, in most of the mastitis samples, ethyl hexanoate was detected. Hexanoic acid is a precursor for the formation of ethyl hexanoate. Possibly, the hexanoic acid in the mastitis samples was converted to ethyl hexanoate. Due to the absence of esterase, ethyl hexanoate was not formed in the inoculated samples, which may explain the presence of its precursor hexanoic acid.

In the inoculated samples, a higher dimethyl sulfide concentration was found compared to the mastitis samples. The amount of dimethyl sulfide found in all inoculated samples (6×10^5 to 1×10^6), except for the sample inoculated with *E. coli*, was similar to what was found in the milk of healthy cows (Hettinga et al., 2008a). The observed difference between inoculated samples and mastitis samples may be due to different sample handling. The tubes containing the mastitis samples were opened after incubation when still warm to take a sample for microbial plating. The inoculated samples had only been opened when the milk sample was cold. As dimethyl sulfide is a highly volatile metabolite (Patton, 1956), it is easily lost upon opening, especially if the sample temperature is relatively high. This does, however, not explain the high concentration of dimethyl sulfide in the samples inoculated with *E. coli*. Dimethyl sulfide most likely is a metabolite formed by *E. coli* and was also lost in the mastitis samples due to sample handling. The formation of dimethyl sulfide by *E. coli* may be useful for the identification of *E. coli* in mastitis samples.

In some of the samples, especially those inoculated with CNS and *Strep. dysgalactiae*, an increased concentration of 2-butanone was detected. However,

the concentration of 2-butanone still falls within the normal range of this component for fresh raw milk (Hettinga et al., 2008a). Also, when comparing inoculated samples amongst each other within the 5 groups, no significant difference in 2-butanone were detected between the samples.
Most inoculated samples showed the absence of certain volatile metabolites which were detected in mastitis samples. As can be seen in table 5.1, these differences were hardly ever significant. This is because for all of these metabolites, only 1 or 2 mastitis samples did contain the metabolite, whereas it was absent in the 8 or 9 other mastitis samples.

5.5 Conclusions

When comparing the whole pattern of volatile metabolites, it can be concluded that most metabolites were present in both inoculated as well as mastitis samples, in similar concentrations. The prediction of the PNN showed that the similarity between the inoculated samples and mastitis samples was sufficient for correct prediction of the pathogen in inoculated samples (93% correct).
The absence of ethyl esters was the most striking difference between the two types of samples, and this could be explained by the absence of transfer of the esterase enzyme from the cow's blood to the milk of a healthy cow.
One of the goals was to investigate the mechanism of volatile metabolite formation in mastitis samples. It can be concluded that the metabolites found in mastitis samples are mainly formed by the pathogens. Only the ethyl esters of free fatty acids are formed by the esterase enzyme from cow's blood.
Finally, because of the similarities between inoculated and mastitis samples, inoculation can be used to obtain samples for further research on the use of volatile metabolites in mastitis diagnosis.

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6

The influence of incubation on the formation of volatile bacterial metabolites in mastitis milk

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The influence of incubation on the formation of volatile bacterial metabolites in mastitis milk
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Abstract

The possibility to detect mastitis causing pathogens based on their volatile metabolites was previously studied. In that study, the mastitis samples were incubated overnight. To minimize the total analysis time, no incubation, or a short incubation, would be preferred. We therefore investigated the effect of the incubation time on the formation of volatile metabolites in mastitis samples. A selection of 6 volatile metabolites, with the highest impact on the prediction model for identifying the mastitis causing pathogen, was compared at different incubation times between 0 and 24 hours. Identification of the pathogens was not possible without incubation. The minimum incubation time for detection of most of the 6 metabolites was 4 to 8 hours. Although a longer incubation time increased the differences between pathogens, after 8 hours all metabolites could be detected and the pathogens could be differentiated. Eight hours was therefore selected as the optimal incubation time. This optimal incubation time was evaluated with a set of 25 mastitis samples, of which 88% were correctly classified after 8 hours of incubation. The total analysis time for this method is therefore considerably shorter than current microbiological culturing.

6.1 Introduction

The most costly disease of dairy cows is mastitis, which is generally caused by bacteria. Although many bacteria can cause mastitis, a limited group of 5 bacteria (*Staphylococcus aureus*, coagulase-negative staphylococci (CNS), *Streptococcus uberis*, *Streptococcus dysgalactiae*, and *Escherichia coli*) is responsible for approximately 80% of the Dutch mastitis cases from which bacteria can be cultured (Barkema et al., 1998; Sampimon et al., 2008). Determination of mastitis causing pathogens is of great interest, both for choice of treatment as well as for herd level management measures to prevent the spread of mastitis. Although other methods like polymerase chain reaction (PCR) are available, routine laboratories generally use bacteriological culturing for mastitis pathogen differentiation (NMC, 2004).

Methods for identification of bacteria based on volatile bacterial metabolites have been published before. These methods are all based on the fact that bacteria have different metabolic pathways resulting in unique patterns of volatile metabolites (Gardner et al., 1998; Marilley et al., 2004; Turner and Magan, 2004).

Previous research (Hettinga et al., 2008b) showed that this method is also suitable for mastitis pathogen identification. Diagnosis based on the analysis of volatile metabolites in milk samples was comparable with bacteriological culturing. For that study, the samples were incubated overnight at 37ºC. After incubation, the volatile metabolites were identified and quantified using gas chromatography/mass spectrometry (GC/MS). Subsequently, a probabilistic neural network (PNN) was used to identify the mastitis causing pathogens based on the pattern of volatile metabolites.

For most previously published methods, samples were incubated before the volatile metabolites were analyzed. The selected incubation time will influence both the result of the analyses as well as the total analysis time. A relatively long incubation time will increase the amount of volatile metabolites formed by the bacteria, which may increase the differences in patterns of metabolites between bacteria. These increased differences may be beneficial for discrimination between pathogens. On the other hand, the incubation time should be as short as possible to minimize the total analysis time. Furthermore, longer incubation times may cause contaminating bacteria to grow and form additional metabolites that complicate pathogen identification.

The formation, and possible subsequent breakdown of metabolites will be a function of time and will depend on the type of bacteria. Breakdown products of the bacterial metabolism (primary metabolites) can already occur in an early

phase of growth, especially if extracellular enzymes are involved. For example, *Staph. aureus* is known to produce extracellular lipase as soon as it starts growing (Smeltzer et al., 1992). This lipase will hydrolyze triglycerides to free fatty acids. Free fatty acids may thus be detected without incubation or after just a short incubation time.
Secondary metabolites, like branched free fatty acids, were detected after incubation of *Staph. aureus* (Ritter and Hanni, 1960; Zechman et al., 1986). The production of branched aldehydes by *Staphylococcus* species has been shown to occur in the exponential growth phase (de Vos Petersen et al., 2004). Depending on a possible lag time before the pathogen starts to grow, these branched aldehydes may thus already be detected after a short incubation. Branched aldehydes can subsequently be enzymatically converted to branched free fatty acids or branched alcohols (Massona et al., 1999; de Vos Petersen et al., 2004). Thus, longer incubation times were needed before these conversion products of the branched aldehydes could be detected. After very long incubation times, this process will also cause the concentration of branched aldehydes to decrease (de Vos Petersen et al., 2004).
Finally, some components are associated with cell lysis and will therefore only be found after long incubation times when high bacterial numbers are reached. An example of such a component is acetoin, which is produced by *Staph. aureus* (Yang et al., 2006).
Previously, we described volatile metabolites that were important for identification of pathogens (Hettinga et al., 2008b). For application of this method for pathogen identification, the incubation time needs to be long enough so that these specific components can be formed. At the same time, the incubation time should be as short as possible to minimize the total analysis time.
The aim of this study was to evaluate the effect of incubation time on the formation of volatile metabolites in mastitis milk samples, to be able to select the optimal incubation time for mastitis pathogen identification.

6.2 Materials and methods

6.2.1 Mastitis milk samples

Incubation samples

Milk samples were collected from 25 quarters of cows with mastitis on 4 dairy herds in the area of Wageningen, The Netherlands. From each affected quarter,

two 10 mL samples were taken. The first sample was used for bacteriological culturing according to NMC protocols (NMC, 2004). The second samples was stored at -45°C. If one of the 5 pathogens of interest (*Staph. aureus*, CNS, *Strep. uberis*, *Strep. dysgalactiae,* or *E. coli*) was cultured, the second sample was subdivided and used for either selecting the optimal incubation time or for evaluating the selected incubation time. For selecting the optimal incubation time, 2 samples per pathogen were collected. For evaluating the optimal incubation time, 3 additional samples per pathogen were collected.

Reference samples
The mastitis milk samples described in our previous publication (Hettinga et al., 2008b) were used as comparison for the GC/MS results of the samples from the incubation test. These 50 mastitis samples, 10 of each pathogen of interest, were selected from the bacteriological diagnostic lab of GD Animal Health Service (Deventer, The Netherlands). Samples were first screened for the presence of bacteria. If one of the 5 pathogens of interest was cultured, the remainder of the milk sample (approx. 5 mL) was frozen at -20°C for later analysis of the volatile metabolites. These samples were treated the same as the samples described above, apart from the fact that these samples were incubated overnight at 37°C. Only milk samples from which one bacterial species was cultured were included and were used here as reference samples.

6.2.2 Incubation test
For the 5 pathogens of interest, 2 samples per pathogen were divided in nine 1 mL samples. These samples were put in either 20 mL sterilized headspace vials sealed with silicon/Teflon septa and magnetic caps (for analysis of volatile metabolites) or in 10 mL sterile tubes (for microbiological counting). For analysis of volatile metabolites, 6 samples were incubated at 37°C for 0, 2, 4, 8, 14, and 24 hours. After incubation, these samples were frozen at -20°C for later analysis of the volatile metabolites. For microbiological counting, 3 samples were incubated at 37ºC for 0, 8, and 24 hours. Microbiological counting was done immediately after incubation, using plate counting. For the number of *Staph. aureus* and CNS in the milk samples, mannitol salt agar (CM0085, Oxoid, Cambridge, UK) was used. For the number of *Strep. uberis* and *Strep. dysgalactiae* in the milk samples, BHI agar (CM0375, Oxoid, Cambridge, UK) was used. For the number of *E. coli* in the milk samples, selective *E. coli* medium (CM1046, Oxoid, Cambridge, UK) was used.

6.2.3 Evaluation test

After selecting the optimal incubation time based on the incubation test, 3 additional samples per pathogen were incubated at 37°C for evaluation of this selected incubation time. After incubation, these samples were frozen at -20°C for later analysis of the volatile metabolites.

6.2.4 Analysis of volatile metabolites

After thawing, the headspace vials were preheated for 1 min at 60°C. Volatile metabolites were extracted from the headspace for 5 min with a 75 µm PDMS-carboxen SPME fiber (Supelco, Bellefonte, PA, USA) using the combiPAL autosampler (CTC Analytics AG, Zwingen, Switzerland). The volatile metabolites were thermally desorbed from the fiber by heating it for 5 min at 250°C in a Best PTV injector with an empty liner. The fiber was subsequently cleaned for 10 min at 290°C. A vial with 5 mL demineralized water was used as blank.

GC separation of the volatile metabolites was performed on a Finnigan Trace GC gas chromatograph (Thermo-Finnigan, San Jose, CA, USA) coupled to a Finnigan DSQ mass spectrometer (Thermo-Finnigan, San Jose, CA, USA). Volatiles were separated on a BPX-5 column of 30 m length, 0.15 mm i.d., and 0.25 µm film thickness (SGE, Austin, TX, USA). Oven temperature was held at -30°C for 3 min, raised to 230°C at 20°C/min, followed by 1 min holding. Helium was used as the carrier gas at a flow rate of 0.6 mL/min. The MS interface and the ion source were kept at 250°C. Acquisition was performed in electron impact mode (70 eV) with 2 scans/s; mass range used was m/z 33-250.

The resulting chromatograms were analyzed using the AMDIS software (NIST, Gaithersburg, MD, USA). To improve peak identification, pure mass spectra were obtained by deconvoluting the data. Identification of volatile metabolites was based on matching mass spectra and retention time with pure standards, if obtainable. Otherwise, spectra were compared to the NIST/EPA/NIH mass spectral database, and the kovats index was compared to data from literature (Acree and Arn, 2004). Peak integration was subsequently performed using the XCalibur software package (Thermo-Finnigan, San Jose, CA, USA). Peak area was corrected for the blank sample.

6.2.5 Probabilistic neural networks

NeuralTools (Palisade, Ithaca, NY, USA) was used to predict the pathogen in the samples based on the previously developed PNN (Hettinga et al., 2008b).

After the incubation test, data from the 10 samples were used for predicting the mastitis causing pathogen. After the evaluation test, 5 samples per pathogen were used: 2 samples from the incubation test which had been incubated for the selected optimal incubation time and 3 samples from the evaluation test.

6.3 Results

6.3.1 Microbiological counting

The number of pathogens in the non-incubated mastitis samples were all below the detection limit (<5×10^3 CFU/mL). After 8 hours, all samples contained approx. 10^7 CFU/mL.
After 24 hours, the number of pathogens had risen to around 10^8 CFU/mL. There were no significant differences in the number of bacteria between the pathogens after 8 and 24 hours of incubation.

6.3.2 Analysis of volatile metabolites

Because of limited available sample volume, smaller samples were incubated and analyzed for their volatile metabolites compared to our previous publications (1 mL instead of 5 mL). Although the sample volume does have some effect on the signal intensity (Abalos et al., 2000), the effect should be relatively small compared to the differences in volatile metabolites between the pathogens. We confirmed this with a separate set of samples (data not shown).
Corresponding with earlier observations, only a limited number of components were found in non-incubated mastitis samples (Hettinga et al., 2008a). No differences were observed between samples containing different pathogens.
After overnight incubation, 19 volatile metabolites were found in the reference samples as reported earlier (Hettinga et al., 2008b). Not all of these 19 metabolites were equally important for the identification of the mastitis causing pathogens. The NeuralTools software used for developing the PNN calculated a variable impact analysis, which showed the relative impact of each variable on the model outcome. Based on this impact analysis, a combination of 6 volatile metabolites, ethanol, 2-methylbutanal, acetoin, butyric acid, 3-methylbutyrate, and ethyl hexanoate, was shown to explain approximately 98% on the model outcome. Thus, for comparing the different incubation times in the incubation test, we focused on these 6 metabolites.

Table 6.1 shows the abundance of the 6 selected volatile metabolites. Only ethanol and butyric acid were found in some of the non-incubated samples.

Table 6.1. Amount (arbitrary unit of area) of volatile metabolites found after different incubation times. Tables a-e correspond to the data for the respective pathogens: a) *Staph. aureus*, b) coagulase-negative staphylococci, c) *Strep. uberis*, d) *Strep. dysgalactiae*, and e) *E. coli*; N.D. = not detected.

a. *Staph. aureus*

Incubation time	ethanol	2-methyl-butanal	acetoin	butyric acid	3-methyl butyrate	ethyl hexanoate
0h	N.D.	N.D.	N.D.	9.0×10^{3}	N.D.	N.D.
2h	N.D.	N.D.	N.D.	8.4×10^{5}	N.D.	N.D.
4h	N.D.	3.2×10^{3}	N.D.	2.7×10^{6}	N.D.	N.D.
8h	2.4×10^{5}	4.7×10^{5}	4.2×10^{4}	2.9×10^{7}	2.1×10^{5}	N.D.
14h	1.4×10^{8}	2.8×10^{6}	1.1×10^{6}	8.5×10^{6}	2.2×10^{6}	N.D.
24h	4.8×10^{8}	4.9×10^{6}	2.1×10^{7}	2.1×10^{7}	2.0×10^{7}	2.9×10^{4}
reference	1.1×10^{8}	3.7×10^{5}	8.2×10^{5}	2.9×10^{6}	1.4×10^{5}	3.7×10^{5}

b. Coagulase-negative staphylococci

Incubation time	ethanol	2-methyl-butanal	acetoin	butyric acid	3-methyl butyrate	ethyl hexanoate
0h	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
2h	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
4h	N.D.	2.7×10^{3}	N.D.	8.7×10^{4}	N.D.	N.D.
8h	5.4×10^{5}	1.5×10^{4}	N.D.	1.8×10^{5}	N.D.	1.1×10^{3}
14h	1.3×10^{6}	1.6×10^{4}	N.D.	1.4×10^{5}	N.D.	2.7×10^{2}
24h	1.5×10^{6}	1.8×10^{5}	5.2×10^{4}	1.1×10^{6}	N.D.	2.2×10^{3}
reference	1.6×10^{5}	5.9×10^{4}	3.2×10^{3}	2.0×10^{5}	N.D.	7.7×10^{2}

c. *Strep. uberis*

Incubation time	ethanol	2-methyl-butanal	acetoin	butyric acid	3-methyl butyrate	ethyl hexanoate
0h	1.6×10^{5}	N.D.	N.D.	N.D.	N.D.	N.D.
2h	1.3×10^{6}	N.D.	N.D.	7.1×10^{4}	N.D.	N.D.
4h	7.3×10^{5}	N.D.	N.D.	6.3×10^{5}	N.D.	N.D.
8h	1.1×10^{6}	N.D.	N.D.	3.1×10^{6}	N.D.	N.D.
14h	5.4×10^{6}	7.7×10^{4}	N.D.	1.4×10^{6}	N.D.	N.D.
24h	7.7×10^{6}	1.9×10^{5}	N.D.	6.5×10^{6}	N.D.	N.D.
reference	1.6×10^{6}	2.7×10^{3}	3.6×10^{3}	7.8×10^{5}	N.D.	6.4×10^{4}

d. *Strep. dysgalactiae*

Incubation time	ethanol	2-methyl-butanal	acetoin	butyric acid	3-methyl butyrate	ethyl hexanoate
0h	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
2h	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
4h	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
8h	1.5×10^{5}	N.D.	N.D.	1.4×10^{5}	N.D.	6.0×10^{3}
14h	5.5×10^{8}	N.D.	N.D.	1.5×10^{6}	N.D.	1.9×10^{4}
24h	5.2×10^{7}	N.D.	1.4×10^{6}	4.0×10^{6}	N.D.	6.6×10^{4}
reference	5.8×10^{6}	2.7×10^{3}	N.D.	1.0×10^{6}	7.2×10^{2}	9.2×10^{4}

e. *E. coli*

Incubation time	ethanol	2-methyl-butanal	acetoin	butyric acid	3-methyl butyrate	ethyl hexanoate
0h	2.9×10^{6}	N.D.	N.D.	3.6×10^{4}	N.D.	N.D.
2h	1.2×10^{6}	N.D.	N.D.	3.1×10^{5}	N.D.	N.D.
4h	3.6×10^{6}	N.D.	N.D.	5.3×10^{6}	N.D.	N.D.
8h	2.0×10^{6}	N.D.	N.D.	6.7×10^{6}	N.D.	1.1×10^{3}
14h	8.3×10^{8}	N.D.	N.D.	9.3×10^{6}	N.D.	1.3×10^{4}
24h	2.4×10^{9}	N.D.	2.8×10^{5}	2.0×10^{7}	N.D.	1.3×10^{5}
reference	2.9×10^{8}	N.D.	N.D.	3.6×10^{4}	N.D.	N.D.

During incubation, the concentration of most components increased considerably. After 8 hours, most components were detectable. After 24 hours, the differences between the milk samples were more obvious, and a quick visual examination of the chromatogram was already enough to identify the mastitis causing pathogens.

Ethanol was formed by all pathogens, although the minimum incubation time before detection was either 0 or 8 hours.

In samples with *Staph. aureus* and CNS, 2-methylbutanal was detected after 4 hours. For the reference samples, this component was one of the main differences between the *staphylococci* and other pathogens. In the other incubated samples, it was not formed at all by *E. coli* and *Strep. dysgalactiae* and only after 14 hours of incubation by *Strep. uberis*.

Acetoin and 3-methylbutyrate are components specifically formed by *Staph. aureus* in the reference samples. In the current study, 3-methylbutyrate was found to be only produced by *Staph. aureus* where it was detected after 8 hours of incubation. Acetoin, on the other hand, was found to be produced by most pathogens, although in samples with *Staph. aureus* it could already be detected

after 8 hours of incubation whereas it could only be detected in the other samples after an incubation time of 24 hours.
Butyric acid could be detected in non-incubated samples with *Staph. aureus* and *E. coli*, although only at a relatively low level. After 4 to 8 hours of incubation, butyric acid was detected in all samples in relatively high amounts. Ethyl hexanoate was formed in considerable amounts only by *Strep. dysgalactiae* and *E. coli*. The amount of ethyl hexanoate formed in the incubated samples was in general lower than in the reference samples.

Table 6.2. Correct classification rate of the prediction by the previously developed probabilistic neural network (Hettinga et al., 2008b) for the mastitis causing pathogen in mastitis samples incubated for 0, 2, 4, 8, 14, and 24 hours.

Incubation time (hours)	Correct classification rate (%)
0	40
2	40
4	50
8	90
14	90
24	60

Table 6.3. Prediction of the mastitis causing pathogen in the sample incubated for 8 hours by the previously developed probabilistic neural network (Hettinga et al., 2008b).

	Predicted by PNN [1]				
Microbiological identification	*Staph. aureus*	CNS [2]	*Streptococcus*	*E. coli*	Correct (%)
Staph. aureus	5	0	0	0	100
CNS	0	4	1	0	80
Streptococcus	0	0	9	1	90
E. coli	0	0	1	4	80
Total correct					88

[1] The number of samples classified in the respective group by the probabilistic neural network (PNN)
[2] CNS = coagulase-negative staphylococci

6.3.3 Probabilistic neural network

The correct classification rate of the PNN after the different incubation times is shown in table 6.2. Samples incubated for 8 and 14h had the highest correct classification rate (90%).
The prediction of the PNN for the 25 samples incubated for 8 hours can be found in table 6.3, showing a correct classification rate of 88%.

6.4 Discussion

6.4.1 Microbiological counting

After 8 hours of incubation time, the number of bacteria was around 10^7 CFU/mL for all pathogens. Table 6.1 shows that most volatile metabolites could be detected after this incubation time. The formation of the whole range of metabolites at a bacterial level around 10^7 CFU/mL is similar to what we showed in a previous study on *Pseudomonas* (Hettinga et al., 2008a). Other authors have also reported that the production of volatile metabolites starts to be noticeable at bacterial levels around 10^7 CFU/mL (Haugen et al., 2006; Ragaert et al., 2006).

6.4.2 Incubation test

Table 6.1 shows that incubation is necessary for pathogen identification. This can be explained by the relatively low number of bacteria found in non-incubated samples.
Compared to the other metabolites, acetoin and 3-methylbutyrate needed a longer incubation time before they could be detected. For acetoin, this was expected, as it is associated with cell lysis (Yang et al., 2006). For 3-methylbutyrate this can be explained by the fact that it is a conversion product of 3-methylbutanal. First, 3-methylbutanal needs to be formed, before it can be converted to 3-methylbutyrate. The later formation during incubation of 3-methylbutyrate compared to its precursor 3-methylbutanal has also been shown by de Vos Petersen et al. (2004). Because these two metabolites are important for the PNN, mainly for identification of *Staph. aureus*, a relatively longer incubation time may thus be required.
Although butyric acid could be detected in samples with *Staph. aureus* and *E. coli* without incubation, the level of butyric acid in these samples was low, and a similar amount of butyric acid has previously been found in samples of milk

from healthy cows (Hettinga et al., 2008a). The amount of butyric acid in these non-incubated samples is therefore too low for mastitis diagnosis.
For identification of mastitis causing pathogen using GC/MS, differences in metabolites must be detectable. When looking at the formation of the 6 metabolites, 4 to 8 hours of incubation time was the minimum for detection of most of them. A longer incubation time had the advantage that differences between pathogens were larger. For example, after 24 hours it was easy to visually inspect the chromatogram and see directly which pathogen was involved, whereas the differences between the pathogens were smaller after 8 hours of incubation.
The PNN was used to help select the optimal incubation time. After 0, 2, and 4 hours of incubation, a maximum of 50% of the samples could be correctly predicted. As already expected based on the amount of volatile metabolites found, up to 4 hours of incubation is too short for pathogen identification. After 8 hours of incubation, 90% of the samples was correctly classified. This correct classification rate did not increase after incubating for 14 hours. After 24 hours the correct classification rate decreased to 60%. This is probably caused by the neural network being developed for samples which had been incubated overnight (Hettinga et al., 2008b). The pattern of volatile metabolites found after 24 hours of incubation was considerably different from the reference samples (see table 6.1). If a PNN would be developed for samples incubated for 24 hours, the correct classification rate is expected to be similar to that for the samples incubated for 8 or 14 hours. Developing a PNN for samples incubated for 4 hours would probably not increase its correct classification rate, as the pathogens show almost no difference in the pattern of volatile metabolites after this incubation time.
Because the correct classification rate after 8 hours is already 90% and total analysis time should be as short as possible, 8 hours was selected as optimal incubation time.

6.4.3 Evaluation test

The correct classification rate of 88% as found for the 25 samples used in the evaluation test is similar to the 93% found for the 50 reference samples (Hettinga et al., 2008b). This confirms that 8 hours of incubation is sufficient for mastitis pathogen identification.

6.5 Conclusions

Incubation was shown to be a necessary step to identify mastitis causing pathogens by detection of their volatile metabolites. After 8 hours of incubation, all important metabolites had been formed. The selected optimal incubation time for this method was thus 8 hours. This was evaluated with a set of 25 mastitis samples, of which 88% were correctly classified after 8 hours of incubation.

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7

Discussion

7.1 Introduction

The objective of the work described in this thesis was twofold. The first aim was to test the suitability of the headspace analysis for quality control of raw milk. The second aim was to further develop the headspace analysis for the identification of mastitis causing pathogens in raw milk.
Before discussing the results of the different experiments described in this thesis, a reflection on the analytical method developed will be given. Then, the suitability of headspace analysis for quality control of raw milk will be discussed. This is followed by a discussion on the results of the experiments related to mastitis milk. Finally, other potential applications of the developed analytical method will be discussed.

7.2 Analytical method

The results of the research described in this thesis was all based on an analytical methodology, which is schematically shown in figure 7.1. Background information on the different steps of this method can be found in chapter 2. These steps have been extensively described in literature, as summarized in the references of chapter 2. The advantage of the developed method is the specific combination of several analytical tools in combination with an effective method for data analysis.

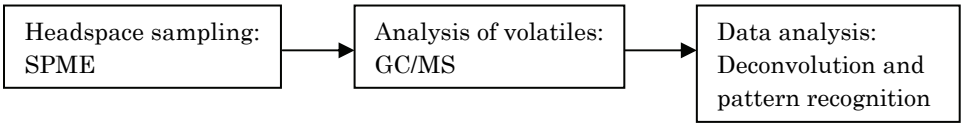

Figure 7.1. Schematic overview of the different steps of the analytical method. SPME: solid-phase microextraction; GC/MS: gas chromatography/mass spectrometry.

Solid-phase microextraction (SPME) for headspace sampling of dairy products combined with gas chromatography/mass spectrometry (GC/MS) for subsequent identification and quantification of the extracted volatiles has been used and discussed extensively by a wide range of authors (Pan et al., 1995; Elmore et al., 1997; Grote and Pawliszyn, 1997; Mariaca and Bosset, 1997; Marsili, 1999a, 1999b, 2000; Roehrig and Meisch, 2000; Peres et al., 2001; Contarini and Povolo, 2002; Fabre et al., 2002; Pinho et al., 2002; Gonzalez-Cordova and Vallejo-Cordoba, 2003; Povolo and Contarini, 2003). In these references, the background of the methodology is sufficiently described and will therefore not be repeated here. However, the methods for data analysis are less frequently

described. This paragraph will therefore focus on the application of the different methods for data analysis. Two methods for data analysis were used: deconvolution for identification and multivariate statistics for pattern recognition and classification.

7.2.1 Deconvolution

Deconvolution using the AMDIS software was performed on all chromatograms (see chapter 2.4.1 for an explanation of deconvolution). Deconvolution was needed for two reasons.

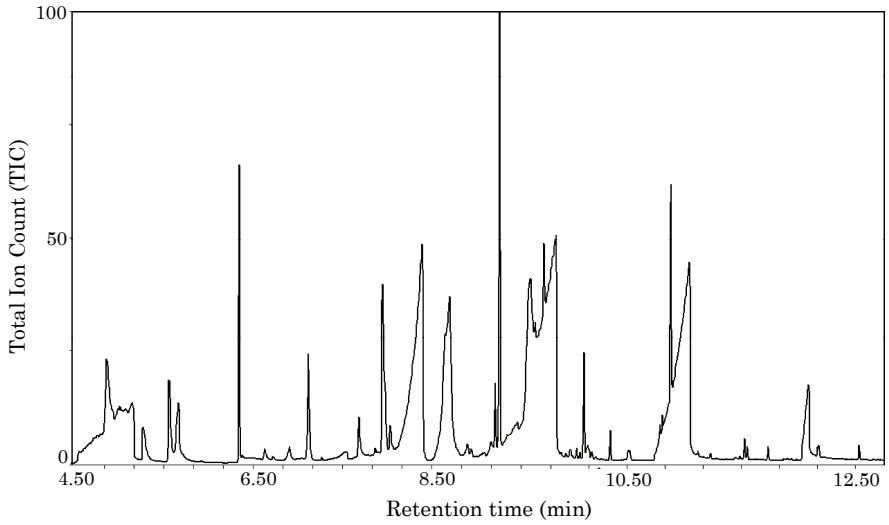

Figure 7.2. Example of a chromatogram showing many coeluting components resulting in overlapping peaks.

First, the mass spectra of coeluting peaks from the GC could be separated using deconvolution. Separate mass spectra were thus obtained for the individual components. Such a separation could not be obtained by manual inspection of the chromatogram, because identification of overlapping peaks due to coelution was very difficult, especially when one of the peaks was much more abundant. An example of a chromatogram with multiple coeluting components is shown in figure 7.2. The mass spectra of the single components in the overlapping peaks could easily be identified using AMDIS. Subsequent analysis of pure standards confirmed the identity of the peaks.

Second, some components were present in only minute quantities, making them invisible in the total ion count (TIC) chromatogram. AMDIS was able to detect multiple low abundance components in the milk samples. The peaks associated with these components could not be found using visual examination of the TIC chromatogram. Again, subsequent analysis of pure standards did confirm the identity of the components. The results presented in this thesis showed the suitability of deconvolution for enhanced GC/MS peak identification.

7.2.2 Pattern recognition

After identification of the volatile metabolites by AMDIS, all peaks were subsequently quantified, followed by univariate statistics. Differences between samples with and without quality defects could be determined using univariate statistics. Using univariate statistics, also differences among pathogens in mastitis samples could be observed. However, these differences were only useful for comparing groups of samples, because individual samples varied considerably in composition of volatile metabolites (both in presence and amount). To be able to identify the mastitis causing pathogen in individual samples, pattern recognition techniques were used. These pattern recognition techniques have also the advantage of automated result interpretation.

Different methods for pattern recognition were tested. Linear Discriminant Analysis (LDA), Quadratic Discriminant Analysis (QDA), and Soft Independent Modelling of Class Analogy (SIMCA) were judged on their ability to identify the mastitis causing pathogen. All had a correct classification rate (based on cross-validation) of no more than 60 to 70%.

Because of the non-linearity of the data, artificial neural networks (ANN) were then tested as a pattern recognition and classification tool. The most widely applied ANN is the multi-layer feedforward (MLF) network. With a MLF network, the correct classification rate was approximately 90%. Another type of ANN, probabilistic neural network (PNN), was also tested for its ability in mastitis pathogen identification. PNN showed a similar correct classification rate of approximately 90%. Probabilistic neural networks have the advantage of being specifically developed for classification purposes. Also, PNN results in a probability distribution over the different classification groups (Specht, 1990). Because of these advantages of PNN, it was chosen as the best method for pattern recognition of volatile metabolites in mastitis milk samples.

7.3 Quality control of raw milk by headspace analysis

Headspace analysis for quality control purposes has been used for a wide variety of food products, including dairy products (Vallejo-Cordoba and Nakai, 1994; Marsili, 1999a, 2000; Peres et al., 2001; Fenaille et al., 2003; Gonzalez-Rodriguez et al., 2005). Based on the results described in literature, it was expected that quality control of raw milk could also benefit from the application of headspace analysis. The first objective of the work described in this thesis was therefore to investigate the ability of the headspace analysis to detect several quality defects in raw milk simultaneously.

Most of the published methods for quality control of dairy products based on headspace analysis report the presence of a wide variety of volatile components. The amount as well as the presence or absence of specific volatile components have been used for detecting quality defects.

In fresh raw milk without quality defects, only 7 volatile components were detected with the developed headspace method, as shown in table 3.1. Results in literature often report a higher number of volatile components which may be due to extra processing steps as is described in chapter 3. A small number of peaks in fresh raw milk may simplify the identification of quality defects. The effect of many quality related parameters on volatile components was studied (chapter 3).

The data presented show that different quality defects can be detected by sensory analysis and by headspace analysis. For example, the presence of high amounts of acetone in milk from ketotic cows or terpenes associated with the feeding of orange peel could be easily detected with the headspace analysis, although there was no difference observed in the flavour or appearance of the milk. On the other hand, oxidation experiments performed by placing milk under sunlight-simulating lamps did produce milk samples with a strong off-flavour, but the headspace analysis did not detect any differences (data not shown). Also, the feeding of onions gave a distinct off-flavour to the milk with only minimal changes in the chromatogram recorded from this milk.

This difference between the headspace analysis and the human nose has been published before (Curioni and Bosset, 2002; D'Acampora-Zellner et al., 2008). In these studies, the gas flow from the GC was split over two outlet ports: a "normal" detector resulting in a chromatogram and a human volunteer smelling the gas flow resulting in a so called olfactogram. When comparing the chromatogram with the olfactogram, quite remarkable differences were described. Some components in the chromatogram could not be seen in the olfactogram. Other components could hardly, or not at all, be seen in the

chromatogram, but gave a strong signal in the olfactogram. These differences between the human nose and an GC detector were similar to the results discussed above. The main reason for the differences found is that the human nose has a widely varying sensitivity for different volatile components, ranging from for example 10 g/L for ethanol to 10^{-11} g/L for p-menth-1-en-8-thiol (Hinterholzer and Schieberle, 1998). At the same time, the detection limit for headspace analysis based on mass spectrometry was in the range of 10^{-4} to 10^{-6} g/L for most volatile substances.

The results described in chapter 3 show that the developed analytical methodology could only detect *Pseudomonas* if it was present in higher numbers (>10^7 CFU/mL) than usually seen in practice. To be able to detect *Pseudomonas* in raw milk, incubation and possibly ethanol addition would be necessary. For routine screening of farm tank samples, this would take too much time. In addition, *Pseudomonas* species are always present in raw milk, making a screening procedure more complicated, as it will have to differentiate between normal and high levels of *Pseudomonas*. Analyzing volatile metabolites produced by *Pseudomonas* as marker for microbiological contamination and growth in raw milk is thus not feasible.

The developed analytical methodology can, however, detect several other quality defects simultaneously. It was shown that chloroform, free fatty acids, and feeding of specific vegetable by-products could be detected. Chloroform and free fatty acids are currently already routinely analysed in The Netherlands using two separate methods. With headspace analysis, these parameters can be quantified simultaneously. The described headspace method therefore is a valuable supplementary method for raw milk quality control.

7.4 Analysis of mastitis milk samples

With the detection techniques described in chapter 3, ketosis (a metabolic disease of dairy cows), as well as the metabolites formed at high bacterial loads gave useful results. Combining the themes of animal health and bacterial growth, mastitis was chosen as subject for further study. This resulted in a method for mastitis pathogen identification (chapter 4). The added value of the developed method and the research questions to be answered before using the method for routine mastitis screening will be discussed in section 7.4.1. The headspace method developed will be compared to other recently developed methods for mastitis detection and mastitis pathogen identification in section 7.4.2.

The mastitis pathogen identification method based on a GC/MS is primarily a method for use in a laboratory. Other applications based on the experimental results described in this thesis, which do not necessarily need to be based on GC/MS, may also be developed. Section 7.4.3 will give an outlook on some possibilities in this area.

7.4.1 GC/MS-based detection methods

Based on the results shown in chapters 4 though 6, the method developed for mastitis pathogen identification has great potential for use in laboratories as a routine screening tool. Screening of volatile metabolites has several advantages compared to standard bacteriological analysis.

The faster analysis gives the farmer a quick indication of the (group of) pathogens causing mastitis which can be used for decisions on the treatment. Sometimes the results are conclusive at this stage, either the specific pathogen or the absence of pathogens is detected. If, however, the headspace analysis is inconclusive, subsequent microbial culturing may be necessary. Based on the headspace results, however, standard microbial culturing can be refined. If for example the presence of a group of pathogens is detected based on the headspace results (e.g. streptococci), specific microbial plates can be selected, limiting the number of microbial plates that need to be used.

At this point in time, it is not expected that the method will completely replace bacteriological culturing. For example, the inability to distinguish the two streptococci species is a limitation. Also, screening of pathogens for antibiotic resistance will probably still rely on bacteriological culturing methods. However, the described headspace method has great potential as a screening tool for mastitis, especially because of its cost and speed.

Before the method can be used as a routine screening tool, some further research questions need to be answered. Based on the results in chapter 5, inoculation can be used as an efficient and cost-effective way to obtain the samples needed for answering an important part of these questions.

Firstly, the required sensitivity of the method needs to be established. This can then be used to minimize the necessary incubation time.

Secondly, the number of samples used for building the PNN should increase. The differences among several strains of the same pathogen, as well as the difference between a wider range of pathogens need to be studied.

Thirdly, the presence of non-pathogenic bacteria can cause specific patterns of volatile metabolites, both in combination with pathogens as well as alone. These results should be included in the PNN model.

Finally, it is of interest to study the effect of the absence of (pathogenic) bacteria, because in over 30% of clinical mastitis cases, no pathogen is detected using bacteriological culturing (Bradley et al., 2007).
To investigate the suitability of the headspace method for a wider range of pathogens and non-pathogenic bacteria it is useful to analyze the volatiles in a large number of samples in parallel with bacteriological culturing.

7.4.2 Mastitis diagnostics

In this thesis, headspace analysis for mastitis detection and mastitis pathogen identification was described. However, a range of other methods exists for mastitis diagnosis.

Mastitis detection

Currently, analytical detection of mastitis is mainly based on the somatic cell count (SCC). In addition to the routine quantification of somatic cells by a laboratory, systems determining the SCC integrated in automatic milking systems exist (Kamphuis et al., 2008). Another approach to mastitis detection integrated in (automatic) milking machines is the use of one or more sensors, e.g. for lactate dehydrogenase (LDH) or *N*-acetyl-ß-*D*-glucosaminidase (NAGase) concentration, electrical conductivity, or temperature of the milk (Norberg et al. 2004; Chagunda et al., 2006a).
When the output of multiple sensors is combined, using multivariate statistics, prediction of mastitis was found to be more reliable than when a single parameter was used (Chagunda et al., 2006b; Kamphuis et al., 2008). All these methods aim at detection of mastitis and do not aim at identifying the individual mastitis causing pathogen.

Mastitis pathogen identification

For identification of mastitis causing pathogens, bacteriological culturing is currently the standard method (NMC, 2004). This method, however, has the disadvantage of being time-consuming. To gain time, on-farm culturing systems have been launched (Silva et al., 2005). Using on-farm culturing, just a few (groups of) pathogens can be differentiated, but this can be sufficient for treatment decisions (Leslie et al., 2005).
Another method that has been developed for mastitis pathogen identification is based on polymerase chain reaction (PCR) of cultured bacteria. This method is mainly used to compare strains of bacteria in order to study their epidemiology (Zadoks and Schukken, 2006). Because milk is a complex medium, PCR

performed directly on milk instead of cultured bacteria is difficult, labour intensive, and thus expensive (Yamagishi et al., 2007). However, recent developments in real-time PCR seem to overcome many of the practical problems (Kosinen et al., 2008). PCR-based identification, however, is still relatively expensive.
A different approach was described by de Haas et al. (2004). In their study, logistic regression was used to differentiate SCC patterns during lactation to predict different types of mastitis, related to different pathogens.
Although multiple techniques for mastitis pathogen identification exist, the developed headspace analysis has two advantages: short analysis time and low analysis cost.
The reduction in analytical time will depend on the minimal incubation time. Based on the results described in chapter 6, an incubation time of 8 hours was selected. However, no incubation times between 4 and 8 hours were tested. Four hours of incubation was insufficient for pathogen identification, because many volatile metabolites had not yet been formed. If the total analysis time needs to be as short as possible, incubation times between 4 and 8 hours need to be further studied for suitability.
Finally the costs of bacteriological culturing plays an important role. When headspace analysis is performed on a large scale, the cost of an analysis may be up to 5 times less compared to classical microbiological culturing.

7.4.3 Non-GC/MS-based detection methods

In this thesis, the detection of volatile metabolites was done with GC/MS. GC/MS-based methods are normally only used in laboratories. However, other techniques like electronic noses may also be able to detect the volatile metabolites produced by mastitis pathogens. An electronic nose may be useful for identification of the mastitis pathogen in veterinary practices or even on-farm.
Electronic noses can be used for detection of volatile metabolites (chapter 2). Electronic noses are cheaper and easier to use compared to GC/MS. Whether these techniques can be used or not needs to be established by studying signal patterns of mastitis samples as found by electronic noses. This pattern should be different between pathogens to be able to use an electronic nose for identification of mastitis pathogens. The use of electronic noses for pathogen identification in general has been described already (Turner and Magan, 2004). This may thus be a promising technique for mastitis pathogens as well.

The incubation of mastitis samples prior to headspace analysis, as has been discussed in chapter 6, is necessary to have enough volatile metabolites for headspace analysis. However, other analytical techniques performed on the milk itself instead of on the headspace, aiming at specific metabolites, may be more sensitive for these metabolites. In that way, a shorter incubation time, or no incubation time at all, may be feasible. If incubation is not necessary, sensors which are able to detect metabolites which are specific for (groups of) pathogens may be used. These sensors may then even be incorporated in a milking machine. Such systems have already been developed for various metabolites in milk, e.g. LDH (Blom and Nielsen, 2008). These methods are only feasible if a limited set of metabolites can be used as markers for (a group of) pathogens. An example of a metabolite which may be used as a marker for a group of pathogens is 2-methylbutanal. Based on the results of chapters 4 and 5, this component is a marker for mastitis caused by staphylococci. A sensor for 2-methylbutanal may thus be developed for identification of this group of mastitis causing pathogens.

7.5 Other applications for the mastitis diagnosis method

The method described in chapters 4 to 6 and discussed in section 7.4 is focused on the identification of mastitis causing pathogens. In this paragraph the suitability of several alternative approaches of mastitis detection and method application will be discussed.

7.5.1 On-farm mastitis detection

In addition to a method for identification of the individual mastitis causing pathogens, a fast and reliable method to detect mastitis could be valuable. With the increasing use of automatic milking systems, the detection of (sub)clinical mastitis should best be performed by the milking machine itself. If just one of the metabolites found could be a marker for mastitis, a rapid test on this single metabolite may be developed.

When looking at the metabolites detected in this study, most were not present in the milk during milking; incubation was necessary for the formation of the metabolites (chapter 6). Only 2-butanone, 2-pentanone, and acetic acid were detected without incubation. 2-Butanone was one of the 7 components found in every fresh raw milk sample (see chapter 3). 2-Pentanone and acetic acid are metabolites which are not unique for mastitis milk samples, they are formed in a variety of bacterial and chemical processes.

Based on these data, volatile metabolites cannot be used for screening of milk for the presence of mastitis during milking.

7.5.2 Detection of the severity of mastitis

In chapter 5, the absence of ethyl esters of free fatty acids in inoculated samples was shown. As discussed in that chapter, the presence of ethyl esters in mastitis samples is probably caused by the esterase enzyme, which transferred from the blood to the milk due to disturbance of the blood-milk barrier (Raulo et al., 2002). Also, Marquardt et al. (1965) showed an association between the severity of mastitis and the level of esterases in the milk. These esterases were associated with the leukocytes in the blood that entered the milk during mastitis. Determining the amount of ethyl esters may thus be useful for screening disturbance of the blood-milk barrier, as indicator for damage to the udder during mastitis. To confirm this, further study will be necessary.

7.5.3 Identification of other bacteria

The developed method for mastitis pathogen identification may also be useful for bacteria other than those associated with mastitis. The formation of a unique set of volatile metabolites by several bacteria has been reported before (Gardner et al., 1998; Marilley et al., 2004). This, however, did not lead to applications on bacterial detection methods directly in food products, which may be caused by the complex patterns of metabolites to be detected. The advantage of the analytical method described in this thesis, is the application of advanced data analysis, as has been discussed in chapter 2 and section 7.1. The method that was developed, may therefore also be applicable to other microorganisms.

In raw milk quality control the detection of *Clostridium tyrobutyricum* by microbial culturing is time consuming. It takes at least 4 days to grow for these bacteria, and visible gas formation is necessary for identification. Additionally, this method is not very specific, because other gas-producing spore-forming bacteria may cause false-positive results.

Besides *C. tyrobutyricum*, identification of pathogens like *Salmonella* and *Listeria monocytogenes* are also time-consuming. They first need to be cultured in an enrichment broth before they can be plated using specific media. These bacteria are examples for which identification based on the pattern of produced volatile metabolites could be useful. The use of the headspace method may not

only reduce detection time, it may also increase the specificity of the identification of these bacteria.

7.6 Conclusions

The main conclusions of the research described in this thesis are:

- Fresh raw milk without quality defects always contains the same 7 volatile components. Headspace analysis can be used for quantifying the extent of lipolysis, the amount of chloroform, as well as the detection of feeding specific vegetable byproducts. Headspace analysis is thus able to detect several quality defects with a single analysis and therefore is a valuable supplementary method for raw milk quality control.
- Volatile metabolites in milk from quarters infected by mastitis pathogens differ significantly from metabolites found in milk from uninfected quarters.
- Different pathogens form different patterns of volatile metabolites. With probabilistic neural networks, the mastitis causing pathogens can be identified based on this pattern of volatile metabolites.
- Except for ethyl esters, the pattern of volatile metabolites formed in mastitis samples is also formed in milk samples inoculated with mastitis pathogens. The probabilistic neural network correctly identified the inoculated pathogen. This shows that inoculated samples can be used as a model for mastitis milk samples in headspace analysis.
- Incubation is a necessary step before analyzing the volatile metabolites used for mastitis pathogen identification. Because after 8 hours of incubation all metabolites could be detected, 8 hours was selected as the optimal incubation time.

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Summary

Samenvatting

Populaire samenvatting

Summary

Chemical analyses are an important tool to monitor the quality of food products. One of the fastest and easiest chemical analyses performed with this goal is based on headspace analysis of volatile components. Analysing volatile components was proven to be useful for quality monitoring of a wide range of food products. Headspace analysis of milk and milk products for quality control was performed using several methods, as explained in chapter 2. Although these previous studies focused on (heated) milk products, changes in raw milk quality may also be detectable by analysing the volatile composition. Besides change in raw milk quality, also animal diseases may be detected in milk. The most obvious animal disease to study is mastitis, because it involves pathogens in the milk, which may be identified based on the metabolites produced by these pathogens. In this thesis, quality control of raw milk and identification of mastitis causing pathogens were described.

Chapter 3 describes the suitability of the headspace analysis for monitoring the quality of raw cows' milk. The detection of different quality defects caused by cows' feed, microbiological and chemical contamination, as well as enzymatic deterioration was studied. Fresh raw milk without quality defects was shown to always contain the same seven volatile components. It was also shown that treatments like heating and homogenization of raw milk drastically changed this basic pattern resulting in as much as a 10-fold increase in the number of volatile components. The growth of *Pseudomonas* could not be detected in an early stage using headspace analysis. Feed was shown to have an effect on the volatile composition if specific vegetable byproducts were fed to the cow. Chloroform contamination can be quantified by the method and the extent of lipolysis by measuring the free fatty acids. For quantification of both chloroform and lipolysis, the sensitivity and reproducibility of the method were sufficient for quality control purposes. This method was thus able to detect several quality defects with a single analysis and is therefore a valuable supplementary method for raw milk quality control.

The possibility to identify mastitis pathogens based on their volatile metabolites has been described in chapter 4. Milk samples from cows with mastitis caused by *Staphylococcus aureus*, coagulase-negative staphylococci (CNS), *Streptococcus uberis*, *Streptococcus dysgalactiae*, and *Escherichia coli* were collected. Also, samples from cows without clinical mastitis and with low somatic cell count (SCC) were collected for comparison. All mastitis samples

were examined with classical microbiological methods, followed by headspace analysis. Culture-negative samples contained fewer metabolites compared to milk from quarters with mastitis. Due to variability between samples within a group, comparisons between groups were not sufficient for identification of the samples by univariate statistics. Therefore, an artificial neural network was trained to classify the pathogen in the milk samples based on their bacterial metabolites. A probabilistic neural network (PNN) was the type of artificial neural network used for this study. The trained PNN differentiated milk from uninfected and infected quarters very well. When comparing pathogens, *Staph. aureus* produced a very different pattern of volatile metabolites compared to the other samples. Samples with CNS and *E. coli* had enough dissimilarities with the other pathogens, making it possible to identify them. The two *streptococcus* species did not show significant differences between each other but could be identified as a group that differed from the other pathogens. Five groups can thus be identified based on the volatile bacterial metabolites: *Staph. aureus*, CNS, *streptococcus* (*Strep. uberis* and *Strep. dysgalactiae* as one group), *E. coli*, and uninfected quarters.

Because the origin of the metabolites described in chapter 4 is unknown, the study of the formation of volatile metabolites by the mastitis pathogens inoculated in milk of healthy cows has been described in chapter 5. The volatile metabolites from inoculated samples were compared to those of mastitis milk samples from which the inoculated pathogens were isolated. Most metabolites found in the inoculated samples were the same as the ones in mastitis samples, both in presence and in amount. Prediction by the PNN showed that the similarity between the inoculated samples and mastitis samples was sufficient for correct prediction of the pathogen in the inoculated sample. The main difference between the inoculated samples and mastitis samples was the absence of ethyl esters of free fatty acids in inoculated samples. A possible explanation for this is disturbance of the milk-blood barrier in mastitis cows, allowing the transfer of esterase from the cows' blood to the milk in cows with mastitis.

For the study described in chapter 4, overnight incubation of the mastitis samples was used. To minimize the total analysis time, no incubation, or a short incubation, would be preferred. Therefore the effect of the incubation time on the formation of volatile metabolites in mastitis samples was studied, as has been described in chapter 6. A selection of the 6 volatile metabolites that had the highest impact on the PNN used for identifying the mastitis causing pathogen, were compared for 6 different incubation times between 0 and 24

hours. No difference between the pathogens could be detected without incubation. Thus it was concluded that incubation was necessary for the identification of pathogens. The minimum incubation time for detection of most of the 6 metabolites was 4 to 8 hours. A longer incubation time had the advantage that the differences between the pathogens were larger. However, minimization of the total analysis time is very important for practical use of the method. Because after 8 hours all metabolites could be detected, 8 hours was selected as the optimal incubation time. This optimal incubation time was evaluated with a set of 25 mastitis samples, of which 88% were correctly classified after 8 hours of incubation.

The main conclusions of the research described in this thesis are:

- Fresh raw milk without quality defects always contains the same 7 volatile components. Headspace analysis can be used for quantifying the extent of lipolysis, the amount of chloroform, as well as the detection of feeding specific vegetable byproducts. Headspace analysis is thus able to detect several quality defects with a single analysis and therefore is a valuable supplementary method for raw milk quality control.
- Volatile metabolites in milk from quarters infected by mastitis pathogens differ significantly from metabolites found in milk from uninfected quarters.
- Different pathogens form different patterns of volatile metabolites. With a probabilistic neural network, the mastitis causing pathogens can be identified based on this pattern of volatile metabolites.
- Except for ethyl esters, the pattern of volatile metabolites formed in mastitis samples is also formed in milk samples inoculated with mastitis pathogens. The probabilistic neural network correctly identified the inoculated pathogen. This shows that inoculated samples can be used as a model for mastitis milk samples in headspace analysis.
- Incubation is a necessary step before analyzing the volatile metabolites used for mastitis pathogen identification. Because after 8 hours of incubation all metabolites could be detected, 8 hours was selected as the optimal incubation time.

Samenvatting

Chemische analyses zijn een belangrijke techniek voor het toezicht houden op de kwaliteit van levensmiddelen. Eén van de snelste en makkelijkste chemische analyses die met dit doel wordt toegepast is gebaseerd op headspace analyse van vluchtige componenten. Het is aangetoond dat het analyseren van vluchtige componenten bruikbaar is voor de kwaliteitsborging van een groot scala aan levensmiddelen. Headspace analyse van melk en melkproducten ten behoeve van kwaliteitsborging is eerder uitgevoerd met verschillende technieken, zoals toegelicht in hoofdstuk 2. Alhoewel deze eerdere studies hebben gefocust op (verhitte) melkproducten, zou ook verandering van de kwaliteit van rauwe melk mogelijk op basis van vluchtige componenten bepaald kunnen worden. Naast veranderingen in rauwe melk kwaliteit, zouden dierziekten mogelijk ook gedetecteerd kunnen worden in melk. De meest voor de hand liggende dierziekte om te bestuderen is mastitis, omdat hierbij pathogenen betrokken zijn die mogelijk op basis van hun vluchtige componenten geïdentificeerd kunnen worden. In dit proefschrift worden zowel kwaliteitsborging van rauwe melk als identificatie van mastitis pathogenen beschreven.

Hoofdstuk 3 beschrijft de geschiktheid van de headspace analyse voor toezicht op de kwaliteit van rauwe koemelk. Het herkennen van verschillende kwaliteitsgebreken veroorzaakt door diervoeding, microbiologische en chemische contaminatie, en enzymatische afbraak zijn bestudeerd. Verse rauwe melk zonder kwaliteitsgebreken bleek altijd dezelfde 7 vluchtige componenten te bevatten. Ook werd aangetoond dat behandelingen zoals verhitten en homogeniseren van rauwe melk dit patroon sterk veranderden, resulterend in een tot 10 maal groter aantal vluchtige componenten. De groei van *Pseudomonas* kon niet in een vroeg stadium worden gedetecteerd met de headspace analyse. Diervoeding had een invloed op de vluchtige componenten indien specifieke plantaardige producten werden gevoerd. Chloroform contaminatie kan worden gekwantificeerd, net als de mate van lipolyse door bepaling van het gehalte vrije vetzuren. Voor het kwantificeren van zowel chloroform als lipolyse was de gevoeligheid en reproduceerbaarheid van de methode voldoende voor kwaliteitsborging. De methode was dus in staat verschillende kwaliteitsgebreken in één analyse te bepalen en is daardoor een nuttige aanvullende methode voor kwaliteitsborging van rauwe melk.

De mogelijkheid om mastitis pathogen te identificeren op basis van hun vluchtige metabolieten is beschreven in hoofdstuk 4. Melkmonsters van koeien met mastitis veroorzaakt door *Staphylococcus aureus*, coagulase-negative staphylococci (CNS), *Streptococcus uberis*, *Streptococcus dysgalactiae* en *Escherichia coli* werden verzameld. Ook monsters van koeien zonder klinische mastitis en met een laag celgetal werden ter vergelijking verzameld. Alle mastitis melkmonsters werden onderzocht met klassiek bacteriologisch onderzoek gevolgd door headspace analyse. Melk van bacteriologisch-negatieve monsters bevatte veel minder vluchtige componenten in vergelijking met melk van kwartieren met mastitis. Als gevolg van variatie binnen groepen was vergelijking tussen groepen onvoldoende voor classificatie op basis van univariate statistiek. Daarom werd een neuraal netwerk getraind voor het identificeren van de pathogenen in melkmonsters op basis van hun vluchtige metabolieten. Een probabilistisch neuraal netwerk (PNN) was het type neuraal netwerk dat is toegepast voor dit onderzoek. Het getrainde PNN kon foutloos het onderscheid maken tussen ongeïnfecteerde en geïnfecteerde kwartieren. Bij het vergelijken van pathogenen bleek *Staph. aureus* een sterk afwijkend patroon van vluchtige metabolieten te vormen ten opzichte van de andere monsters. Monsters met CNS en *E. coli* verschilden voldoende van de andere monsters om ze te identificeren. De 2 soorten Streptokokken verschilden niet significant van elkaar maar konden als groep wel onderscheiden worden van de andere pathogenen. Vijf groepen kunnen dus onderscheiden worden op basis van vluchtige metabolieten: *Staph. aureus*, CNS, Streptokokken (*Strep. uberis* en *Strep. dysgalactiae* als groep), *E. coli* en ongeïnfecteerde kwartieren.
Omdat de oorsprong van de vluchtige metabolieten beschreven in hoofdstuk 4 onbekend is werd de vorming van vluchtige componenten door mastitis pathogenen in geïnoculeerde melk van gezonde koeien onderzocht, zoals beschreven in hoofdstuk 5. De vluchtige componenten in geïnoculeerde monsters werden vergeleken met die in mastitis monsters waaruit het pathogeen geïsoleerd was dat gebruikt was voor inoculatie. De meeste metabolieten die gevonden werden in geïnoculeerde monsters waren dezelfde als in mastitis monsters, zowel in aanwezigheid als in hoeveelheid. Voorspelling door het PNN toonde aan dat de overeenkomst tussen geïnoculeerde monsters en mastitis monsters groot genoeg was voor de correcte voorspelling van het mastitis veroorzakende pathogeen in geïnoculeerde monsters. Het belangrijkste verschil tussen de geïnoculeerde monsters en mastitis monsters was de afwezigheid van ethylesters van vrije vetzuren in de geïnoculeerde monsters. Een mogelijke verklaring hiervoor ligt in de verstoring

van de melk-bloed barrière bij koeien met mastitis, waardoor esterase van het koeienbloed naar de melk kan komen.
Tijdens de proef beschreven in hoofdstuk 4 waren de monsters overnacht geïncubeerd. Om de totale analysetijd te minimaliseren zou geen incubatie, of een korte incubatie, de voorkeur verdienen. Daarom werd het effect van de incubatietijd op de vorming van vluchtige metabolieten in mastitis monsters bestudeerd, zoals beschreven in hoofdstuk 6. Een selectie van de 6 vluchtige metabolieten met de hoogste impact op het PNN voor mastitis pathogeen identificatie zijn vergeleken bij 6 incubatietijden tussen 0 en 24 uur. Zonder incubatie kon geen verschil worden aangetoond tussen de pathogenen, waaruit geconcludeerd werd dat incubatie noodzakelijk is voor pathogeen identificatie. De minimale incubatietijd voordat de meeste van de 6 vluchtige metabolieten gedetecteerd kon worden was 4 tot 8 uur. Een langere incubatietijd had als voordeel dat de verschillen tussen de pathogenen toenamen. Het minimaliseren van de incubatietijd is echter belangrijk voor praktische toepassing van de methode. Omdat na 8 uur alle metabolieten konden worden gedetecteerd werd 8 uur gekozen als optimale incubatietijd. Deze optimale incubatie tijd werd geëvalueerd met een set van 25 mastitis monsters, waarvan 88% goed werd geclassificeerd na 8 uur incubatie.

De belangrijkste conclusies van het onderzoek beschreven in dit proefschrift zijn:

- Verse rauwe melk zonder kwaliteitsgebreken bevat altijd dezelfde 7 vluchtige componenten. Headspace analyse kan gebruikt worden voor het detecteren van het voeren van specifieke plantaardige bijproducten en daarnaast voor de kwantificering van de mate van lipolyse en de hoeveelheid chloroform. Headspace analyse is dus in staat verschillende kwaliteitsgebreken te detecteren met een enkele analyse en is daarom een nuttige aanvullende methode voor kwaliteitsborging van rauwe melk.
- Vluchtige metabolieten in de melk van kwartieren die besmet zijn met mastitis pathogenen verschillen significant van de metabolieten gevonden in melk van ongeïnfecteerde kwartieren.
- Verschillende pathogenen vormen verschillende patronen van vluchtige metabolieten. Met een probabilistisch neurale netwerk kunnen mastitis veroorzakende pathogenen worden geïdentificeerd op basis van dit patroon van vluchtige metabolieten.

- Met uitzonder van ethyl esters word het patroon van vluchtige metabolieten gevormd in mastitis monsters ook gevormd in met mastitis pathogenen geïnoculeerde melkmonsters. Het probabilistisch neuraal netwerk identificeerde het geïnoculeerde pathogeen correct. Dit laat zien dat geïnoculeerde monsters als model kunnen dienen voor mastitis monsters in headspace analyse.
- Incubatie is een noodzakelijke stap voorafgaande aan de analyse vluchtige componenten ten behoeve van de mastitis pathogeen identificatie. Omdat na 8 uur incubatie alle metabolieten gedetecteerd konden worden werd 8 uur geselecteerd als optimale incubatietijd.

Populaire samenvatting

Om te bepalen of uw voedsel nog goed is, zult u er vaak aan ruiken. Dit principe wordt ook toegepast in de levensmiddelenindustrie, met behulp van apparaten die aan het voedsel ruiken. Alhoewel het principe al veelvuldig wordt toegepast, is nooit eerder geprobeerd hiermee de kwaliteit van boerderijmelk vast te stellen. Het idee van mijn onderzoek was daarom om kwaliteitsafwijkingen in boerderijmelk vast te stellen door er met een apparaat dat geurstoffen meet aan te ruiken.

Eerst is het patroon van geurstoffen bepaald in melk waar niks mis mee is, om als vergelijkingsmateriaal te dienen bij het meten van melk met kwaliteitsafwijkingen. Bij deze metingen bleek dat in melk van goede kwaliteit slechts zeven verschillende geurstoffen voorkomen (ter vergelijking, in kaas worden meer dan honderd verschillende geurstoffen aangetroffen).

Vervolgens heb ik mij gericht op zaken die op de boerderij mis kunnen gaan. Na analyse van de geurpatronen in de melk bleek dat verschillende problemen goed te detecteren waren. Zo kon in melk onder andere het voeren van groente en fruit aan koeien, de aanwezigheid van een hoog aantal bacteriën, residuen van reinigingsmiddelen en de mate van vetafbraak gedetecteerd worden.

Omdat bleek dat zowel dierziekten als grote aantallen bacteriën goed gedetecteerd konden worden is vervolgens onderzoek gedaan naar melk van koeien met uierontsteking (mastitis). Uierontsteking is één van de belangrijkste ziekten die bij de melkkoeien voorkomt en die voor de koe vaak veel pijn en voor de veehouder grote kosten met zich meebrengt. Om vast te stellen welke bacterie de ontsteking veroorzaakt wordt melk op kweek gezet, waardoor de bepaling enkele dagen duurt. Nu is van verschillende bacteriën bekend dat deze geurstoffen uitscheiden. Het bepalen van het patroon aan geurstoffen in de melk van koeien met uierontsteking zou dus een snel alternatief kunnen zijn voor de huidige kweekmethodes.

In een eerste experiment werden 50 monsters verzameld van koeien met uierontsteking. Al snel bleek dat de geurstoffen in deze melkmonsters een bijzonder patroon vertoonden, waarbij het patroon uniek leek te zijn voor de bacterie die in het monster zat. Met behulp van speciale software (kunstmatige neurale netwerken) kon de computer op basis van het patroon aan geurstoffen voorspellen welke bacterie de veroorzaker van de uierontsteking was.

Vervolgens is bepaald of deze geurstoffen gevormd werden door de bacterie zelf, of dat ze het gevolg waren van het ziekteproces. Hiervoor zijn bacteriën

toegevoegd aan de melk gevolgd door het laten groeien van de bacteriën bij 37°C. Hierbij bleek dat het patroon van geurstoffen in deze melkmonsters vrijwel hetzelfde was als het patroon van geurstoffen in de melk van zieke koeien: de geurstoffen worden dus door de bacteriën zelf gevormd.
Tijdens het eerste experiment is de melk altijd een nacht bij 37°C bewaard om te zorgen dat de bacteriën voldoende geurstoffen aanmaken. In het laatste experiment is gekeken hoe lang de bacteriën bij 37°C moeten groeien om een goed patroon aan geurstoffen te krijgen. Hierbij bleek dat 8 uur groeitijd voldoende was, en 4 uur te kort. De minimale groeitijd ligt hier dus tussen.
De belangrijkste conclusies van het onderzoek dat ik heb gedaan zijn:

- Verse rauwe melk bevat een constant patroon van 7 geurstoffen.
- Melk van koeien met uierontsteking vormt een patroon aan geurstoffen dat gebruikt kan worden om vast te stellen welke bacterie de uierontsteking veroorzaakt.
- De geurstoffen worden door de bacterie zelf aangemaakt.
- De bacteriën in de melk van een zieke koe moeten tussen de 4 en 8 uur kunnen groeien om de geurstoffen goed te kunnen detecteren.

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Zo'n 6 jaar geleden begon ik serieus na te denken over wat ik na het afronden van mijn opleiding levensmiddelentechnologie wilde gaan doen. Terwijl ik daarover aan het nadenken was werd mij gevraagd serieus na te denken over het doen van promotieonderzoek. Aangezien ik het doen van onderzoek leuk vond ben ik toen met Prof. Jos Lankveld gaan praten. Ik kan mij het gesprek nog altijd goed herinneren en al snel was de keus gemaakt: ik werd promovendus bij de leerstoel zuivelkunde. Na 4 jaar van onderzoek doen, met de nodige ups en downs, en veel schrijfwerk is het nu zover: het proefschrift is af. Gedurende deze tijd zijn veel mensen betrokken geweest bij mijn onderzoek die ik op deze plek graag wil bedanken.

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Abbreviations

AMDIS	Automatic Mass spectral Deconvolution and Identification System
ANN	Artificial Neural Network
ANOVA	Analysis of Variance
BDI	Bureau of Dairy Industries
CFU	Colony Forming Units
CI	Chemical Ionization
CNS	Coagulase-negative Staphylococci
E. coli	Escherichia coli
ECD	Electron Capture Detector
EI	Electron Ionization
eV	Electronvolt
FFA	Free Fatty Acids
FID	Flame Ionization Detector
FTIR	Fourier Transform Infrared
GC	Gas Chromatograph
KNN	k-Nearest Neighbours
LDA	Linear Discriminant Analysis
LDH	Lactate dehydrogenase
LOD	Limit of Detection
m/z	Mass-to-charge ratio
MLF	Multi-Layer Feedforward
MS	Mass Spectrometer
NAGase	*N*-acetyl-ß-*D*-glucosaminidase
NMC	National Mastitis Council
NIST	National Institute of Standards and Technology
P&T	Purge and Trap
PCR	Polymerase Chain Reaction
PFPD	Pulsed Flame Photometric Detector
PNN	Probabilistic Neural Network
QDA	Quadratic Discriminant Analysis
SAFE	Solvent Assisted Flavour Evaporation
SCC	Somatic Cell Count
SIMCA	Soft Independent Modeling of Class Analogy
SPME	Solid-Phase Microextraction
Staph.	*Staphylococcus*
Strep.	*Streptococcus*
TIC	Total Ion Count

Curriculum vitae

Kasper Arthur Hettinga werd geboren op 5 januari 1980 in Groningen. In 1998 behaalde hij zijn VWO diploma aan het Christelijk College Nassau-Veluwe in Harderwijk. In september van dat jaar begon hij aan zijn opleiding levensmiddelentechnologie aan Wageningen Universiteit. Na twee afstudeervakken en stages bij Unilever (Vlaardingen) en Nutricia (Zoetermeer) studeerde hij in september 2003 af met als specialisatie geïntegreerde levensmiddelentechnologie. In oktober van datzelfde jaar begon hij als promovendus bij de leerstoelgroep productontwerpen en kwaliteitskunde, leerstoel zuivelkunde. Zijn promotieonderzoek richtte zich op de toepassing van headspace analyse voor kwaliteitsborging van rauwe melk en mastitis pathogeen identificatie. Dit werk vormde de basis voor dit proefschrift, getiteld "Quality control of raw cows' milk by headspace analysis, a new approach to mastitis diagnosis". Sinds november 2007 is hij werkzaam als postdoc bij de leerstoel zuivelkunde van Wageningen Universiteit.

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PhD training activities

Discipline specific activities

Courses

Masterclass AMDIS, Interscience, Breda, 2003
Workshop "Thermische desorptie", Interscience, Breda, 2004
Short course "Biochemical sensors", University of Salamanca, 2004
Management of microbiological hazards in foods, VLAG, Wageningen, 2005
Bionanotechnology, VLAG, Wageningen, 2006
Reaction kinetics in food science, VLAG, Wageningen, 2006

Meetings

Euroanalysis XIII, Salamanca, 2004
International conference "Analytical chemistry&chemical analysis", Kiev, 2005
International congress on Analytical Sciences ICAS, Moscow, 2006
World Dairy Summit, Dublin, 2007
1st Annual meeting Dutch Mastitis Research Workers, Utrecht, 2007
International Conference "Mastitis 2008", The Hague, 2008

General courses

Organising and supervising MSc thesis projects, Wageningen, 2005
Techniques for writing and presenting a scientific paper, Wageningen Business School, 2005
Scientific English, Centa, Wageningen, 2005
VLAG PhD week, Bilthoven, 2005
Philosophy and ethics of food science and technology, VLAG, Wageningen, 2006
PhD competence assessment, Wageningen Graduate School, 2007

Optionals

Preparation PhD research proposal
PhD-meetings of the Dairy Science and Technology group, 2003-2007
Meetings "Netherlands Association for the Advancement of Dairy Science", 2003-2007
MSc course "Chemometrics 1", Radboud Universiteit, Nijmegen, 2004
PhD trip of the Product Design and Quality Management group, USA, 2007
NZO research meeting, Wageningen, 2007

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