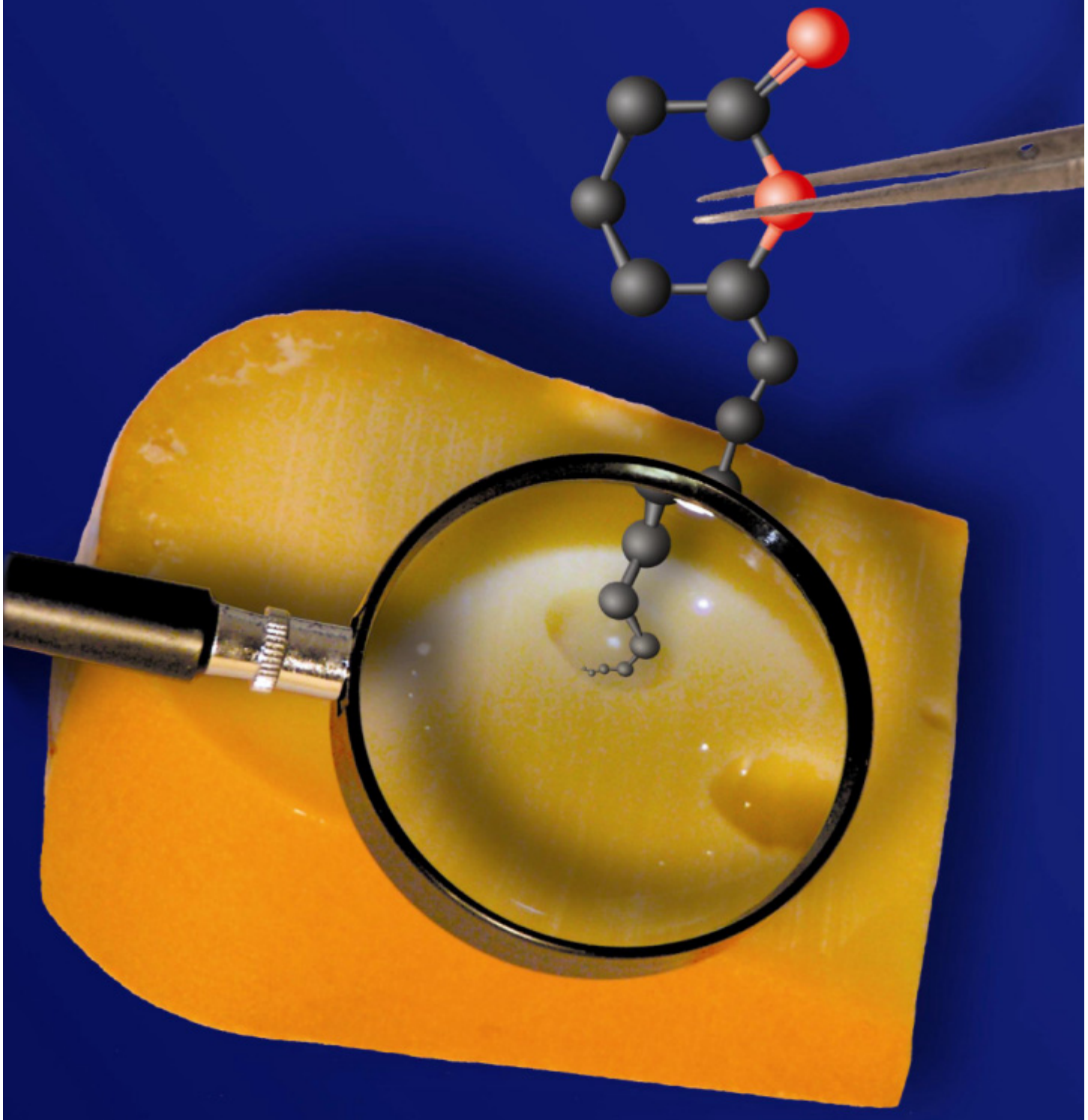
The formation of fat-derived flavour compounds during the ripening of Gouda-type cheese



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Abstract

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The aim of this research was to investigate the routes to the formation of fat-derived (aroma) compounds during the ripening of Gouda-type cheeses, with the ultimate goal to obtain control on their development during cheese ripening. To easily investigate the presence of these compounds, a method was developed to measure all relevant compounds simultaneously. This method was based on a simple liquid/liquid extraction and GC/MS, and was able to quantify fat-derived fatty acids, lactones, ketones, esters, alcohols and aldehydes. The formation of said compounds during the ripening of several batches of Gouda cheese was followed for almost two years, and showed that the formation of long-chain free fatty acids, lactones and 2-ketones was fairly similar across all batches of cheese, while short-chain free fatty acids and esters showed a wide variation. A second ripening study included foil-wrapped cheeses, which showed a slower formation of fat-derived aroma compounds than their conventionally ripened counterparts, but confirmed the formation profiles of these compounds. The formation of long-chain fatty acids was attributed mainly to the action of native milk lipoprotein lipase, while the formation of short-chain fatty acids and fatty ethyl esters was attributed to the action of esterases from the cheese (secondary) flora. The formation of lactones and ketones was studied in more detail, and appeared to be predominantly a non-enzymatic event in Gouda-type cheeses. The formation kinetics for lactones and ketones were studied, and reaction mechanisms that started from esterified hydroxy- and keto-fatty acids in milk fat were proposed. The kinetic data obtained enabled the prediction of the formation of lactones and ketones during the ripening of the cheeses presented earlier. The data presented in this thesis may lead to tools for cheese producers to control and influence the formation of fat-derived compounds in their cheeses.

Contents

1. General introduction

1. Cheese and cheese production

Cheese is a well-known, highly nutritious foodstuff that is produced all over the world. The history of cheese goes back many centuries. The recipe for cheese preparation was probably discovered accidentally, but it proved to be a good manner of conserving milk, which is a food readily susceptible to spoilage. Having such a long history, the preparation of cheese has developed into many different deviations, many of which have become traditional processes to produce a certain cheese variety. These artisanal cheese varieties are still being produced today, in many cases in small-scale production facilities (Stanley, 1998).
However, cheese is also being produced on large industrial scale. In 2004, the world cheese industry processed roughly $1.3x10^{11}$ litres of milk into 13,358,000 tonnes of cheese (www.usdec.org). This makes cheese also an important product in economical sense. Some countries, including the Netherlands, export substantial amounts of the cheese produced, which is obviously beneficial for their economies. For economic reasons, cost and quality are the most important cheese characteristics. In the more artisanal field, the traditional background and cheese quality will be the more important determinants of the product success. Cheese quality is determined by a number of factors as the appearance, the structure, and of course the taste and aroma, or 'flavour' of the cheese.

Cheese flavour is very much affected by the materials and processes used. Cows' milk is probably the most commonly used milk for cheese production, especially in the Western world. However, almost any milk can be used to produce cheese, and indeed cheeses are also prepared from milk derived from goats, sheep, buffaloes, camels, yaks, and llamas. The vast majority of cheeses is prepared by adding (mostly animal) rennet to the milk, a crude protease that causes the coagulation of casein, and thus clotting. Coagulation can also be promoted by acid (pH 4.6, achieved by lactic acid bacteria or added acid), which is frequently used for fresh cheeses, or acid and heat (pH 5.2 and 80-90°C) (Fox & Grufferty, 1991). After that, the coagulum is cut, and under influence of heat and acid syneresis occurs, allowing whey and curd to be separated. After pressing and usually salting, the cheese is left for a short or long ripening period (Fox et al., 1991). It is during the ripening period that most flavour develops, under the influence of time, spontaneous reactions, reactions catalysed by enzymes present in milk, and mainly by the action of microbial enzymes. The vast diversity of microbes used, intentionally or not, around the world is the main reason for the many different flavoured cheeses that exist.

2. Cheese flavour

2.1 General cheese flavour considerations

One of the main characteristics of cheese is its flavour. Flavour consists of aroma, taste, and texture. Aroma, or odour, is generally the sensation that is perceived by olfactory receptors in the nose, and is mainly determined by the nature of volatile and semi-volatile compounds. Taste is perceived by the gustatory senses, i.e. the taste buds on the tongue. They are sensitive to sweet, sour, salt, bitter, and umami. Texture is sometimes also included with "taste", and is perceived by the tactual senses. Hardness, viscosity, melting behaviour of the fat, feel of (salt)-crystals, elasticity of the body, distribution of the eyes in the cheese, and many physical characteristics alike determine the texture of cheese, and also play a role during taste sensation when a cheese is eaten (Badings, 1984).

The differences between taste and aroma perception are not absolutely clear, as some compounds will both have a taste and an aroma. In fact, in common language the terms "flavour", "aroma" and "taste" are used somewhat interchangeably. Although this thesis is not about perception of flavour, research on the formation of flavour compounds is presented. Most of the compounds described can be predominantly perceived by the olfactory senses, and in this sense they should be considered aroma compounds. However, some of the compounds possibly also play some role in the taste perception, or maybe even the texture (fatty feel, astringent), and therefore in this thesis, the most broad term "flavour" will be used.

The degree of participation of a single compound to flavour perception will vary, according to its flavour strength and its concentration. The frequently used descriptor to assess the contribution of a compound to flavour is its flavour threshold. A flavour threshold is the concentration that can just be perceived by a person. This threshold varies considerably between individual persons, and is therefore usually expressed in a concentration at which half of the population can just perceive the compound (Delahunty & Piggott, 1995). In its most elementary form, the aroma threshold describes the minimum amount of a compound that triggers a detectable response by the relevant sensory receptors (Hall & Andersson, 1983). For a compound trapped in a foodstuff, it is easy to see that several factors influence this amount. These include the volatility of the compound, but also person-dependent characteristics as mouth cavity shape, chewing and swallowing movements and degree of mastication. Furthermore, the matrix is important, as it determines how strong a flavour compound is bound to the polar protein/water phase or the fat phase, decreasing the actual volatility (Piraprez, Herent & Collin, 1998). The headspace concentration of a relatively lipophilic compound (fat derived flavour) in an aqueous-and-fatty matrix (cheese) can be relatively easily predicted using the partition coefficients of the compound between water

and fat, and between water and air (Guyot, Bonnafont, Lesschaeve, Issanchou, Voilley & Spinnler, 1996).

Cheese flavour is a complex mixture of many chemical compounds, usually derived from precursors present in the milk. While the flavour (aroma) of some foods is largely determined by one or two 'character impact' compounds (i.e. banana: isoamyl acetate, raspberry: *p*-hydroxybenzylacetone, cucumber: (E,Z-2,6-nonadienal), most cheeses do not have such a compound. There is maybe an exception for the blue mould-type cheeses, which heavily rely on 2-heptanone and 2-nonanone for their aroma. Also, pure samples of 2-heptanone and 2-nonanone will to most individuals smell like those cheeses. In most other cheeses, there is no single compound responsible for the total flavour, but instead, many compounds are perceived together to form a final cheese flavour. While there are no distinct character impact compounds, there are some compounds that contribute substantially to the total flavour. Others can hardly be observed individually, though play a role in the total flavour, resulting in a full, well-balanced overall flavour. This balance is different in all cheese varieties. In six different cheese varieties, most known cheese-related aroma compounds were present, but in different quantities (Bosset & Gauch, 1993). In the large variety of cheeses around the world, nearly all possible aroma compounds are present, it is the balance that defines the particular flavour of the cheese. This generally accepted idea is called the component balance theory (Delahunty et al., 1995).

Another difficult aspect of aroma perception is the synergistic effects that compounds may have (Siek, Albin, Sather & Lindsay, 1969). Chemical homologues will generally have similar, but not equal, aromas, as they will generally stimulate the same receptor due to the presence of the same functional group. Therefore, they will have a more or less additional effect on the individual aroma threshold.
Taken all this into account, aroma threshold is a rough estimate for the odour activity of a certain compound in a certain foodstuff, but whether a compound is finally perceived upon consumption is dependent on a large number of additional variables.

2.2 Flavour in cheese

Of the many cheese flavour compounds, some are already present in the milk (Forss, 1979), but most of the flavour is formed during ripening (McSweeney & Sousa, 2000). Flavour formation is a combination of enzymatic and non-enzymatic events. The predominant activity is usually enzymatic flavour formation, caused by lactic acid bacteria (Olson, 1990). Other sources of enzymes are non-lactic acid bacteria and endogenous enzymes. Also

yeasts and moulds can contribute to the pool of flavour-forming enzymes, but usually play an important part only in some specific cheese types.
Many cheese types use lactic acid bacteria to acidify the curd by converting lactose to lactic acid, and thus contribute to the prevention of microbial spoilage of the end product. Apart from the ability of converting lactose, the starter culture, whether single-strain or mixed-strain, is capable of producing many other enzymes, some of which can lead to the formation of flavour compounds.
However, usually the starter bacteria are somewhat limited with respect to their diversity of flavour formation (Ayad, Verheul, Jong, Wouters & Smit, 1999). Amongst other factors, non-starter lactic acid bacteria can contribute to cheese flavour formation by intensifying the reaction, or make new reactions possible. Their contribution is reflected in the final cheese flavour. Series of cheeses made with pasteurised versus non-pasteurised milk showed that using non-pasteurised milk yielded better tasting cheese, partly due to more free fatty acids and ester development (McSweeney, Fox, Lucey, Jordan & Cogan, 1993; Shakeel Ur, Banks, McSweeney & Fox, 2000a; Shakeel Ur, McSweeney, Banks, Brechany, Muir & Fox, 2000b). Other authors report a better tasting cheese when non-pasteurised milk was used for the preparation of Cheddar (Chin & Rosenberg, 1997), Swiss-type cheese (Beuvier et al., 1997), and Austrian Bergkäse (Ginzinger, Jaros, Lavanchy & Rohm, 1999). It should be noted that both non-starter lactic acid bacteria and indigenous milk enzymes may play a role in this effect.

As most cheeses are ripened for a certain amount of time, also non-enzymatic reactions may take place. These are usually not fast at temperatures cheese is ripened, but (auto)oxidation, (de)esterification, hydrolysis and Maillard reactions are examples of possibly flavour-forming non-enzymatic reactions that can occur to some extent during cheese ripening.
Apart from the flavour that is already present in the milk and transferred to the curd, flavour is produced from material that is present in the milk. The three major flavour precursors are carbohydrates, protein and fat, and these will be shortly outlined in sections 2.4 to 2.6.

2.3 Detection of cheese flavour compounds

Detection and quantification of cheese flavour compounds is not an easy task. Volatiles as such are relatively easy separated by gas chromatography, after which they can be detected by a general-purpose detector, as a flame ionisation detector (FID), or more frequently by mass spectroscopy (MS), which with some degree of confidence enables identification of the compound. For GC-analysis, the matrix "cheese" is rather difficult, as the components of the matrix are quite incompatible with this analysis. Fat, protein and peptides, salt and water

are present in much higher quantities than the volatiles of interest. Therefore, the major problem is to (quantitatively) separate the volatiles from the matrix components, and as the matrix covers a wide range from hydrophobic to hydrophilic properties, this is not an easy task. However, there are many methods to extract volatiles from cheese, all with different advantages and drawbacks. Furthermore, some methods are specially tailored for one or a few specific (class of) compounds, which are generally more effective for that class than a generic method.

A nice example of a specific method is the extraction and quantification of free fatty acids. This method combines acid liquid/liquid extraction with a solid phase extraction (SPE) cartridge to selectively retain the fatty acids entrapped in the lipophilic extract. The fatty acids are then determined by GC-FID (Jong & Badings, 1990; Woo & Lindsay, 1982).

Most methods use the volatility of the compounds as a separation mechanism. Steam distillation extraction (SDE) (Buchgraber & Ulberth, 1999), solid phase microextraction (SPME) (Adahchour, Vreuls, Heijden & Brinkman, 1999), static and dynamic headspace analysis (Thierry, Maillard & Quere, 1999), high-vacuum distillation and molecular distillation are examples of these methods (Mariaca & Bosset, 1997). Because of the matrix, it is almost impossible to quantitatively extract all volatiles of interest. In some cases, it is possible to correct for the amount of volatiles that is retained in the cheese matrix, but in most cases the amounts of extracted volatile (Gonzalez-de-Llano, Ramos, Polo, Sanz & Martinez-Castro, 1990; Shakeel Ur et al., 2000b; Villasenor, Valero, Sanz & Martinez, 2000) is used. Even dimensionless integration units, directly from the detector used are reported (Bosset et al., 1993; Lawlor, Delahunty, Wilkinson & Sheehan, 2002). When method and matrix are kept constant, these methods are quite suitable for comparison of compound levels across cheese samples, but give only little information about the distribution of different compounds within a cheese, and are also unsuitable if another method or matrix is used.

Other methods to liberate volatiles from their (cheese) matrix include liquid/liquid or liquid/solid extraction, supercritical fluid extraction, dialysis, and solid-phase extraction (Coulibaly & Jeon, 1992), and are reviewed by Mariaca et al. (1997); Vandeweghe & Reineccius (1990). Methods for cheese volatile determination are also further discussed in Chapter 2 of this thesis.

To illustrate the many different flavours that contribute to cheese flavour, the next table contains a selection of compounds that were found to be flavour-active in a range of cheeses. Note that many more possible flavour compounds are present in most cheeses, they are so far not detected as single compounds by the gas chromatography/olfactometry method used. The compounds are ordered by their chemical group, and their origin, when known, is indicated.

Table 1.1. Compounds found by gas chromatography-olfactometry to be flavour-active in different cheeses, from Curioni & Bosset (2002), with their chemical origins, from various sources.

Compound	origin[1]	Compound	origin[1]	Compound	origin[1]
primary alcohols		***ketones***		***pyrazines***	
ethanol	C	acetone	C	2-acetylpyrazine	H
1-propanol	C	2-butanone	C	2,3-dimethylpyrazine	H
1,2-butanediol	C	2-pentanone	F	2-ethyl-3,5-dimethylpyrazine	H
1-pentanol	F	2-heptanone	F	3-ethyl-2,5-dimethylpyrazine	H
hexanol	F	2-octanone	F	3-ethyl-2,6-dimethylpyrazine	H
primary branched alcohols		3-octanone	F	2-isopropyl-3-methoxypyrazine	H
2-methyl-1-propanol	P	2-nonanone	F	3-isopropyl-2-methoxypyrazine	H
2-methyl-1-butanol	P	2-undecanone	F	2,3-diethyl-5-methylpyrazine	H
3-methyl-1-butanol	P	2-tridecanone	F	3,5-diethyl-2-methylpyrazine	H
2-ethyl-1-hexanol	F	2-pentadecanone	F	2-isobutyl-3-methoxypyrazine	H
secondary alcohols		***unsaturated ketones***		2-*sec*-butyl-3-methoxypyrazine	H
2-propanol	C	1-octen-3-one	F	***S-compounds***	
2-pentanol	F	8-nonen-2-one	F	methional	P
2-heptanol	F	*ß*-damascenone	M	methanethiol	P
2-nonanol	F	***other ketones***		methylsulfide	P
unsaturated alcohols		2,3-butadione	C, P	dimethylsulfide	P
3-methyl-2-buten-1-ol	P	3-hydroxy-2-butanone	C, P	dimethyldisulfide	P
1-octen-3-ol	F	3-hydroxy-2-pentanone	C, P	dimethyltrisulfide	P
1,5-octadien-3-ol	F	2-hydroxy-3-pentanone	C, P	dimethyltetrasulfide	P
aromatic alcohols		3-methyl-2-pentanone	C, P	dimethylsulfone	P
phenylethanol	P	***lactones***		***N & S-compounds***	
2-phenylalcohol	P	δ-octalactone	F	2-acetylthiazoline	H
straight-chain aldehydes		δ-decalactone	F	***terpenes***	
acetaldehyde	C, P	δ-decalactone	F	*alfa*-pinene	M
pentanal	F	δ-dodecalactone	F	*L*-carvone	M
hexanal	F	γ-undecalactone	F	linalool	M
heptanal	F	γ-dodecalactone	F	geranyl acetate	M
octanal	F	***unsaturated lactones***		***phenolic compounds***	
nonanal	F	(*Z*)-6-dodecen-γ-lactone	F	toluene	M
decanal	F	***straight-chain esters***		4-methylanisole	M
branched aldehydes		ethyl acetate	C	2-methoxyphenol (guaiacol)	M, P
2-methylpropanal	P	ethyl butyrate	C, F	vanillin	M, P
2-methylbutanal	P	pentyl acetate	P, F	4-methylphenol (*p*-cresol)	M, P
3-methylbutanal	P	propyl butyrate	P, F	***straight chain FFA***	
unsaturated aldehydes		ethyl hexanoate	F, C	acetic acid	F, C
(*Z*)-4-heptenal	F	ethyl octanoate	F, C	propanoic acid	F, C, P
(*E*)-2-nonenal	F	ethyl dodecanoate	F, C	butanoic acid	F, C, P
(*Z*)-2-nonenal	F	***branched esters***		pentanoic acid	F, P
trans-4,5-epoxy-2-(*E*)-decenal	F	methyl-2-methylbutanoate	P, C	hexanoic acid	F, P
(*E*,*E*)-2,4-nonadienal	F	ethyl isobutanoate	P, C	heptanoic acid	F
(*E*,*Z*)-2,6-nonadienal	F	3-methylbutyl actetate	P, C	octanoic acid	F
(*E*,*E*)-2,4-decadienal	F	ethyl-2-methylbutanoate	P, C	nonanoic acid	F
trans-trans-2,4-decadienal	F	ethyl-3-methylbutanoate	P, C	decanoic acid	F
decadienal	F	isobutyl butanoate	P, F	dodecanoic acid	F
2,4-dodecadienal	F	methyl octanoate	F	tetradecanoic acid	F
aromatic aldehydes		3-octyl acetate	F	***branched-chain FFAs***	
phenylacetaldehyde	P	2-methylbutyl hexanoate	F,P	isobutyric acid	P, F
furans		***aromatic esters***		2-methylbutanoic acid	P, F
3-OH-4,5-diMe-2-furanone (sotolon)	H	ethyl benzoate	P	3-methylbutanoic acid (isovaleric	P, F
4-OH-2,5-diMe-3-furanone (furaneol)	H	phenethyl acetate	P	4-methyloctanoic acid	F
4-OH-2-Et-5-Me-3-furanone	H	***N-compounds***		4-ethyloctanoic acid	F
5-Et-4-OH-2-Me-3-furanone	H	2-acetyl-1-pyrroline	H	***aromatic FFAs***	
miscellaneous compounds		indole	M	phenylacetic acid	P
dodecane	M	skatole	M	phenylpropanoic acid	P
chloroform	M				

[1]Origin abbreviations:
C=Carbohydrate
P=Protein
F=Fat
H=Heat treatment (usually Maillard-type compounds, and derived from amino acid/reducing sugar)
M=Milk (or milk contaminant)

2.4 Cheese flavour precursors: carbohydrates

The principal carbohydrate in milk is lactose. Starter bacteria use this as an energy source, and it is generally broken down via the glycolytic route, producing lactate. This lactate is the principal acidifier in cheese. In most cheeses, part of the lactate can be metabolised by lactococci, via pyruvate into formate, acetaldehyde, ethanol and acetate. In Swiss type cheeses, propionibacteria metabolise lactate into mainly propionate, acetate and CO_2 (McSweeney et al., 2000). Novak & Loubiere (2000) give an overview of the carbon flow in a model system with growing *Lactococcus lactis* cells.
Milk also contains citrate, a part of which is included in the curd. This compound is also metabolised by lactococci, and broken down in acetate and lactate, but also in the important flavour compounds acetoin, 2,3-butanediol and diacetyl (McSweeney et al., 2000).

2.5 Cheese flavour precursors: protein

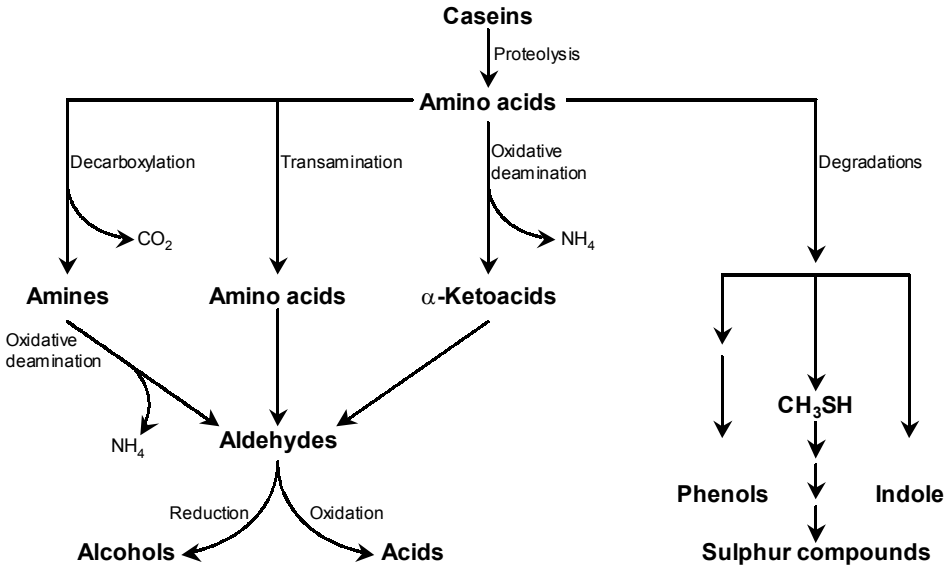


Fig. 1.1. General formation of flavour compounds from protein fraction, modified from McSweeney et al. (2000).

Protein breakdown is usually the most important route for cheese flavour formation. Due to the large number of degradation possibilities, it is a very complex route. The first step of protein degradation is the conversion of protein (mainly caseins) into peptides and free amino acids. Free amino acids can be degraded into a multitude of secondary products.
The breakdown of protein starts already during cheese preparation, as the coagulant – usually rennet, chymosin, pepsin, or plant or fungal proteinases– start breaking down the caseins. Also native milk enzymes as plasmin and pepsin may play a role. Later, proteinases and peptidases from the starter culture and from non- and secondary starter can break

down parts of the protein (Fig. 1.1). By this process, peptides and free amino acids are formed, both of which have some flavour effect – some peptides are bitter, and some amino acids have sweet, sour or bitter notes.
The most important flavours are usually produced from free amino acids, mostly in enzymatic ways.
Further information on general cheese flavour formation is available in many reviews: (Adda, Gripon & Vassal, 1982; Berg & Exterkate, 1993; Collins, McSweeney & Wilkinson, 2003b; Crow, Coolbear, Holland, Pritchard & Martley, 1993; Curioni et al., 2002; Downey, 1980; Dufosse, Latrasse & Spinnler, 1994; El-Soda, Madkor & Tong, 2000; Forss, 1979; Fox, 1993; Hemme, Bouillanne, Metro & Desmazeaud, 1982; Hettinga & Reinbold, 1972; Janssens, Pooter, Schamp & Vandamme, 1992).

2.6 Cheese flavour precursors: fat

Cheese aroma is predominantly determined by compounds derived from protein and carbohydrates. However, when Cheddar cheese was prepared with vegetable oil instead of milk fat, proteolysis and peptidolysis seemed to have proceeded as usual, although no characteristic (Cheddar) cheese flavour was obtained, even with prolonged maturation (Wijesundera & Drury, 1999). Other studies showed that the peptides formed in low-fat cheeses differed from those in normal fat cheese, indicating that (bio)chemical processes as proteolysis are affected by fat content (Fenelon, O'Connor & Guinee, 2000). Experiments in which milk fat was replaced by mineral oil also yielded poor sensory quality cheeses (Foda, Hammond et al. 1969). When instead of mineral oil deodorised milk fat was re-added to skim milk the resulting cheese showed normal aroma production. This suggests that aroma is not only present in milk fat, but is actually produced during ripening (Foda, Hammond, Reinbold & Hotchkiss, 1969; Wijesundera, Watkins & Wijesundera, 2000). The above findings show that also fat is crucial for total cheese flavour development. The nature of milk fat, and the formation of possible flavour compounds is outlined in the next section.

3. Fat-derived flavour

3.1 Cheese fat composition

Dependent on the season and the stage of lactation, cow's milk contains 3.5 to 5 % fat. The fat is typically present in 0.1 to 10µm diameter globules (Collins et al., 2003b), surrounded by a highly complex membrane (Mather, Weber & Keenan, 1977), emulsifying the fat

globule in the aqueous milk plasma phase. Milk fatty matter consists mainly of triglycerides. The majority of the further fatty matter consists of diglycerides (0.36% to 1.8%), monoacylglycerols (0.03%) and free fatty acids (0.03%), and of phospholipids (0.6%), free cholesterol (0.3%) and traces of cholesterol esters (Mariani, Contarini, Zucchetti & Toppino, 1990; Walstra & Geurts, 1982).

Most fatty matter consists of triglycerides, the vast majority of which is built up with glycerol and even-numbered saturated fatty acids. Characteristic for bovine milk fat is the presence of relatively large amounts of short and intermediate chain (even-numbered) fatty acids. Furthermore, the distribution of fatty acids in milk triacylglycerides is non-random (Angers, Tousignant, Boudreau & Arul, 1998). The main fatty acids in a typical bovine milk fat, and their positions are given in Table 1.2.

Table 1.2. Global and positional distributions of major fatty acids in the triacyl-sn-glycerides from cows' milk, in mole%, from Christie & Clapperton (1982).

Fatty acid	Triglyceride	sn-1	sn-2	sn-3
C4:0	11.8			35.4
C6:0	4.6		0.9	12.9
C8:0	1.9	1.4	0.7	3.6
C10:0	3.7	1.9	3.0	6.2
C12:0	3.9	4.9	6.2	0.6
C14:0	11.2	9.7	17.5	6.4
C15:0	2.1	2.0	2.9	1.4
C16:0	23.9	34.0	32.3	5.4
C16:1	2.6	2.8	3.6	1.4
C17:0	0.8	1.3	1.0	0.1
C18:0	7.0	10.3	9.5	1.2
C18:1	24.0	30.0	18.9	23.1
C18:2	2.5	1.7	3.5	2.3
total (%)	100	100	100	100

Apart from the major fatty acids reported in Table 1.2, many more different fatty acids are present in "trace" levels. About 1% (w/w) of the fatty acids is branched, most of them fall in the range of C12-C18. Apart from the unsaturated fatty acids mentioned in Table 1.2, trace levels of unsaturated C10 and longer are also present in bovine milk fat. Furthermore, there are keto and hydroxy (both about 0.3% w/w) fatty acids present, esterified in triglycerides (Walstra et al., 1982).

These numbers are taken for an "average" fat. There are a number of factors that can cause milk fat to be different from this average, such as cow-to-cow differences, stage of lactation and season. Also feed can have a clear influence on the composition of milk fat, which can affect the flavour of milk and derived dairy products by influencing lactone,

ketone and aldehyde productions, presumably through altering the fatty acid residues in the milk fat (Urbach, 1990). Sections 3.4, 3.5 and 3.6 go into this in more detail.
In milk and milk products, especially during cheese ripening, fat might be altered by enzymatic and non-enzymatic processes, which may yield a number of different flavour compounds. The known routes and compounds are given in Fig. 1.2.

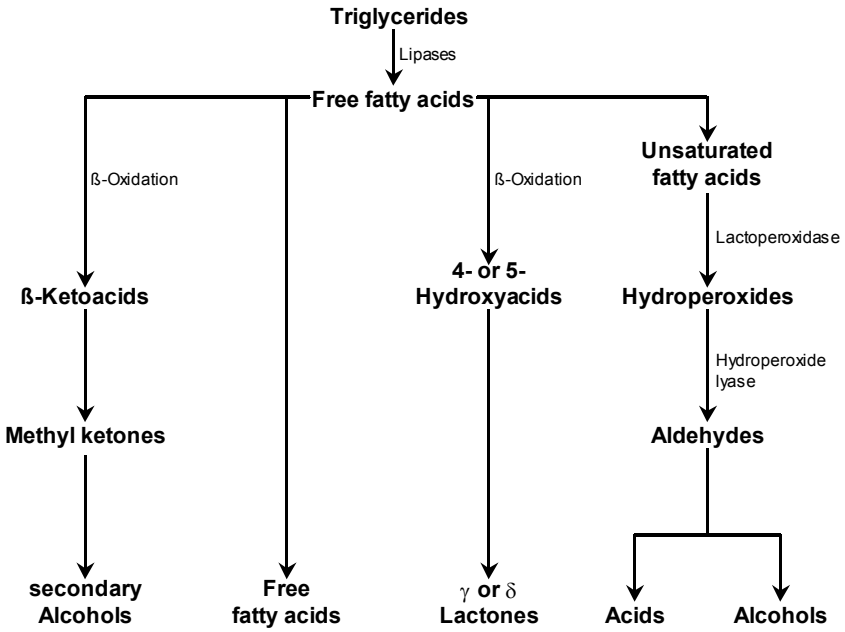
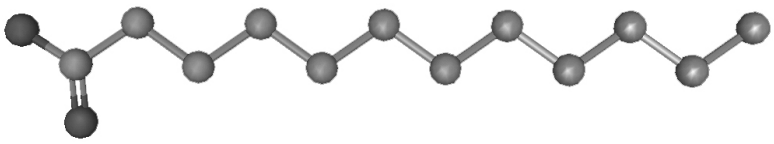


Fig. 1.2. Possible routes to form fat-derived flavour compounds. Adapted from Molimard & Spinnler (1996).

The compounds mentioned in Fig. 1.2, their relevance to cheese flavour, occurrence, formation and unknowns are further separately reviewed in the next sections.

3.2 Fatty acids



As mentioned above, cows' milk fat triglycerides predominantly consist of esterified straight chain, saturated and even-numbered fatty acids, ranging from 2 to 20 carbon atoms in chain length. After hydrolysis, the resulting free fatty acids (FFA) can be important flavour compounds. Especially butyric acid has a "cheesy" flavour note. Butyric acid and other short chain fatty acids (C2 up to C6) can be produced by fat hydrolysis, but also from protein catabolism by lactic acid bacteria.
The aroma threshold of free fatty acids is influenced substantially by the composition of the medium and its pH. Within the group of fatty acids there is considerable variation in polarity,

which is reflected by the taste threshold differences between aqueous and fatty media. The pH has an influence on the taste, as the dissociated fatty acid anions at high pH are less flavour active and less volatile, and are perceived as soapy flavours. Compared to Dutch type and Cheddar cheeses, Camembert and Blue type cheeses contain extremely high levels of free fatty acids. Because of the high pH in the latter cheese types, flavour perception is reduced and fatty acids are not immediately associated with rancid off-flavours, although the risk of soapy off-flavours becomes apparent under those circumstances.
Flavour thresholds in a fatty environment range from about 0.7 (butyric acid) to 15000 ppm (stearic acid) (Siek et al., 1969). In water, thresholds are from 0.5 (octanoic acid) to 50 (acetic acid) (Rothe, Woelm, Tunger & Siebert, 1972). In a fatty medium, a fatty acid mixture consisting of C2 to C12 acids was perceivable, despite that the concentrations were at least 10 times below their individual aroma thresholds, (Siek et al., 1969). Thus, free fatty acids show a strong synergistic effect in fatty media, an effect which will certainly play a role in cheese.
Between different cheeses, enormous differences in FFA levels exist, totalling from 356 mg kg^{-1} in US-made Edam cheese, up to about 32 g kg^{-1} in French Roquefort (Woo, Kollodge & Lindsay, 1984). In contrast to cheese, freshly drawn milk contains only a small amount of free fatty acids (Ahrne & Björck, 1985; Olivecrona & Bengtsson, 1984), ≤ 0.5 µmol FFA per ml (Deeth & Fitz-Gerald, 1994). During storage and by handling the milk, a little hydrolysis will unavoidably occur (Ahrne et al., 1985), and is mostly a reaction catalysed by enzymes – especially milk lipoprotein lipase (LPL) (Olivecrona et al., 1984), see section 3.2.2.

3.2.1 Lipolysis

Lipolysis, the hydrolysis of free fatty acids from triglycerides, can be carried out by two different hydrolytic enzymes: esterases and lipases. Both enzymes catalyse the same reaction: hydrolysis of the ester bond in a glyceride, yielding an acid, the fatty acid, and an alcohol (glycerol), which may or may not be still esterified with one or two other fatty acid residues.

In fact, lipases are a sub-family of the esterases. The difference between the two groups is sometimes based on the chain length of the fatty acid which is split off: esterases are said to liberate fatty acids with chain lengths of 10 carbon atoms and less, while lipases release longer fatty acids. A more fundamental difference is the mode of activation of both enzymes, as described by Verger (1997). In this distinction, esterases (carboxylesterases EC 3.1.1.1) catalyse ester hydrolysis in aqueous environment, with both enzyme and substrate present in the water phase. Lipases (EC 3.1.1.3), on the other hand are present in the water phase, but are designed to direct the active site towards a non-polar plane, and thus work

on the interface between aqueous and fat phase (Arpigny & Jaeger, 1999). Frequently, lipases possess a structural element that covers the (mainly hydrophobic) active site of the enzyme in aqueous solutions. This "lid" may open when the enzyme is in contact with a non-polar substrate as a fat globule. No esterases have been described that posses a similar lid, but while most true lipases are facilitated with such an "interfacial activation" mechanism, some lipases do not, which makes this a less appropriate criterion (Verger, 1997). Other authors argue that there are many exceptions to this division between esterases and lipases, and state that both are carboxylesterases, the lipases splitting off longer chain fatty acids (Beisson, Tiss, Riviere & Verger, 2000; Verger, 1997). In the water phase, the most likely glycerides to be present are monoglycerides, some diglycerides and triglycerides with short chain acid substituents. In practice, this will mean that esterases preferentially liberate short chain fatty acids. Lipases, which work on the lipid/water interface will encounter most frequently the longer chain fatty acids, and therefore will in practice release mostly longer chain fatty acids.

Apart from being present and being active in milk, all hydrolytic enzymes have one other requirement for performing the hydrolytic activity, which is reaching the milk fat. Milk fat is generally present in milk fat globules, whereas the enzymes are primarily present in the aqueous phase, sometimes even bound to or associated with protein. Therefore, the immobility of the enzyme hampers the actual activity of the enzyme. With the removal of calcium ions from the milk by adding a chelating agent, the casein breaks up and LPL activity increases (Olivecrona & Bengtsson, 1991). Furthermore, the milk fat globule membrane protects the fat from enzymatic activity. This layer is about 8 to 10 nm in thickness, and surrounds the fat droplet completely as it passes into the lumen upon synthesis. This membrane consists of protein and phospholipids, and acts as a bridge between the fat and the hydrophilic milk serum phase. This membrane causes the low surface tensions values of 2.5 to 1 mN/m of the milk fat globule, which stops enzymes to penetrate this layer. If the membrane is broken, the membrane reformed will also include serum proteins and casein that have some emulsifying action. As a result, the surface tension will be higher, which enhances the possibilities of LPL to penetrate the membrane and access the fat in the globule, leading to lipolysis (Mulder & Walstra, 1974). In addition, lipase product inhibition, i.e. the association of the produced free fatty acids with the enzyme can also occur (Markweg-Hanke, Lang & Wagner, 1995). Calcium ions, usually abundant in milk, may interact with the free fatty acids and counteract this product inhibition (Olivecrona et al., 1991). Hydrolysis can be catalysed by endogenous milk enzymes, and by enzymes from microorganisms in contact with the milk fat, for example during cheese ripening. The most common endogenic and exogenous enzymes are briefly described below.

3.2.2 Endogenous milk enzymes

Milk lipoprotein lipase (LPL, EC 3.1.1.34) is the most well known fat hydrolysing enzyme from milk. It is produced in relatively large quantities by the cow, having a biological function in the metabolism of plasma lipoproteins at the capillary endothelium, but also in the mammary gland where it affects uptake of blood lipids for milk fat synthesis (Olivecrona et al., 1984). It exists in milk probably only by spilling over from the mammary gland (Olivecrona et al., 1984). It is a true lipase since it is active at the aqueous/lipid interface. LPL is selective for the fatty acids at position one of the *sn*-triacylglycerol, but not specific. Also the 3-position can be hydrolysed, but LPL has a very low activity on the 2-position (Smith & Pownall, 1984). Ovine LPL has a preference for long chain fatty acids, and liberates preferentially C16:0 from ewe's milk fat, while tricaprylin (tri-C8:0) is the preferred model substrate (Chavarri, Santisteban, Virto, Renobales & De-Renobales, 1998). In bovine milk, LPL causes the release of a mixture of fatty acids, mostly depending on their position in the triglycerides (Olivecrona et al., 1991).

In fresh milk, however, the enzyme is largely bound to casein, and usually its substrate is therefore unreachable (Deeth et al., 1994). The substrate is also surrounded by the fat globule membrane, which in practice prevents most LPL action (Walstra, Geurts, Noomen, Jellema & van Boekel, 1999). The membrane causes a very low interfacial tension of the fat globules so that the enzyme cannot penetrate into the fat (Walstra & Jenness, 1984). Therefore, only small amounts of free fatty acids can be found in fresh milk (Ahrne et al., 1985). The protein from the milk fat globule membrane can even inhibit LPL action (Sundheim, 1987b).

Activation of LPL can be accomplished by mechanically damaging the milk fat globule membrane, for example by rough transport, fast pumping or homogenisation of the milk. Even normal handling of milk can cause parts of the milk fat globule membrane to be substituted by plasma proteins, increasing the interfacial tension and the ability of LPL to access the fat (Walstra et al., 1984). These processes are sufficient to cause a substantial LPL activity, and can produce rancid milk, i.e. relatively large quantities of free fatty acids, in a short time, even in minutes (Cartier & Chilliard, 1989; Walstra et al., 1999). Cold storage will cause partial dissociation of the LPL from the casein it was bound to, enabling association with the milk fat globule (Grappin & Beuvier, 1997; Hohe, Dimick & Kilara, 1985). The biological way of activating is the addition of heparin, which releases the bond between casein and LPL, and allows the LPL to migrate to the fat globules (Sundheim,

1987a; Sundheim & Bengtsson-Olivecrona, 1985). Also apoprotein CII stimulates the activity of LPL in milk by enabling LPL to bind to the fat globule membrane (Jensen & Pitas, 1976).
Like most other enzymes, LPL can perform its reaction both ways, the reverse reaction being esterification of free fatty acids to an acyl receptor. For LPL, mono- and diglycerides are suitable receptor molecules, glycerol is not (Olivecrona et al., 1991). LPL binds relatively well to (long chain) free fatty acids, and thus the action of LPL can be hindered by its products, if they are not effectively removed from the proximity of the enzyme (Olivecrona et al., 1984).
LPL is inactivated by pasteurisation, but it is uncertain whether the enzyme is inactivated completely, as some authors suggest (Andrews, Anderson & Goodenough, 1987), or if still a small portion of the enzyme remains active in the pasteurised milk (Pande & Mathur, 1990).
In the cheese environment, even in raw milk cheese, LPL seems to play a role in fat hydrolysis only in the very beginning. Vlaemynck (1992) found that LPL caused a 10% increase in free fatty acids versus a heat-treated non-LPL control cheese, and found this change to affect flavour. Geurts, Lettink & Wouters (2003), showed that the enzyme rapidly loses its activity as the pH of the developing curd drops to 4.0-4.5. It was not shown whether the enzyme is destroyed at this pH, or if it is reversibly rendered inactive, regaining activity if pH rises to a value more suitable for LPL, but it is known that below pH 6, LPL is essentially inactive (Cartier, Chilliard & Bout, 1989).
In conclusion, despite of its high potential activity in fresh milk, the largest effect of milk LPL will be the release of free fatty acids in milk that has been mechanically handled in some way. A substantial fraction of the free fatty acids, the more lipophilic the better, will be retained in the curd and will be present in the resulting cheese. In the first hours, or maybe first day of cheese ripening, some activity can be expected, but after that period the enzyme will not play a role in free fatty acid formation in Gouda-type cheeses, mainly because of the low pH throughout ripening.

3.2.3 Psychrotrophic lipases

Cold storage of raw milk on the farm has effectively removed the spoilage of milk by lactic acid bacteria, but the longer time before collection and pasteurisation of the milk has produced another possibility of microbiological spoilage. These so-called psychrotrophic bacteria grow at temperatures below 7°C and can produce extracellular enzymes, including lipases (Stead, 1986). The main psychrotrophic bacteria that most frequently produce extracellular lipases in milk are *Pseudomonas fluorescens* and *Pseudomonas fragi*, but also other species (particularly *Serratia* and *Acinetobacter*) have been described to produce lipases that can be active in milk (Deeth et al., 1994). Only little lipase is produced if these organisms are present below $10^6 – 10^7$ cells per ml milk, and pasteurisation will eliminate the

living psychrotrophic bacteria from the milk. Once formed, the notorious heat-stability of the (lipolytic) enzymes causes problems in their inactivation (Deeth et al., 1994). Most enzymes retain activity after pasteurisation, and even UHT treatments can be insufficient to inactivate these lipases (Chen, Daniel & Coolbear, 2003; Downey, 1980). Due to their association with caseins or fat globules, the lipases will not be removed with the whey if cheese is prepared from the milk, and will thus be concentrated in the curd. They can remain active for 6 to 8 months in Cheddar cheese (Stead, 1986). In contrast to LPL, it seems that these lipases can act on milk fat globules with intact membranes, but it is unsure whether this is due to the properties of the enzyme or the activity of accompanying enzymes, such as glycosidases, proteases or phospholipases (Deeth et al., 1994).

The lipases from *Pseudomonas* spp. have a preference for the fatty acids at the primary position of the triglyceride, and for substrates at the water/lipid interface, whereas its acyl specificity is very low (Deeth et al., 1994). A culture isolated from milk, a *Pseudomonas fluorescens*-like strain, produced mainly butyric, palmitic, stearic and oleic acid when incubated in milk (McKay & Beacham, 1995). The optimum conditions for these lipases are not found in cheese ripening conditions, as the pH optima are around 7-9 and the temperature optima are 40-50°C (Law, 1979).

Due to their long-lasting activity and their ability to act on membrane-covered fat, these enzymes are likely to play a role in fat hydrolysis during cheese ripening, but only if they have formed in the milk. Using proper hygienic conditions, formation of these psychrotrophic enzymes can usually be avoided, and except for some individual cases, no large influence can be expected.

3.2.4 Exogenous lipolytic enzymes

Pre-gastric esterase (PGE) is a lipolytic enzyme produced by calf and other mammals, and is also called oral lipase. This lipase is traditionally present in the rennet used in the preparation of hard Italian cheeses such as Provolone and Romano. The enzyme is called an esterase, which implies it works on substrates in solution. However, it was shown that a PGE preparate could liberate both long and short chain fatty acids, both from substrates in solution as well as emulsified substrates, and it would thus qualify as lipase. Since the enzyme has a strong preference for hydrolysis of butyric acid and other short chain fatty acid residues from emulsified triglycerides, it is regarded as an esterase. The enzyme has a preference for the *sn*-3 position of the triglyceride, but also works well on substrates in solution like mono and di-glycerides, tributyrin (tri-C4:0) and synthetic fatty acid esters (Nelson, Jensen & Pitas, 1977). When applied to cheese, flavour development is significantly altered, and the flavour is usually described as "piccante", indeed due to substantial amounts of butyric and other short chain fatty acids from milk fat triglycerides (Fox et al.,

1991). An alternative for PGE is a lipase from *Mucor miehei*, which shows strong similarities to PGE (Fox et al., 1991). In most other cheese types, PGE is not used. The rennet used for most other cheese types is usually a preparation from calf stomachs, mainly containing chymosin, a proteolytic enzyme. Because of its source, it is not unthinkable that traces of the strongly lipolytic PGE are present. However, normally commercial rennet preparations contain no lipolytic activity (Collins et al., 2003b).

3.2.5 Lipases from microorganisms

3.2.5.1 Lipases from typical Gouda cheese starter organisms

It has long been known that lactic acid bacteria are only weakly lipolytic (Fryer, Reiter & Lawrence, 1967) (Khalid & Marth, 1990; Stadhouders & Veringa, 1973). However, in Dutch-type cheeses, studies showed that despite the generally low lipolytic and esterolytic activities of the lactic acid bacteria used, they are still the major free fatty acid producing agents (Collins et al., 2003b), and thus the most important agents to consider in this section. The reason that the enzymes from lactic acid bacteria are so important consists of multiple arguments. The most important reason is that there is no other significant fat hydrolysing system present throughout the period of cheese ripening. Furthermore, despite their relatively low activity, they have the ability to act. Meyers, Cuppett & Hutkins (1996) showed that some strains of lactic acid bacteria are weakly lipolytic, possessing esterases, active on short chain fatty acid residues, and true lipases, active on insoluble long-chain triacylglycerides, which confirms earlier studies (Fryer et al., 1967). In general, starter bacteria are not very active against triglycerides, but they do have a significant effect on the liberation of fatty acids when mono- and diglycerides are present. These can be prepared due to prior action of LPL or other lipases (Stadhouders et al., 1973). It was shown that lactic acid bacteria in fresh cheese are mainly localised in direct contact with the fat globule membrane, or at the fat/casein interface. In older cheese, the bacteria seemed to be even embedded in the fat globule membrane (Laloy, Vuillemard, El-Soda & Simard, 1996), enhancing the chance of cell wall associated esterases or lipases to access the substrate. Most lactic acid bacteria will show cell lysis at some point of cheese ripening, and lactic acid bacteria that contain an intracellular esterase thus contribute to some extra possible lipolysis (Crow et al., 1993).

The esterases and lipases from lactic acid bacteria may vary by strain, and quite some research has been carried out to characterise the different enzymes. It was shown that different lactococcus strains usually contain similar but low levels of esterases (Crow, Holland, Pritchard & Coolbear, 1994). A substantial part of the esterase activity was located on the cell surface (Crow et al., 1994), but this varies with different strains. An esterase from *Lactococcus lactis* subsp. *lactis* was characterised by Chich, Marchesseau & Gripon

(1997), but this was reported to be an intracellular enzyme, and was presumed to play a minor role in cheese fat lipolysis. An esterase from *Lactococcus lactis* subsp. *cremoris* E8 was purified and further characterised by Holland & Coolbear (1996), and it was concluded that this enzyme was a tributyrin esterase. Fernandez et al. (2000) reported that the tributyrin esterase from *Lactococcus lactis* was active on tributyrin, but to some extent also on medium and long chain fatty acid residues from milk fat. Because of the intermediate characteristics, it was difficult to classify the enzyme into either esterase or lipase. In general, it can be said that lactococci produce esterases, and that some strains produce an enzyme inclined to behave lipase-like.

Perhaps more interesting is the actual action of the bacterial enzymes in cheese. Early studies by Kleter (1976) showed that aseptically prepared Gouda cheeses with only *Lactococcus cremoris* E8 showed essentially no FFA development in 6 months of ripening. However, other studies showed that *Lactococcus* strains could contribute significantly to the lipolysis in aseptic Cheddar cheese slurries. Some of the strains investigated were capable of liberating FFA up to C12, whereas others were not (Wijesundera, Roberts & Limsowtin, 1997), indicating that the difference in the FFA profiles was actually caused by the bacteria and not by residual LPL or other factors. *Lactococcus cremoris* and *Lactococcus lactis* showed true lipase activity in model systems with emulsified fat. The cell free extracts showed a preference for short-chain FFA, but were also active on C18 residues in the emulsion (Kamaly, Takayama & Marth, 1990).

Collins, McSweeney & Wilkinson (2003a) conducted an experiment using 2 strains of *Lactococcus lactis*, which cell free extracts showed similar activity against milk fat, but one of the strains was more susceptible towards cell lysis. The cheese with the easily lysing strain showed more FFA (C8-C18), which indicates that intracellular *Lactococcus lactis* esterase may play a role in FFA formation during cheese ripening, but not before cell lysis (Collins et al., 2003a). If this esterase causes formation up to C18, it might almost be considered having a lipase-like activity.

3.2.5.2 Lipases from non-starter and non-Gouda cheese organisms

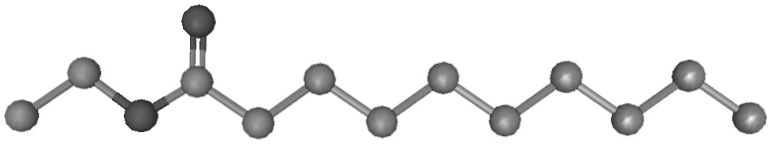
Non-starter dairy-related microorganisms are also capable of fat hydrolysis. For example, *Lactococcus thermophilus* contained about double the amount of esterases compared to several strains of *Lactococcus lactis* subsp. *lactis* and *cremoris* (Crow et al., 1994). Lipolytic activities were also found in some *Lactobacillus* strains. Some of the cultures investigated were active on trilaurin (tri-C12:0), and on emulsified milk fat (El-Soda, Korayem, Ezzat & Soda, 1986), suggesting a lipase-like activity. Another *Lactobacillus*, *Lactobacillus casei* subsp. *casei* IFPL731 produced an intracellular esterase that was active on naphtyl-esters from C4 up to C14 (Castillo, Requena, Fernandez-de-Palencia, Fontecha & Gobbetti, 1999). Fenster, Parkin & Steele (2000) showed that a cell free extract from *Lactobacillus helveticus*

was mainly active on water-soluble short chain FFA synthetic esters. Gobbetti, Fox & Stepaniak (1997) purified several esterases from *Lactobacillus plantarum*. These proved to be most active on tributyrin, but were to a lesser extent also capable of hydrolysing tricaprylin and milk fat.
A characterised esterase from *Propionibacterium freudenreichii* subsp. *shermanii*, which is a characteristic starter species for Swiss-type cheeses, hydrolysed tributyrin, but was inactive against long chain fatty acid residues (Suoniemi & Tynkkynen, 2002). Lipolysis is rather low in Emmental cheese, also due to the 'cooking' step, inactivating LPL in a very early stage. However, Chamba & Perreard (2002) concluded that the lipolysis that occurred was due to the propionic acid bacteria, that predominantly produced free long chain fatty acids. This discrepancy shows that there are large strain-to-strain differences, and maybe even differences in lipolysis due to differences in cultivation, or perhaps large differences in lysis behaviour.
Yeasts and moulds possess usually a much stronger lipolytic activity than bacteria. *Penicillium camemberti* and *Penicillium roqueforti* cause very characteristic flavours in the cheeses they are used in, due to the production of methyl ketones from free fatty acids (see section 3.5). To liberate these fatty acids, both produce potent lipases, two related alkaline lipases, operating optimally around pH 8, while *Penicillium roqueforti* also produces an acid lipase, having an optimum around pH 6.5. Their activity is highest against tributyrin, but also works on larger triglycerides (Cerning, Gripon, Lamberet & Lenoir, 1987). These species are used only in specific cheese types, and will not be further discussed here. Lipases exist also from many other (micro)organisms, and can posses all thinkable activities and selectivities. They are generally not used in cheese production, and information about these enzymes can be found elsewhere (Ha & Lindsay, 1993; Law & Wigmore, 1985; Villeneuve & Foglia, 1997).
Some authors suggest the possibility of interpreting the free fatty acid profile in the dairy product to determine the lipase or esterase that has been active (Deeth et al., 1994; Woo & Lindsay, 1983). Indeed, some of the potent lipases have clear and different characteristics, mainly with respect to stereo-selectivity (Rogalska, Nury, Douchet & Verger, 1997). However, the situation in milk, with protein-derived short chain fatty acids, and possibly multiple lipases/esterases with rather low activity and low acyl specificity is probably too complex for certain conclusions.

From these reports, it may be concluded that lactococci and lactobacilli contribute to FFA formation in cheese, as all strains will produce an esterase, or maybe even an esterase with affinity for long-chain fatty acids, capable of (slowly) accessing its substrate and perform hydrolysis. However, there is a strong strain-to-strain difference in enzyme production,

activity and selectivity. Apart from that, the variance in cell lysis, causing the release of intracellular enzymes, seems to be an important characteristic.

3.3 Esters



Esters are common volatiles in a very wide range of cheeses. Ethyl esters occur most commonly, but most other combination between available carboxylic esters and alcohols, as well as thioesters can be found in cheese (Liu, Holland & Crow, 2004b). Low levels of esters generally contribute to a positive overall cheese flavour, but too high concentrations can cause a fruity-type off-flavour. Short chain ethyl esters are described as apple or pineapple-like (Liu, Holland & Crow, 2003a). Other (ethyl) esters have been described as "fruity", "wine-like", "brandy", "floral", and "waxy/soapy" for the longer chain ethyl esters (Liu, Baker, Bennett, Holland, Norris & Crow, 2004a). In water, ethyl ester thresholds are at or slightly below ppm levels, for the whole series from ethyl acetate to ethyl dodecanoate (Rothe et al., 1972). Due to their lipophilic nature, their aroma threshold values in fatty systems and cheese are expected to be higher. Esters show considerable synergy with respect to their flavour (Liu et al., 2004b).

The formation of an ester from an acid and an alcohol is a reversible process, and depends heavily on the water activity of the system. Low and high pH speed up the reactions, and lead to a faster equilibrium. While it is possible that esters are formed non-enzymatically, the main ester formation in cheese ripening will be the enzymatically catalysed, simply because this formation is faster at ambient temperatures. In cheese, bacterial esterases play a main role in the formation and hydrolysis of esters. Liu, Holland & Crow (1998) showed that ethyl butanoate was produced by many starter and non-starter lactic acid bacteria, but in widely varying concentrations. This indicated that different enzymes had noticeable effects over the non-enzymatic ester formation or hydrolysis. Thus, many lactic acid bacteria produce esterases that are capable of ester synthesis, with varying formation rates. For ester formation, water activity is also important, as it influences the balance between esters and alcohols and acids. The water activity in cheese can be as low as 0.70, but is generally above 0.90 in most cheeses, which makes ester formation unfavourable. As cheese is not a homogeneous system, it is suggested that "low-water" zones exist, in which ester formation is more advantageous (Liu et al., 2004b). For both ester synthesis and hydrolysis, obviously esterases can play a role, but also a true lipase (from *Rhizomucor miehei*) can synthesize ethyl butanoate from ethanol and tributyrin in an aqueous medium (Liu et al., 2003a).

The production of esters can be done by esterification of a free carboxylic acid and an alcohol. It is also known that esters may be modified by alcoholysis (transfer of the acid group in an ester to another alcohol), acidolysis (transfer of the alcohol group in an ester to another free acid), and transesterification (exchange of the alcohol/acid group between two esters). These ester formation pathways are visualised in Fig. 1.3. In cheese, the most predominant esters are in fact the (tri)acylglycerols, and if alcoholysis (usually with ethanol) or transesterification occur, this may result in the production of volatile esters.

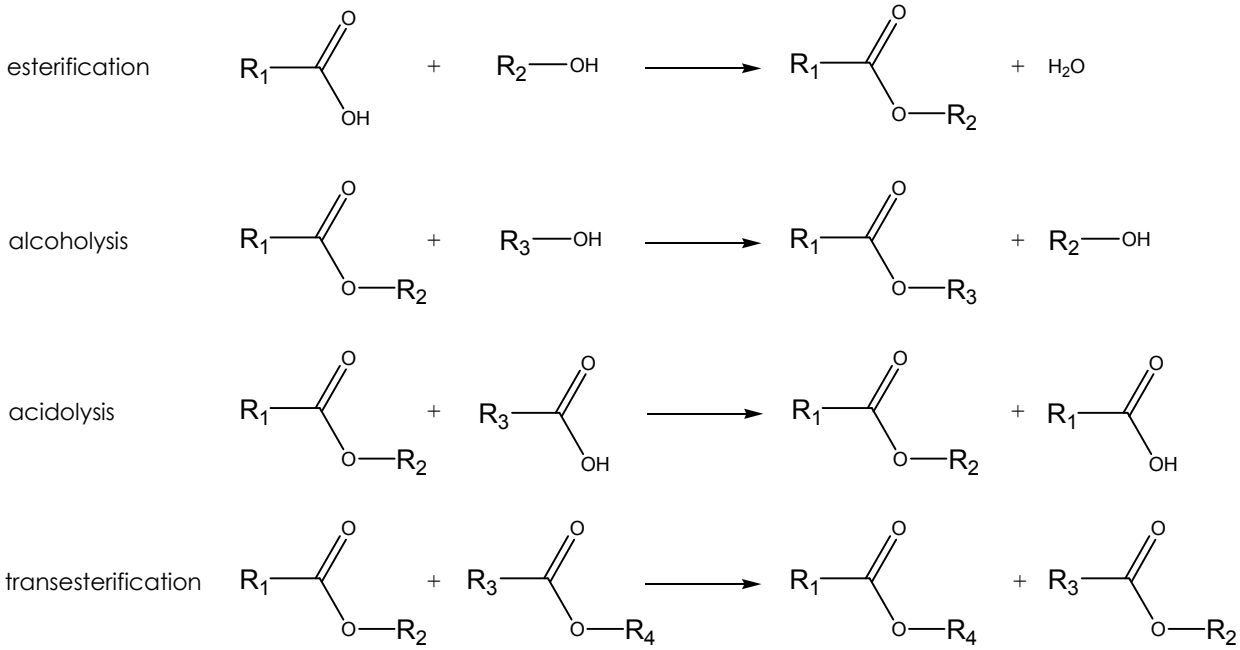
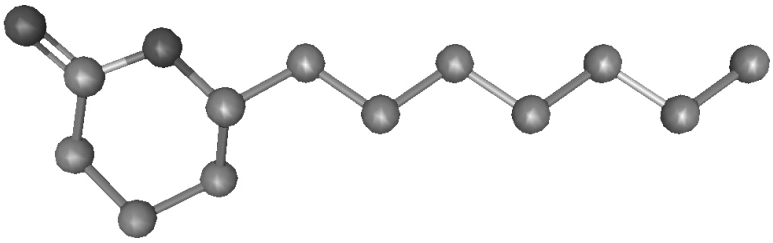


Fig. 1.3. Mechanisms for ester formation and modification. From Liu et al. (2004b).

Recently, it was shown that *Lactococcus thermophilus* and *Lactococcus lactis* subsp. *cremoris* possess esterases that can catalyse both hydrolysis, liberating free fatty acids, and alcoholysis (Liu et al., 2004a; Liu, Holland & Crow, 2003b). It is likely that esterases from some other possible cheese organisms possess similar characteristics. In aqueous environment, ester synthesis through alcoholysis gives much higher esters concentration than esterification, and it is likely that this process is the main responsible for ester formation in semi-hard cheese types (Liu et al., 2004b). Despite of this enzymatic potential, in semi-hard type cheeses as Cheddar and Gouda, ethanol or other alcohols are generally the limiting factor in ester synthesis (Liu et al., 2004b), as acid(-precursor) and enzymes are usually present.

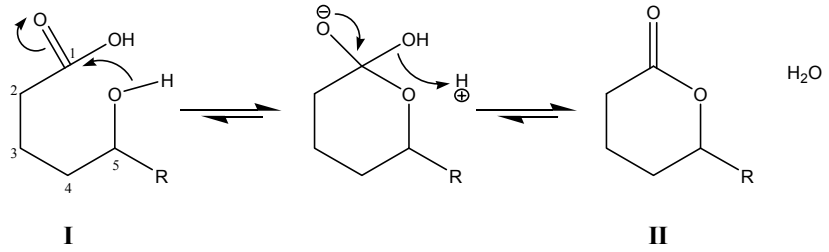
3.4 Lactones



Lactones are intramolecularly esterified hydroxy fatty acids, as shown in the figure above. In milk and milk products (butter), the lactone species commonly found are 5- and 6-membered rings, γ- and δ-lactones, respectively, which are the lactonisation (or esterification) products of 4- and 5-hydroxy fatty acids, respectively (Dufosse et al., 1994). The length of the aliphatic side chain is variable, up to 18 total carbon atoms in lactones have been reported. Lactones are flavour-active compounds. Sensory descriptions include coconut-like (Mattick, Patton & Keeney, 1959), fruity (Dufosse et al., 1994), peach (Derail, Hofmann & Schieberle, 1999) and peach-abricot (Moio, Dekimpe, Etievant & Addeo, 1993).
Aroma thresholds are low in water systems, from 0.02 (γ-C11) to 0.5 (γ-C7) and 0.1 (δ-C12) to 0.5 (δ-C8) ppm (Siek, Albin, Sather & Lindsay, 1971). In the fatty environment of deodorised butter oil, Siek et al. (1969) found thresholds from 0.95 (γ-C11) to 8 (γ-C6) and 1.4 (δ-C10) to 500 (δ-C14) ppm. Especially for the longer lactones, reported thresholds in fatty media vary, and can vary by a tenfold (Kinsella, 1975; Urbach, Stark & Forss, 1972). Also, lactones in fatty media show some degree of synergism, lowering their combined aroma threshold (Kinsella, 1975; Siek et al., 1969).

Lactones are known compounds in butter (Boldingh & Taylor, 1962; Kinsella, 1975) and in cheese, and in fact in many more foods and food products, such as beer, wine, spices, fruits, meat and some vegetables (Dufosse et al., 1994). Lactones are virtually absent in fresh milk (Moio et al., 1993; Moio, Etievant, Langlois, Dekimpe & Addeo, 1994), and as lactones are present in cheese, they must be formed during ripening (Wong, Ellis & LaCroix, 1975).
It is also known that lactones are formed when milk fat is heated (Parliment, Nawar & Fagerson, 1966; Moio et al., 1994). However, after extensive heating the formation slows down to finally stop at a certain level of lactones. The concentration thus obtained has been known as the lactone potential (Urbach & Stark, 1978). The diet of a cow is of influence on the lactone concentrations that are formed during heating (Stark, Urbach, Cook & Ashes, 1978; Urbach, 1982; Urbach et al., 1978), as well as the season (Dimick & Walker, 1968) and the stage of lactation (Dimick, Walker & Patton, 1969).
While it is known that extensive heating yields lactones, the formation mechanism is not yet known. Moreover, it is unclear whether the formation mechanism at the relatively low temperature of cheese ripening (5-20°C) is the same reaction that occurs at temperatures of typically 160°C, at which the full lactone potential can be released into free lactones. As

stated before, free hydroxy fatty acids may spontaneously lactonise into lactones, an equilibrium reaction which lies to the lactone side (Boldingh et al., 1962; Kinsella, 1975), as drawn as an example in Fig. 4.



I **II**

*Fig. 1.4. Spontaneous lactonisation of a 5-hydroxy fatty acid (**I**) to a δ-lactone (**II**). R=H, or $(CH_2)_n$-CH_3.*

The source of free hydroxy fatty acids is then still unknown. Most authors assume that free hydroxy fatty acids are incorporated in milk fat triglycerides (Jurriens & Oele, 1965; Wyatt, Pereira & Day, 1967), and that lipases or esterases release them from the fat, yielding a lactone (Mattick et al., 1959). It is known that hydroxy fatty acids exist in triglycerides from various sources (Ahmad, Maier, Mukherjee & Mangold, 1987; Schwartz & Rady, 1992). It was shown that hydroxy acid triglycerides are normally synthesised from acetate, in a similar manner as normal saturated fatty acid triglycerides (Walker, Patton & Dimick, 1968). In milk fat, due to the position of the hydroxy group, only a fraction of the hydroxy fatty acids is suitable to form γ- or δ-lactones (Chance, Gerhardt & Mawhinney, 1998). Also, lipolytic enzymes are known that can catalyse hydroxy acid deesterification from triglycerides, although no dairy related organisms are mentioned (Hayes, 1996). When a lipase from *Penicillium roqueforti* was added to cheese curd, the development of lactones was faster in the ripening cheese than in the control (Jolly & Kosikowski, 1975). Hydroxy fatty acids can also be formed through oxidation of unsaturated fatty acids. While this can be a source of hydroxy fatty acids, unless the position of the hydroxy group could shift freely across the aliphatic chain, the positions of the double bonds in the unsaturated fatty acids in milk fat make it quite impossible to obtain the distribution and quantities of lactones that are found in butter and cheese.

Milk fat naturally contains oxo-fatty acids (Brechany & Christie, 1992; 1994), and in theory it is possible that these are reduced into hydroxy acids, which, in turn, might lactonise.

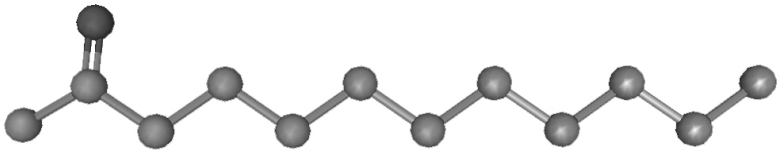
However, lactones are chiral compounds, and occur both in butter and in cheese predominantly in the R-conformation (Palm et al., 1991), except for δ-octalactone, which is found in the S-conformation (Palm et al., 1991). As most non-enzymatic reactions yield racemic mixtures, the formation mechanism is probably of biological nature. As the lactonisation (esterification) of a free hydroxy fatty acid will retain its conformation, the stereo-preferential requirement is probably also fulfilled if the hydroxy fatty acids are formed

enzymatically. This all agrees with the theory that lactones in cheese and butter are formed after a hydroxy fatty acid residue is liberated from the milk fat by a lipase or esterase, which then (spontaneously) lactonises.

One other possibility of lactone formation was reported by Wanikawa, Hosoi & Kato (2000) and Wanikawa, Shoji, Hosoi & Nakagawa (2002). In these reports is shown that oleic acid is oxidised by either yeasts (baker's yeast) or lactic acid bacteria (*Lactobacillus casei*, *Lactococcus casei*, *Lactobacillus plantarum*, and via a number of steps (including ß-oxidation steps, (Schulz, 1991)) to hydroxy fatty acid and lactone. This would be a possibility of lactone formation, while still the variety of lactones found in cheese and butter would be difficult to explain based on the unsaturated fatty acids present in milk fat. A similar reaction was described by Gocho, Tabogami, Inagaki, Kawabata & Komai (1995), using an unknown Gram-positive rod to convert oleic acid to 10-hydroxystearic acid, and baker's yeast to convert this to γ-dodecalactone (Gocho et al., 1995). Haffner & Tressl (1996) described the transformation of oleic acid into γ-decalactone, γ-dodecalactone and 6-γ-dodecelactone by *Sporobolomyces odorus*, and it is also reported that *Penicillium roqueforti*, well known for its formation of methyl ketones, can also produce lactones (γ-dodecalactone and 6-γ-dodecelactone) from soybean and copra oil free fatty acids. No pathways were proposed (Chalier & Crouzet, 1992), but it is likely to be similar to the previously mentioned pathway from oleic acid (Wanikawa et al., 2002).

To conclude, lactones are known possible flavour-active compounds, common in cheese and other dairy products, but their formation is yet unknown. An unknown non-enzymatic formation at high temperature is known, but also a range of enzymatic pathways have been described.

3.5 Ketones



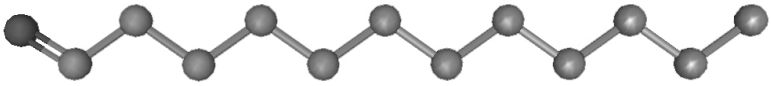
Ketones, and especially 2-ketones or methyl ketones are predominant and well known in Blue type cheeses, but occur in most other cheeses. Also heated milk (UHT) contains 2-ketones, which have even been described as important sensory compounds (Contarini & Povolo, 2002; Moio et al., 1994). They have also been described in milk that has been stored for long times (Arnold & Lindsay, 1969; Hall, Andersson, Lingnert & Olofsson, 1985; Tamsma, Kontson & Kurtz, 1974). 2-Ketones have been described as "fruity", "musty", "green", and "herbaceous" (Molimard et al., 1996). A specific ketone, 2-heptanone, is described by "sweet-bitter with anise note" (Gibka, Gora, Glinski & Kijeinski, 1999), and by "Blue cheese", or "Roquefort cheese" (Molimard et al., 1996), which tells a great deal about where to find these compounds and their importance.

Methyl ketone aroma thresholds are low in water, ranging from 0.03 (C13on) to 3 (C5on) ppm (Rothe et al., 1972). In deodorised butter oil, 2-nonanone is detectable at 9 ppm, while longer and shorter ketones show aroma thresholds around 100 ppm (Kinsella, 1975). Ketones show a very high degree of synergy with respect to aroma thresholds, a mixture of ketones was detectable when the ketones were present at 10 to 100 times below their individual threshold in butter oil (Siek et al., 1969).

Two possible routes for the formation of 2-ketones has have been described, an enzymatic route and a non-enzymatic route. The most well known route is the enzymatic route, which has extensively been reviewed by Kinsella & Hwang (1976). In moulds, free fatty acids are toxic probably due to acidifying effect of the diffusion of undissociated free fatty acids through the cell wall membrane, which then splits off a proton in the intracellular environment. This effect has been described for *P. roqueforti* (Dartey & Kinsella, 1973), and for bacterial systems, for example in *Lactococcus lactis* (Costilow & Speck, 1951). Unlike most lactic acid bacteria, moulds possess a detoxification mechanism for free fatty acids (King & Clegg, 1979; Okumura & Kinsella, 1985). The (unsaturated) free fatty acid is converted into a ß-ketoacid, via ß-ketoacyl-coenzyme A. This complex can be used in the ß-oxidation pathway (Schulz, 1991), which shortens the acyl chain by removing an acetyl moiety from the chain. The remainder can eventually be decarboxylated to form a 2-ketone (Urbach, 1997b). Apparently, this process can also been done by spores, as spores from *Penicillium roqueforti* could produce odd numbered methyl ketones ranging from C3 up to C11 from ^{14}C labeled lauric acid (C12:0) (Dartey et al., 1973). Formation from free fatty acids by encapsulated spores of *Penicillium roqueforti* (Pannell & Olson, 1991), but also from *Penicillium camemberti* in a model system with milk fat yielded free fatty acids, and mainly 2-nonanone and 2-heptanone (Okumura et al., 1985). Addition of a non-specific lipase to this model system increased the level of ketones produced, but it is clear that the majority of the ketones produced are produced from aliphatic fatty acids by the *Penicillium*, rather than from milk fat's esterified ß-keto acids (Okumura et al., 1985), which may also be precursors of the ketones. Production of ketones in model systems eventually becomes limited by the absence of intracellular free fatty acid (Larroche, Besson & Gros, 1996). Methyl ketones, in turn, can be reduced into their alcohol derivates by several heterofermentative lactobacilli and *Leuconostoc* species (Maconi & Aragozzini, 1989). These alcohols have aroma notes comparable to their ketone precursors (Urbach, 1997b).
As mentioned above, 2-ketones are also formed during storage of (UHT)-milk and during heating itself. This formation is unlikely to be enzymatic, and therefore another formation mechanism must be considered. As with lactones (section 3.4), heating of milk (fat) yields a certain concentration of ketones that does not further increase after intensive heating. This concentration is described as the ketone potential (Urbach et al., 1978). This potential is

unique for each portion of milk fat, and might vary with the diet of a cow (Stark et al., 1978; Urbach, 1982; Urbach et al., 1978), The potential is also dependent on the season, and is highest in mid-winter, lowest in summer (northern hemisphere) (Dimick et al., 1968). Furthermore, the potential increases with the stage of lactation (Dimick et al., 1969).
While this is known, it is not really recognised how the potential can be used to from 2-ketones. It is unsure if the formation also occurs at temperatures normally applied in cheese ripening. While the enzymatic formation of ketones is fairly well known and will occur when the proper organisms are applied to the cheese, the kinetics of the non-enzymatic formation are yet unknown.

3.6 Aldehydes



Aldehydes are important aroma compound in ageing (oxidising) butter (Widder, Sen & Grosch, 1991). Furthermore, cheese prepared from milk with a high unsaturated fatty acid content was more susceptible to oxidation, but could still produce a cheese acceptable in flavour (Lightfield, Baer, Schingoethe, Kasperson & Brouk, 1993).

Aldehydes are very strong flavour compounds. Their aroma thresholds in water range from 7 to 50 ppt (parts per trillion) (Hall et al., 1983). In butter oil the thresholds are at 0.04 to 1 ppm (Kinsella, 1975). Unsaturated aldehydes in butter, such as the 2-enals have thresholds around 1 ppt, while 2,4-dienals have reported aroma thresholds even below 0.1 ppt (Kinsella, 1975).

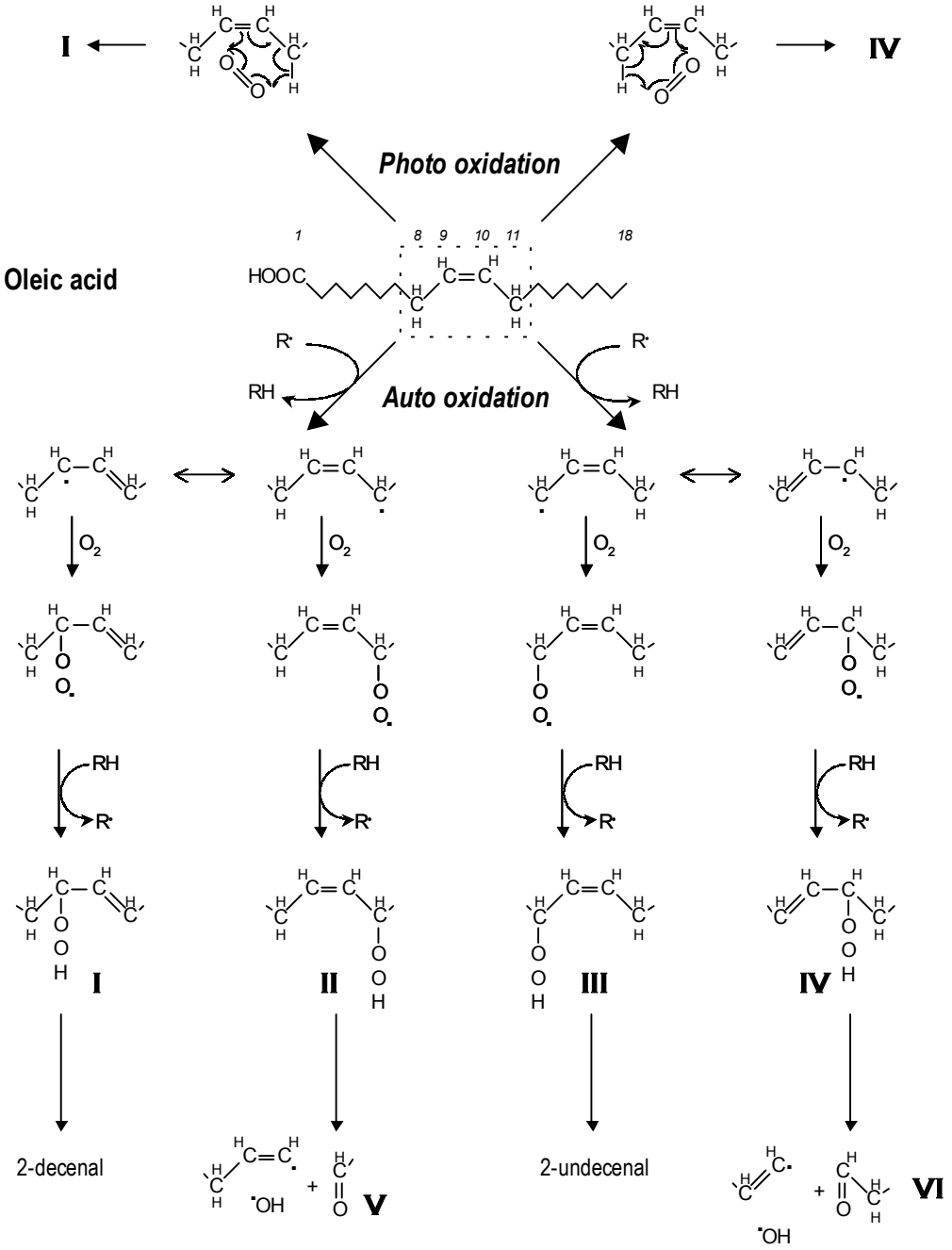


*Fig. 1.5. Simplified reaction scheme for photooxidation and autooxidation of unsaturated fatty acids, using oleic acid as an example. After production of the intermediate hydroperoxides (**I-IV**), four possible aldehydes are formed; 2-decenal and 2-undecenal, and octanal (**V**) and nonanal (**VI**). Adapted from Belitz & Grosch (1987).*

Unsaturated fatty acids, both free and esterified, are susceptible to oxidation (Walstra et al., 1984). A simplified reaction scheme with free oleic acid as an example is given in Fig. 1.5. This shows the two possible modes of oxidation. Photooxidation is the cycloaddition of oxygen to the double bond of oleic acid or other unsaturated fatty acids. Molecular oxygen

in its ground state will not react with unsaturated fatty acids, and needs to be in its high-energy singlet state, which can be accomplished directly by light absorption, or through the action of an activated sensitiser, such as riboflavin for example. This yields the relatively unstable hydroxyperoxides (**I** and **IV** in the example for oleic acid), the same compounds that can be formed during autooxidation.

Autooxidation is a quite complex phenomenon, and will be only briefly discussed here. In essence, it initially relies on the formation of free radicals, generally peroxy (RO_2•), alkoxy (RO•), or alkyl (R•) radicals. These reactive species will react with molecules they encounter, forming a new molecule and a new radical (propagation), until a reaction with another radical occurs (termination). H-atoms next to a double bond are easily abstracted by a radical, and the radical formed is slightly stabilised. This radical may react with molecular oxygen, resulting in a hydroperoxide (the four possible hydroperoxides from oleic acid **I** – **IV** are shown in Fig. 1.5. Hydroperoxides can further react in several ways, yielding several classes of compounds (alkanes, alkenes, acids), but especially aldehydes are interesting with regard to flavour. The major aldehydes from oleic acid are octanal (**V**) and nonanal (**VI**) (Belitz et al., 1987), which is shown in Fig. 1.5.

Short chain aldehydes such as acetaldehyde and 2 or 3-methylbutanal can also be formed by microbial activity, but are not derived from fat or fatty acids (sections 2.4 and 2.5).

The formation of fat-derived, long-chain aldehydes in dairy products seems to be exclusively a non-enzymatic process (Forss, 1979). Photooxidation can be largely avoided with proper precautions, but oxidation can play a role in products that are stored, or ripened, for longer times. The fat in cheese can be susceptible to oxidation, and especially in Blue type cheeses aldehydes occur, since these cheeses have an open structure to supply the mould with oxygen, as this is required for mould growth. In most hard and semi hard type cheeses, the semi-permeable rind and the low redox potential (Urbach, 1997a) limit the available oxygen, which means that fat oxidation will not play a major role. However, with prolonged storage time fat oxidation will occur to some extent.

4. Outline of this thesis

From the above it is clear that many aspects of cheese flavour are not fully understood. Flavour itself, the interactions of all compounds, and the perception during consumption as such is a science on its own. Although in this thesis aroma thresholds will be used to indicate the importance of compounds under consideration, no sensory type of research has been done. From the possible cheese flavour compounds, the fat-derived compounds were subject to study. The occurrence of these compounds has been described in different cheeses, but in many cases the origin and formation kinetics are unknown. The aim of this thesis was to study the changes in amounts of fat-derived flavour compounds during cheese ripening, in order to better understand and possibly control the formation of these compounds.

In chapter 2, a method is described that is able to identify and quantify fat derived flavour compounds from cheese. This chapter also describes the occurrence of the compounds detected in a variety of cheeses. As the occurrence alone gives little information about the actual formation, the formation of these compounds during the ripening of a number of Gouda cheeses was followed up to 2 years of ripening time. The results from this study are reported in chapter 3.

Questions arose if the formation of fat-derived compounds was also influenced by external factors as oxygen, temperature and humidity. Therefore, a smaller ripening study was conducted on cheeses that were packed in air-tight foil during ripening. This packaging has several advantages over the traditional packaging, and a question in this chapter was if the packaging is of influence on the flavour of the cheeses, based on development of fat-derived flavour compounds. This is addressed in chapter 4.

Based on the previous chapters, especially lactones were regarded interesting compounds, with respect to their formation kinetics and possible sensory relevance. It was known that lactones are formed upon extensive heating of milk and milk fat, and that lactones are present in cheese. It was studied whether the formation of lactones during cheese ripening is an enzymatic or a non-enzymatic process, and chapter 5 reports the new insights regarding the lactone formation.

While the formation of 2-ketones in Gouda-type cheeses is only mediocre, it can be important in milk pre-treatment, and in storage of milk-based products. The formation of ketones was studied, and chapter 6 reports the results, along with the consequences for the understanding of ketone formation during cheese ripening.

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Chapter 2

A fast and simple method for quantitative determination of fat-derived medium and low-volatile compounds in cheese

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Abstract

Cheese flavour is a mixture of many (volatile) compounds, mostly formed during ripening. The current method was developed to qualify and quantify fat-derived compounds in cheese. Cheese samples were extracted with acetonitrile, which led to a concentrated solution of potential flavour compounds, mainly derived from milk fat. The solution was virtually free from triglycerides, protein and salt from the cheese matrix. Therefore, such an extract could be analysed directly by gas chromatography/mass spectroscopy (GC/MS). In the samples of the three cheese varieties analysed, 61 different compounds were identified, including 23 fatty acids, 14 lactones, 9 esters, 5 ketones, 10 alcohols, and several miscellaneous compounds. Furthermore, most compounds could be quantified by determining their distribution coefficients and thus correcting for their loss during extraction. This method was shown to be suitable for both qualitative and quantitative analysis of medium and low-volatile compounds.

Keywords: Cheese, flavour, fat, extraction, GC/MS, lactones, ketones, fatty acids, esters, quantification, acetonitrile

1. Introduction

Cheese flavour consists of many different compounds, most of them being formed during cheese ripening. According to their origin, flavour compounds can be divided into three groups, i.e. compounds derived from carbohydrates (lactose), protein and fat, respectively (Walstra, Geurts, Noomen, Jellema & van Boekel, 1999). In ripened cheeses such as Gouda and Cheddar, the pathway of flavour formation for the sugar- and protein-derived compounds has received quite some scientific attention (McSweeney & Sousa, 2000). However, to achieve a balanced cheese flavour, fat-derived compounds are crucial also (Urbach, 1993), and it has been shown that fat is indispensable to the development of the usual flavour in Cheddar cheese (Wijesundera & Drury, 1999). Sensory experiments confirmed the contribution of fat-derived compounds to cheese flavour (Kubickova & Grosch, 1998; Rychlik, Warmke & Grosch, 1997; Buchin et al., 1998; Arora, Cormier & Byong, 1995). Known flavour compounds, presumably derived from fat, include fatty acids, alcohols, methyl ketones, aldehydes, lactones and esters (Molimard & Spinnler, 1996; McSweeney & Sousa, 2000). However, the amounts of these compounds, their development during cheese ripening, and their formation mechanism are poorly understood, due to the limited interest in lipolysis (Walstra et al., 1999), which hardly occurs in cheeses like Cheddar and Gouda. On the other hand, cheese varieties such as Romano, and blue mould cheeses such as Roquefort and Danish Blue, exhibit an extensive degree of lipolysis (Fox, 1993). A satisfactory method to detect all compounds formed from fat has not been available. Due to their high perception threshold, it can be argued that the higher-molecular fat-derived compounds play a small direct role in the total cheese flavour. However, for the elucidation of the pathways for the formation of the lower-molecular weight compounds, detection of all fat-derived compounds would be valuable.

Numerous authors have analysed cheese flavour, and a variety of methods have been used for the extraction of flavour compounds (Mariaca & Bosset, 1997). The most important methods are discussed briefly below.

Dynamic headspace techniques have been used in several studies (Valero, Villasenor, Sanz & Martinez Castro, 2000; Neeter & de Jong, 1992; Bosset, Buetikofer, Gauch & Sieber, 1997; Thierry, Maillard & le Quere, 1999; Izco & Torre, 2000; Villasenor, Valero, Sanz & Martinez Castro, 2000; Barbieri et al., 1994; Careri, Manini, Spagnoli, Barbieri & Bolzoni, 1994; Bosset & Gauch, 1993). In these studies, only the highly volatile compounds were detected. Generally, the presence of acids with up to 8 carbon atoms, and neutral compounds with up to about 12 carbon atoms were reported. It is expected that the heavier molecules have a low recovery using these techniques. Hence, this characteristic makes dynamic headspace techniques less suitable for the analysis of fat-derived compounds.

Static headspace analyses led to a more reproducible quantification, but yielded a very low number of compounds (Partidario, Barbosa & Vilas Boas, 1999; Milo & Reineccius, 1997). For soft-type cheeses, high vacuum distillation has been used, and many neutral compounds could be found. Free fatty acids could not be detected using this method (Gallois & Langlois, 1990; Dumont, Roger, Cerf & Adda, 1974). Simultaneous distillation extraction (SDE) has been used to identify cheese flavour by several authors (Dirinck & de Winne, 1999; Buchgraber & Ulberth, 1999; Barbieri et al., 1994; Careri et al., 1994; Gonzalez de Llano, Ramos, Polo, Sanz & Martinez Castro, 1990). This method tends to extract higher molecular weight compounds from cheese than dynamic headspace techniques, at the expense of losing some of the highly volatile compounds. Yet, the higher molecular weight compounds seem to have a very low recovery, which was not corrected for in the studies cited. Comparisons between dynamic headspace and SDE methods to detect cheese flavour compounds have been made by Barbieri et al. (1994), Careri et al. (1994), and Larráyoz, Addis, Gauch and Bosset (2001). Solid phase extraction has been used to analyse flavour compounds in butter (Adahchour, Vreuls, van der Heijden & Brinkman, 1999). Solid phase micro extraction (SPME) is a relatively new method to detect volatiles, and has been applied to cheese samples (Chin, Bernhard & Rosenberg, 1996). Conditions can be set to extract either highly volatile compounds (Pérès, Viallon & Berdagué, 2001) or fatty acids (Tomaino, Parker & Larick, 2001), but only a relatively narrow range of compounds is extracted. Also, quantification is difficult. Solvent extraction is a very rapid and simple method, and has been used previously by Wong and Parks (1968) and by Vandeweghe and Reineccius (1990). The main disadvantages of this extraction method were the relatively low concentration of compounds in the extract, and the loss of highly volatile compounds due to the overlap with the solvent peak. Virtually all fat-derived compounds dissolve, at least partly, in acetonitrile, while the insoluble cheese matrix can be discarded, enabling gas chromatography. Therefore, if solvent extraction is used to analyse cheese flavour, despite its toxicity, acetonitrile is the preferred solvent.

The objective of this study was to test both the qualitative and quantitative analysis of the fat-derived compounds in cheese, using acetonitrile solvent extraction.

2. Materials and methods

2.1. Cheeses

The cheeses (Gouda, Cheddar and Danish Blue) represented medium aged cheeses within their type, and were purchased at local retailer stores.

2.2. Extraction procedure

Fifteen grams of cheese, after discarding at least 2 cm of the rind, was mixed with 15 g of sodium citrate (Merck, Darmstadt, Germany) and ground in a mortar. The mixture was transferred into a 50 mL Greiner-tube, and 4 mL of acetonitrile (Biosolve, Valkenswaard, the Netherlands) was added. The tube was placed in a water-bath at 45°C for 10 min, with occasional shaking, subsequently centrifuged at 700x*g* for 4 min at 4°C, and kept at 4°C for 15 min. Then the top layer (acetonitrile) was transferred to a 2 mL cup and kept at -20°C for 30 min. The last traces of undissolved triglycerides were removed by a brief centrifugation (2500x*g*, 5 s), and 1 mL of the supernatant was transferred into a GC-vial.

2.3. Gas Chromatography / Mass Spectroscopy analysis

After addition of an internal standard (100 µL, 20µg mL^{-1} myristylbromide, Sigma, St. Louis, MO, USA) to the extract in the GC-vial, 2 µL of the extract was analysed on a 8000^{top} gas chromatograph (CE Instruments, Milan, Italy), equipped with a 25m, 0.15 mm ID FFAP GC-column (d_f = 1.0 µm) (Chrompack, Middelburg, the Netherlands). The gas-flow (helium) was constant at 0.50 mL min^{-1}, and after a splitless time of 100s, the split flow was 15 mL min^{-1}. The initial temperature of 60°C was maintained for 6 min, then raised to 250°C at a rate of 8°C min^{-1}. The final temperature was maintained for 15 min. The injector and transfer line temperatures were 250°C and 280°C, respectively. The mass spectroscopy was carried out with a Finnigan Automass II (Finnigan, Bremen, Germany). Electron impact (EI) spectra were recorded at 70eV. Full spectra (m/z 20-300) were recorded at a scan time of 500ms. Data reprocessing was performed using Xcalibur™ (ThermoFinnigan, San Jose, CA, USA) software.

2.4. Determination of distribution coefficients

To determine the distribution coefficients, enabling quantification in cheese, an extraction as described above was carried out. After removing the first extract, the resulting residue was extracted again (with 2 mL of acetonitrile). Using the data recovered from every two

successive extractions, the distribution coefficient (K) could be calculated, as explained below.

The extraction process was considered a simple two-phase extraction, where each compound was distributed between acetonitrile and the residue (fat, water, salt, protein) in a ratio as described by Equation 1:

(eq 1) $$\mathrm{K} = \frac{C_E}{C_R}$$

where K is the distribution coefficient of a given compound, C_E and C_R are the concentrations of the compound in the acetonitrile extract and residue, respectively. The unknown concentration in the residue (C_R) was given by Equation 2

(eq. 2) $$C_R = M_R \times (V_R)^{-1}$$

where M_R is the mass of a compound in the residue and V_R is the volume of the residue. Using equations 1 and 2, the distribution coefficient for the *n*-th extraction (K_n) was calculated based on the results from two successive extractions (*n* and *n+1*):

(eq. 3) $$K_n = \frac{C_{E_n}}{M_{R_n} \times (V_{R_n})^{-1}} = \frac{C_{E_{n+1}}}{M_{R_{n+1}} \times (V_{R_{n+1}})^{-1}} = K_{n+1}$$

The mass balance for the extraction yielded Equation. 4

(eq. 4) $$M_{R_n} = M_{R_{n+1}} + M_{E_{n+1}}$$

Combination of equations 3 and 4 enabled the elimination of the last unknowns (M_{Rn} and M_{Rn+1}). Assuming that V_R remained constant led equations 3 and 4 to be arranged into an equation for K_n based only on known (measurable) parameters:

(eq. 5) $$K_n = \frac{V_R}{V_{En+1}} \times \frac{(C_{E_n} - C_{E_{n+1}})}{C_{E_{n+1}}} \quad .$$

For all compounds, the K_n-values were calculated from every two successive extractions. K was calculated by averaging K_1, K_2 and K_3. The total masses (M) of the individual compounds in cheese could be calculated, based on the concentration in the first extract, given by Equation 6:

(eq. 6) $$M = C_{E_1} \times (\frac{V_R}{K} + V_{E_1})$$

2.5. Validation of extraction characteristics

To check if there were concentration effects on the value of K, a total of four successive extractions were made. To examine the influence of water and fat content, 5 g of purified sunflower oil or 5 mL of water, was added to 15 g of the same cheese type, and the K-values were determined again by carrying out four sequential extractions.

3. Results and discussion

3.1. Identification of compounds

After analysis of the acetonitrile extracts of the three cheeses a total of 61 different compounds was identified. These were comprised of 23 fatty acids, 14 lactones, 9 esters, 5 ketones and 10 alcohols, besides several miscellaneous compounds. It appeared that the method is suitable for the detection of low to medium volatility compounds. A typical chromatogram of the extract of Gouda cheese, with the major peaks highlighted, is shown in Fig. 2.1. The peaks were generally well separated and gave good responses. Minor peaks, such as those of the methyl ketones or esters, are not visible using the intensity scale in Fig. 2.1. However, these minor peaks are clearly discernible on examination of the chromatogram over a smaller range of intensity values. Only the compounds listed in Tables 2.2 through 2.7 were quantified. Many more compounds have been detected and identified, but were not present at concentrations high enough to enable quantification.

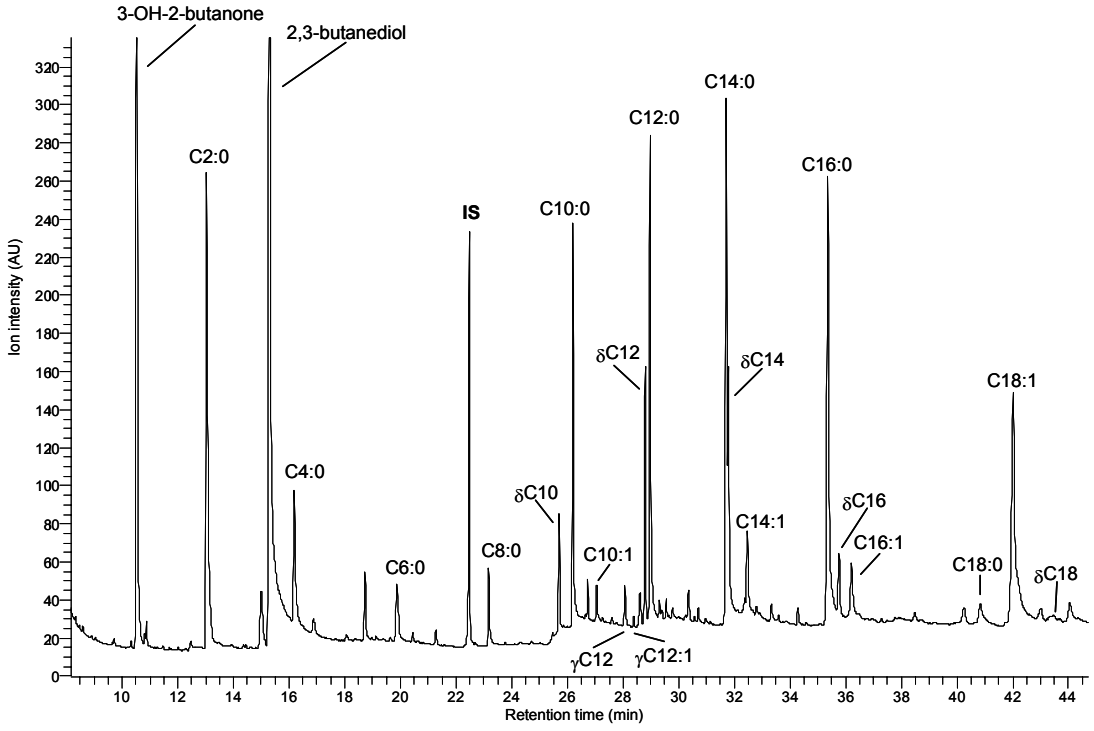

Fig. 2.1. Typical chromatogram of an acetonitrile extract of Gouda cheese. IS = internal standard (myristylbromide), Cx:y = fatty acid with x C-atoms in the chain and y double bonds, γCx or δCx = γ or δ lactone with x C-atoms.

3.2. Validation of extraction

Two fatty acids, two methyl ketones and one lactone were added to Gouda cheese before the extraction, at levels which were approximately twice those naturally present in the

cheeses. After extraction from these cheeses, the concentrations of these compounds were determined and compared with those in the non-spiked samples. After subtracting the latter from the former, the recovery of the added compounds was determined by using the respective K-values as described below. The recovery of the added compounds ranged from 95-102%, with a maximum of 5% error (Table 2.1).

Table 2.1. Recovery of spikes (%) added to medium aged Gouda cheese, calculated after subtraction of the amount of these compounds in the non-spiked cheese. The mass was calculated using the internal standard and the K-values[a] determined for each compound[b].

	Recovery (%)	error (%)
pentanoic acid	101.0	4.3
tridecanoic acid	94.0	2.0
2-nonanone	100.3	2.1
2-tridecanone	101.1	4.9
δ-dodecalactone	97.1	0.5

[a] The distribution coefficient (K-value) of a given compound between organic phase and cheese (see text for details)
[b] Presented values are the means of two independent replicates

To determine the influence of the cheese composition on the extraction performance, the dry matter percentage was set at 45 or 70% (w/w) by adding either water or purified sunflower oil to the sample before extraction. In comparison with the normal cheese, which contained 60% (w/w) dry matter, the extracted amount was for each flavour compound within 80-110% of the concentration found in normal cheese, which was considered to be acceptable (results not shown).

3.3. Fatty acids

Fig. 2.2 shows the distribution coefficients of fatty acids versus their chain length. When extracting with acetonitrile, short-chain fatty acids have a relatively high affinity for water and long-chain fatty acids have a higher affinity for fat. This is reflected by the lower distribution coefficients for both short and long chain fatty acids in Fig. 2.2.

Therefore, medium chain fatty acids are extracted most efficiently, although quantification can be achieved for all fatty acids for which the distribution coefficients were established. Propanoic acid (C3:0) and octadecanoic acid (C18:0) were the limits of the detectable range of fatty acids. Being slightly more polar, unsaturated fatty acids have a little more affinity for the acetonitrile phase during extraction, which results in a slightly higher distribution coefficient than their saturated counterparts. The free fatty acid concentrations in Table 2.2

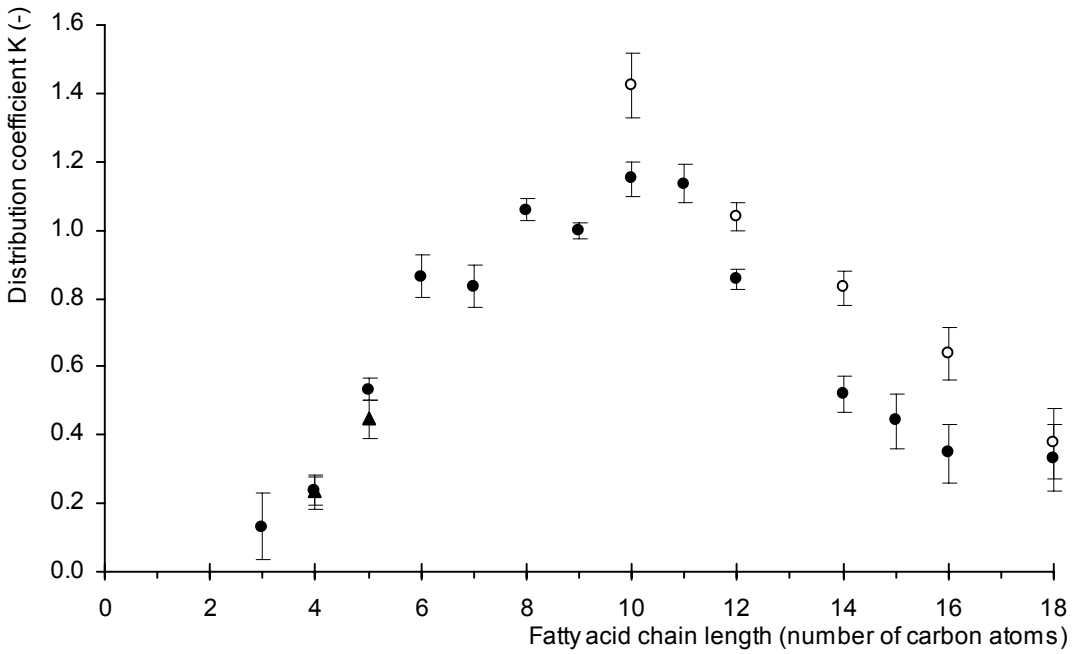

Fig. 2.2. Distribution coefficient (K-value) of free fatty acids versus chain length (number of carbon-atoms) for: (•) saturated acids, (o) unsaturated acids, (▲) branched (iso) acids. Values presented represent the means of two independent replicates; the error bars show the standard deviations of the mean.

were compared with results obtained with the method of de Jong & Badings (1990), which is a method developed exclusively to determine free fatty acids in dairy products. The results presented here showed free fatty acids from C:4 to C16:0 in very similar proportions, although the absolute levels were lower than in the one year old cheese in the former study.

Table 2.2 shows the concentrations of free fatty acids in three different cheese types, Gouda, Cheddar and Danish Blue. In all, even-numbered fatty acids were found in much higher concentration than those with odd-numbered chains. This trend reflects the composition of the fatty acids in the milk fat. The lipolytic activity that liberated these fatty acids was presumably not specific. Concerning free fatty acid content, Cheddar and Gouda cheeses were quite similar. The latter contained a higher concentration of short chain fatty acids (butyric and acetic), which might have sources other than fat (e.g. lactate). On dry matter basis, Danish blue contained higher quantities of all free fatty acids than Gouda and Cheddar. The higher levels in the former are presumably caused by the lipolytic activity of the moulds in this cheese type (Cerning, Gripon, Lamberet & Lenoir, 1987; Molimard & Spinnler, 1996).

Table 2.2. Concentration of fatty acids found in various cheese varieties [a] [b]

	Retention time (min)	K(-)	Gouda		Cheddar		Danish Blue	
acetic acid (C2:0)[c]	13.02	0.06	133	*(7.73)*	15	*(0.11)*	556	*(267)*
propanoic acid (C3:0)[c]	14.64	0.13	0.34	*(0.08)*	0.18	*(0.04)*	419	*(397)*
2-methyl propanoic acid (C4:0i)	15.11	0.23	2.6	*(0.18)*				
butanoic acid (C4:0)[c]	16.17	0.24	12	*(0.94)*	3.9	*(0.12)*	718	*(14.6)*
3-methyl butanoic acid (C5:0i)	16.92	0.45	11	*(0.91)*			37	*(2.18)*
pentanoic acid (C5:0)[c]	18.12	0.53	0.09	*(0.01)*	0.03	*(0.00)*	5.8	*(0.10)*
hexanoic acid (C6:0)[c]	19.86	0.86	3.4	*(0.19)*	1.7	*(0.07)*	469	*(14.0)*
heptanoic acid (C7:0)	21.58	0.83	0.02	*(0.00)*	0.02	*(0.00)*	5.4	*(0.09)*
octanoic acid (C8:0)[c]	23.19	1.06	1.5	*(0.02)*	2.3	*(0.03)*	145	*(2.41)*
nonanoic acid (C9:0)	24.73	1.00	0.06	*(0.00)*	0.08	*(0.01)*	8.3	*(0.08)*
decanoic acid (C10:0)[c]	26.19	1.15	8.0	*(0.19)*	11	*(0.11)*	543	*(6.80)*
undecanoic acid (C11:0)	27.61	1.14	0.09	*(0.00)*	0.13	*(0.00)*	13	*(1.40)*
dodecanoic acid (C12:0)[c]	28.97	0.86	9.9	*(0.12)*	8.6	*(0.03)*	522	*(39.8)*
tetradecanoic acid (C14:0)[c]	31.70	0.52	27	*(0.06)*	27	*(0.19)*	1810	*(163)*
pentadecanoic acid (C15:0)	33.34	0.44	1.5	*(0.06)*	1.6	*(0.04)*	94	*(17.8)*
hexadecanoic acid (C16:0)[c]	35.37	0.35	69	*(0.87)*	76	*(0.03)*	1697	*(323)*
octadecanoic acid (C18:0)[c]	40.82	0.33	2.4	*(0.14)*	2.1	*(0.03)*	59	*(14.2)*
decenoic acid (C10:1)	27.05	1.42	0.80	*(0.02)*	1.3	*(0.00)*	93	*(1.1)*
dodecenoic acid (C12:1)	29.55	1.04	0.89	*(0.02)*	0.72	*(0.05)*	10.0	*(2.5)*
tetradecenoic acid (C14:1)	32.47	0.83	4.2	*(0.16)*	6.4	*(0.24)*	301	*(47.7)*
9-hexadecenoic acid (C16:1)	36.20	0.64	4.5	*(0.09)*	3.9	*(0.18)*	434	*(109)*
11-hexadecenoic acid (C16:1)	36.75	0.39	0.15	*(0.15)*	0.05	*(0.01)*	9.9	*(2.6)*
oleic acid (C18:1)[c]	42.02	0.38	36	*(0.20)*	43	*(0.35)*	5057	*(1228)*
octadeca-di-enoic acid (C18:2)	44.05	0.65	4.5	*(0.06)*	3.4	*(0.24)*	259	*(73.3)*

[a] Values (mg kg^{-1}) are means of two independent replicates
[b] Values in parenthesis represent standard deviation of the means
[c] MS-Identification confirmed by comparison with retention time of authentic compound.

3.4. Lactones

Fig. 2.3 shows the distribution coefficients determined for the lactones versus chain length. Their extractability is rather similar to that of the fatty acids, decreasing towards larger molecular weights due to increasing affinity for fat. However, the decrease in extractability is not observed towards the smaller lactones, because they are less hydrophilic than the fatty acids with comparable chain lengths. Furthermore, no significant difference was observed between the K-values of γ- and δ-lactones. Table 2.3 shows that 14 lactones were identified in the three different cheese types, and that the majority were delta isomers. The presence of the long chain lactones, δ-hexadecalactone and δ-octadecalactone has not been previously reported in cheese. The quantity of total lactones formed in cheese was

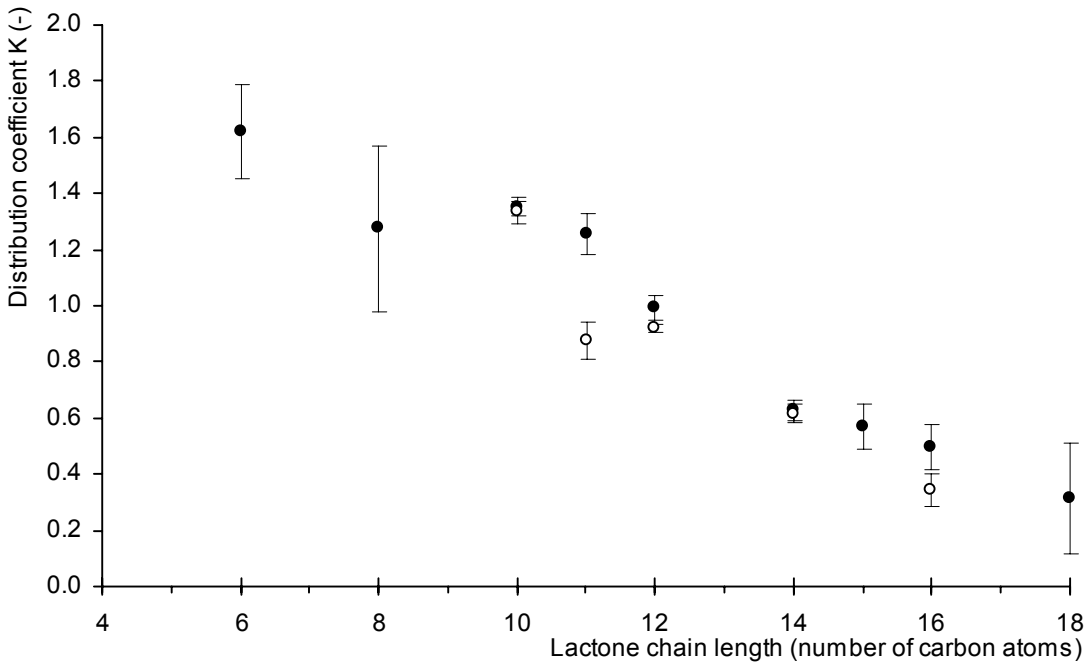

Fig. 2.3. Distribution coefficient (K-value) of lactones versus chain length (number of carbon-atoms) for: (•) delta-lactones, (o) gamma-lactones. Values presented represent the means of two independent replicates; the error bars show the standard deviations of the mean.

Table 2.3. Lactones found in various cheese varieties [a b]

	Retention time (min)	K(-)	Gouda		Cheddar		Danish Blue	
δ-hexalactone	19.64	1.62	0.02	*(0.00)*	0.04	*(0.00)*		
δ-octalactone	22.40	1.28			0.04	*(0.01)*	0.03	*(0.00)*
δ-decalactone	25.70	1.35	1.5	*(0.07)*	1.6	*(0.01)*	0.67	*(0.03)*
δ-undecalactone	27.28	1.26	0.03	*(0.00)*	0.03	*(0.00)*		
δ-dodecalactone[c]	28.79	0.99	3.2	*(0.03)*	3.2	*(0.02)*	2.6	*(0.13)*
δ-tetradecalactone	31.79	0.63	3.9	*(0.01)*	3.6	*(0.05)*		
δ-pentadecalactone	33.59	0.57	0.31	*(0.02)*	0.31	*(0.02)*	2.7	*(0.46)*
δ-hexadecalactone	35.76	0.49	3.1	*(0.17)*	2.9	*(0.12)*	6.6	*(0.28)*
δ-octadecalactone	41.73	0.31	0.24	*(0.04)*	0.27	*(0.11)*		
γ-decalactone[c]	24.93	1.34	0.03	*(0.00)*			0.16	*(0.00)*
γ-undecalactone	27.50	0.88					0.15	*(0.03)*
γ-dodecalactone	28.06	0.92	0.51	*(0.00)*	1.58	*(0.02)*	3.8	*(0.08)*
γ-6-dodecelactone	28.39	1.33	0.09	*(0.00)*	0.15	*(0.00)*	0.44	*(0.02)*
γ-hexadecalactone	24.58	0.34	0.50	*(0.01)*	0.29	*(0.03)*	1.6	*(0.02)*

[a]Values (mg kg^{-1}) are means of two independent replicates
[b]Values in parenthesis represent standard deviation of the means
[c]MS-Identification confirmed by comparison with retention time of authentic compound.

substantially higher than that previously reported (Wong, Ellis & Lacroix, 1975; Shakeel Ur Rehman, Banks, Brechany, Muir, McSweeney & Fox, 2000).

The amount of γ-lactones in Danish blue was clearly higher than that in the other cheese types. Remarkably, the levels of some δ-lactones in the Danish Blue were lower than those in Gouda and Cheddar, despite the much higher levels of possible precursors (free fatty acids) in the former.

3.5. Esters

Table 2.4 shows the occurrence of a variety of esters in the different cheese types, mostly methyl and ethyl esters. Esters were present in largest quantities in Danish Blue, whereas Gouda and Cheddar contained only minor quantities. The majority of esters consisted of the most-likely predominant alcohols (methanol and ethanol) and free fatty acids (even numbered medium to long fatty acids), suggesting a non-specific formation process. Therefore, it is noteworthy that some esters, such as the ethyl and methyl esters of dodecanoic acid, have not been found, probably due to coelution with the much larger fatty acid peaks, especially in Danish Blue.

Table 2.4. Esters found in various cheeses (mg kg^{-1}) [a b]

	Retention time (min)	K(-)	Gouda		Cheddar		Danish Blue	
2hydroxy4methyl pentanoic acid methyl ester	14.45	1.17	0.01	*(0.00)*				
hexadecanoic acid methyl ester[c]	25.46	1.27	0.03	*(0.00)*	0.01	*(0.00)*	0.94	*(0.05)*
octadecanoic acid methyl ester	28.28	1.30	0.00	*(0.00)*			0.08	*(0.00)*
oleic acid methyl ester[c]	28.56	1.99	0.12	*(0.03)*	0.06	*(0.00)*	4.46	*(0.08)*
hexanoic acid ethyl ester	9.35	0.69					1.23	*(0.08)*
decanoic acid ethyl ester[c]	16.38	0.08					11.48	*(0.66)*
tetradecanoic acid ethyl ester[c]	23.01	0.04	0.14	*(0.00)*			4.01	*(0.02)*
hexadecanoic acid ethyl ester[c]	26.00	0.10	0.05	*(0.00)*			1.29	*(0.08)*

[a]Values (mg kg^{-1}) are means of two independent replicates
[b]Values in parenthesis represent standard deviation of the means
[c]MS-Identification confirmed by comparison with retention time of authentic compound.

3.6. Ketones

Table 2.5 shows the 2-ketones found in the different cheese types. All odd-numbered ketones with 7 to 15 carbon atoms were found. In Gouda and Cheddar, the concentrations of methyl-ketones were very low, and quite similar. In Danish Blue however, the concentrations were 100 to 1000 fold higher. The moulds present in blue-type cheese are known to produce enzymes which convert free fatty acids into CO_2 and methyl ketones (Cerning et al., 1987). These ketones are known to contribute significantly to the flavour of Danish Blue cheese (Gallois & Langlois 1990).

Table 2.5. Ketones found in various cheeses (mg kg^{-1}) [a b]

	Retention time (min)	K (-)	Gouda		Cheddar		Danish Blue	
2-heptanone[c]	8.60	0.30	0.15	*(0.00)*	0.21	*(0.05)*	21.8	*(3.49)*
2-nonanone[c]	12.03	0.53	0.06	*(0.01)*	0.12	*(0.03)*	65.1	*(5.43)*
2-undecanone[c]	15.80	0.31	0.07	*(0.00)*	0.05	*(0.00)*	41.6	*(2.91)*
2-tridecanone[c]	19.37	0.16	0.12	*(0.00)*	0.07	*(0.01)*	5.53	*(0.28)*
2-pentadecanone	22.69	0.13	0.21	*(0.01)*	0.13	*(0.00)*	3.70	*(0.09)*

[a]Values (mg kg^{-1}) are means of two independent replicates
[b]Values in parenthesis represent standard deviation of the means
[c]MS-Identification confirmed by comparison with retention time of authentic compound.

Table 2.6. Miscellaneous compounds found in various cheeses (mg kg^{-1}) [a b]

	Retention time (min)	K(-)	Gouda		Cheddar		Danish Blue	
3-methyl-1-butanol	9.04	1.25	0.13	*(0.01)*				
3-methyl-3-buten-1-ol	9.70	1.04	0.01	*(0.00)*	0.01	*(0.00)*	0.08	*(0.00)*
1-pentanol	9.64	0.89	0.02	*(0.00)*	0.03	*(0.01)*		
thiocyanic acid methyl ester	10.23	1.36	0.07	(0.01)	0.05	(0.00)		
3-hydroxy-2-butanone	10.52	0.66	56.2	*(0.93)*	7.6	*(0.30)*	0.75	*(0.18)*
1-hydroxy-2-propanone[c]	10.70	0.45	0.22	*(0.01)*	0.28	*(0.02)*		
2-hydroxy-3-pentanone	11.69	2.01	0.02	*(0.00)*				
2,3-butanediol	15.30	0.62	90.1	*(3.09)*				
benzaldehyde	14.82	0.56	0.01	*(0.00)*	0.02	*(0.00)*	0.98	*(0.09)*
dimethylbenzaldehyde	19.91	0.17	0.11	*(0.00)*				
benzyl alcohol	20.63	1.49	0.02	*(0.00)*	0.01	*(0.00)*		
butanamide	20.75	0.78	0.01	*(0.00)*			0.09	*(0.02)*
phenyl ethyl alcohol	21.22	1.84	0.03	*(0.00)*	0.00	*(0.00)*	1.45	*(0.32)*
dimethyl sulfone	21.28	1.21	0.28	*(0.01)*	0.22	*(0.00)*	2.01	*(0.02)*
2acetylaminopropionic acid	21.75	1.85	0.01	*(0.00)*				
dehydromevalonic lactone	23.44	2.20					0.89	*(0.00)*
2-methyl-phenol	23.76	1.05	0.02	*(0.00)*	0.07	*(0.00)*	0.75	*(0.01)*
2,4-bis-(1,1-dimethylethyl)-phenol	26.73	0.90	0.03	*(0.00)*	0.03	*(0.00)*	0.33	*(0.04)*
benzoic acid[c]	28.62	0.22	1.39	*(0.02)*	2.76	*(0.01)*	10.80	*(0.98)*
phenyl propanedioic acid	30.18	0.36	0.32	*(0.04)*			11.55	*(1.33)*
mevalonic acid lactone	30.36	1.28	0.52	*(0.08)*	0.13	*(0.01)*		

[a]Values (mg kg^{-1}) are means of two independent replicates
[b]Values in parenthesis represent standard deviation of the means
[c]MS-Identification confirmed by comparison with retention time of authentic compound.

3.7. Miscellaneous compounds

In addition to the fat-derived compounds listed above, a considerable number of other compounds was detected after extraction and analysis. These were either i) compounds not derived from fat, and therefore out of the scope of this paper, ii) fat-derived compounds which were not quantifiable, or iii) compounds that could not be identified. Table 2.6 shows a range of miscellaneous compounds from the first category. These and the other compounds will not be further discussed here.

4. Conclusions

The method described is suitable to detect a broad range of fat-derived compounds in the cheeses, including fatty acids, lactones, ketones, and esters, which can be important flavour compounds. Some non-fat derived compounds were also detected. Because of the properties of the solvent used, highly volatile compounds could not be detected. A mathematical method has been developed to calculate the concentrations of these compounds in cheese, although quantification was only possible for compounds present in a concentration high enough to enable the determination of a distribution coefficient (K-value). The cheese types investigated in this study generally contained the same fat-derived compounds, but their quantities were different. These compounds were apparently formed during cheese ripening, and it would be interesting to monitor the changes of fat-derived compounds during ripening of some selected cheese types, which will be the subject of further studies.

Acknowledgements

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Production of fat-derived (flavour) compounds during ripening of Gouda cheese

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Abstract

Fat-derived flavour compounds influence the overall flavour of cheese. While the quantitative occurrence and formation mechanisms of fat-derived compounds are generally known for soft-type and mould-ripened cheeses, relatively little is known about the occurrence and the formation mechanisms of these compounds in (semi)-hard cheeses. Therefore, fat derived flavour compounds (free fatty acids (FFA), lactones, ketones, and esters) in four different batches of Gouda cheese were monitored for 2 years of ripening time. Total FFA concentrations rose from 200-400 to 700-1200 mg kg^{-1} dry matter in 2 years, in a fairly linear manner. In curds, long chain FFA proportions were predominant, but during ripening relatively more short and intermediate chain fatty acids were released. The production of δ-lactones was fast from the beginning, but slowed to a near standstill at a total concentration of 55 mg kg^{-1} dry matter in about 20 weeks. The production of γ-lactones was slower and decreased also, but it was noticeable over a longer time, leading to levels of 5.5 mg kg^{-1} dry matter in 90 weeks. Ethyl ester formation varied substantially in some raw-milk cheeses, and was very low in pasteurised-milk cheeses. Ketone levels increased only very slightly during ripening, whereas long chain alcohols and aldehydes were not found. Some individual FFA and lactones exceeded previously reported flavour thresholds, and are expected to influence Gouda cheese flavour.

Keywords

Gouda cheese, ripening, free fatty acids, lactones, ketones, esters

1. Introduction

It is well-known that most cheese aroma is mainly formed during ripening. Besides proteins and carbohydrates, fat has been shown to be important for flavour formation as well (Foda, Hammond, Reinbold & Hotchkiss, 1969; Badings, Walstra & Jenness, 1984; Collins, McSweeney & Wilkinson, 2003a). Fat-derived compounds such as free fatty acids, esters, lactones and ketones, generally have distinct flavour characteristics and low aroma thresholds (Siek, Albin, Sather & Lindsay, 1971; Rothe, Woelm, Tunger & Siebert, 1972; Kinsella, 1975). Therefore, it is expected that these compounds play a role in the overall cheese flavour. Furthermore, fat plays a physical role in cheese ripening, by acting as a flavour solvent, or by providing water/protein/fat interfaces that help flavour-forming reactions to occur (Wijesundera & Drury, 1999). Also the fatty mouthfeel it provides can be considered to be part of the cheese flavour.

Lipolysis of fat yields fatty acids, which are flavour compounds that have "cheesy" notes or cheese-like aromas, especially the shorter ones (Kinsella, 1975). Lipolysis can occur when fat is accessible for lipases or esterases. The origins of these enzymes include the milk itself (lipoprotein lipase) (Jensen & Pitas, 1976), psychrotrophic bacteria that grow in raw milk before preparation of cheese (Law, 1979; Chen, Daniel & Coolbear, 2003), possible contamination with lipases during cheese making (Nelson, Jensen & Pitas, 1977), and microbes that develop during cheese ripening, either intentionally (starter bacteria, specific moulds or yeasts) or unintentionally (non-starter lactic acid bacteria, moulds, yeasts) (Collins et al., 2003a). Fatty acids, in turn, may be the precursors of other compounds such as esters, lactones, ketones, and aldehydes. These secundary fat-derived compounds can be very potent flavour compounds. In some cases, as for methyl ketones in blue type cheeses, the formation mechanism and the enzyme sources are known (Gehrig & Knight 1963; Molimard & Spinnler, 1996). Bacterially ripened cheeses as Gouda and Cheddar lack the fat and FFA degrading enzyme systems that occur in mould ripened cheeses and therefore, the fat degradation that occurs in these cheeses is different and less pronounced.

Concentrations of free fatty acids (FFA) during ripening of various bacterially ripened cheeses have been studied by several authors (Umemoto & Sato, 1975; Macedo & Malcata, 1996; Sousa & Malcata 1997; Partidário, Barbosa & Vilas Boas, 1999; Buffa, Guamis, Pavia & Trujillo, 2001; Chamba & Perreard, 2002; Mallatou, Pappa & Massouras, 2003). These studies concerned different, mainly Mediterranean cheeses from cows', ewes' or goats' milk. Due to the different milk types and cheese preparations used, free fatty acid concentrations, compositions and ripening profiles were inconsistent. Because these studies reported only a limited number of data points, there is a lack of detailed data of the formation of FFA in semi-hard cheeses. Additionally, secundary fat-derived compounds were not determined in these studies. Little, and mostly semi-quantitative data is available on the occurrence of

secundairy fat-derived compounds (Urbach, 1993; Rehman, Banks, Brechany, Muir, McSweeney & Fox, 2000; Curioni & Bosset, 2002), and their formation during the ripening of semi-hard cheese types is not fully known.
The aim of the current research was to obtain quantitative data on the development of individual FFA and secondary fat-derived compounds (lactones, ketones and esters) in Gouda cheeses. Therefore, these compounds were monitored simultaneously in four different batches of Gouda cheese during 2 years of ripening. In most cases the obtained data permitted simplification in the form of summation within the class of compounds, and these summations were reported. This data may lead to more insight in the factors that influence the formation in cheese, and the formation mechanisms of fat-derived flavour compounds.

2. Materials and methods

Four batches of Gouda-cheese were prepared as described previously (Walstra, Noomen & Geurts, 1999a). Three batches of milk and three different starters were used, as given in Table 3.1. To minimise the effect of differences in milk composition due to seasonal variation, all cheeses were prepared within one week during early spring. Starter, milk pre-treatment, shape and weights are listed in Table 3.1. The starters used were all mesophilic, mixed-strain starters, consisting of mixtures of different strains of *Lactococcus lactis,* subsp. *lactis* and *cremoris*, *L. lactis* subsp. *lactis* biovar *diacetylactis*, and *Leuconostoc* sp., and obtained from CSK Food Enrichment, Ede, The Netherlands. Curds samples were taken just before pressing and kept frozen. All cheeses were ripened in the same room at 85% RH and 13°C, and turned regularly. Initial moisture, fat and salt content (measured after 2 weeks) ranged from 41.0 to 41.9% (w/w), 29.9 to 30.6 %(w/w), and 1.96 to 2.03 %(w/w), respectively, while the initial pH ranged from 5.24 to 5.30.

Table 3.1. Process variables and coding used for the experimental cheeses.

Cheese	Starter	Milk pre-treatment	Shape	Weight (kg)
A	A	15 sec, 72°C [a]	round	13
B	BK2	15 sec, 72°C [a]	round	13
C	BK2	raw	round	13
D	BOS/Fr18	15 sec, 72°C	block	15

[a]Milk used comes from the same batch.

Cheese samples were taken every 2 to 6 weeks up to 42 weeks, and at 96 weeks. About 100 g of cheese sample was taken longitudinally, halfway between edge and centre, and the holes were immediately filled with paraffin. Subsequent samples within the same cheese were taken at 15 cm distance from previous sample positions. Eight commercially available

raw milk Gouda cheeses of different origins in the region of North-Holland, and different ripening times were sampled similarly and used for additional ester measurements.
Determination of the fat-derived compounds from the cheeses was carried out as described earlier (Alewijn, Sliwinski & Wouters, 2003). Independent duplicates (2x 15g cheese) were mixed with 4mL of acetonitrile, and put at 40°C for 10 minutes, with regular shaking to obtain distribution equilibrium. After a short centrifugation run the organic top layer was transferred into a GC vial, and the extract was analysed for fat-derived compounds by GC/MS, consisting of a 8000^{top} GC (CE Instruments, Milan, Italy) equipped with a FFAP GC column (J&W Scientific, USA) and a Finnigan Automass II (Finnigan, Bremen, Germany). Myristylbromide (Sigma, St. Louis, MO. USA) was used as an internal standard, and extraction coefficients were used to correct for extraction losses, as described earlier (Alewijn et al., 2003).

3. Results and discussion

3.1. Free fatty acids

At 96 weeks of ripening time, the quantity of free fatty acids (FFA) in the current cheeses was 700 to 1200 mg kg^{-1} dry matter, which is similar to that in other bacterially ripened cheeses (Woo, Kollodge & Lindsay, 1984; de Jong & Badings, 1990). This quantity was reached mainly during ripening, as discussed below, but also depended on the FFA concentration in the curds. Fig. 3.1 shows that the levels in the Gouda cheese curds were already at 30 to 35% of the levels reached after 96 weeks of ripening. During the preparation of the curds there was some time for the formation of FFA, but it is likely that most of the FFA in curd originated from milk. Thus, a part of the total FFA in a ripened cheese originated from the FFA available in the cheese milk. In milk, FFA may develop after milking, mainly by action of native lipoprotein lipase (LPL) (Borgstrom & Brockman, 1984). Since process conditions (eg. transport, pumping, homogenisation) are known to enhance the action of LPL (Downey, 1980), the substantially higher FFA level of the curd produced from raw milk (cheese C) can be attributed to prolonged action of LPL in the milk during storage and the first hours of the cheese preparation process. Therefore, milk storage and processing conditions are well worth considering, as they influence the FFA levels in the resulting cheese. The FFA compositions in the current curds, however, were quite different from the compositions of FFA in milk (de Jong et al., 1990), and from the composition of fatty acids in milk triglycerides (Walstra, Geurts, Noomen, Jellema & van Boekel, 1999b). The molar percentage of short chain FFA in the curds ranged from 2.0 to 3.6 (Table 3.2), which is substantially lower than the percentage in milk, or in milk triglycerides. This loss of short chain FFA in curd occurring during cheese preparation was possibly due to the loss of these relatively more polar FFA with the whey.

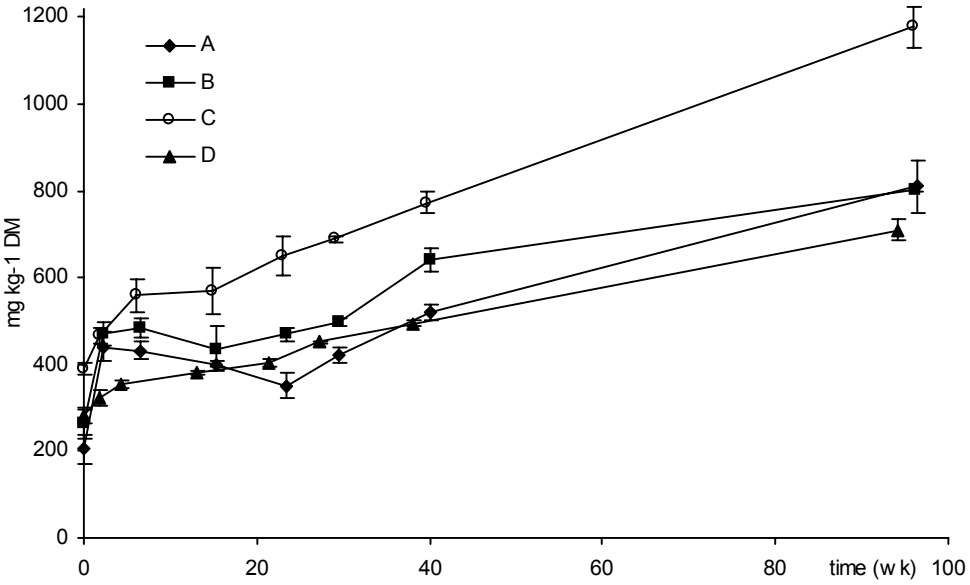

Fig. 3.1. Total free fatty acid concentrations during the ripening of 4 Gouda type cheeses, in mg kg^{-1} dry matter (DM). Values are a summation of even- and odd numbered saturated FFA from C6:0 till C18:0, and C10:1, C12:1, C14:1, C16:1, C18:1 and C18:2 free fatty acids. Error bars represent standard deviations.

Throughout the 96 weeks of cheese ripening investigated, FFA levels generally increased in all cheeses at an average rate of about 6 mg kg^{-1} dry matter $week^{-1}$ (Fig. 3.1). Therefore, it can be assumed that both lipolytic activity and fat susceptible for enzyme action were present throughout the ripening phase. To investigate whether certain FFA were generated preferentially during the ripening period, the individual FFA were arbitrarily classified into short, intermediate and long chain FFA, and their combined mole percentages were calculated (Table 3.2). During ripening, short chain FFA, and intermediate chain FFA to a lesser extent, proportionally increased as compared to the long chain FFA (Table 3.2). This showed that lipolysis during ripening favoured the release of short and intermediate chain fatty acids over long chain fatty acids. A lipolytic preference for short chain FFA has been found in goats' milk cheese (Buffa et al., 2001). As shown in Fig. 3.2, short chain FFA production was constant throughout ripening time for the four cheeses studied. Such a selectivity of lipolysis is both determined by the selectivity of the lipolytic enzymes involved and the approachability of the fat for the enzymes, neither of which were studied in detail. It is likely that the hydrolytic activity can be attributed to enzymes produced by microbial sources in cheese (e.g. lactic acid bacteria). In general, esterases from lactic acid bacteria (LAB) are intracellular, and these enzymes only become available upon (auto-)lysis. A positive correlation between a more autolysing starter and FFA production has been found in Cheddar cheese (Collins, McSweeney & Wilkinson, 2003b). Also, non-starter lactic acid bacteria (NSLAB) possess esterases, and it has been postulated that these contribute to the

release of especially short chain fatty acids (Rehman et al., 2000). The production of FFA was higher in raw milk cheese (C) (Figs. 3.1 and 3.2), which could be due to higher initial LPL levels due to the absence of a pasteurisation step. However, because the higher production was mainly due to increased formation of short chain FFA, it might therefore be caused by esterolytic activity of NSLAB, which are presumably present in this cheese, but not in cheeses made from pasteurised milk. LAB are known to produce esterases which are able to release fatty acids (Crow, Holland, Pritchard & Coolbear, 1994; Meyers, Cuppett & Hutkins, 1996; Collins et al., 2003a). Possibly, these enzymes have a substrate preference for mono and diglycerides (Fernandez et al., 2000), which are likely to be produced in the initial stages of cheese ripening by LPL or other truly lipolytic enzymes. The presence of mono and diglycerides in milk and cheese has been shown (Mariani, Contarini, Zucchetti & Toppino, 1990; Walstra et al., 1999a). Although mono and diglycerides are only present in small amounts in milk and cheese, it is worth noting that the quantity of FFA in the current cheeses corresponded to at most 0.3% of the total fatty acid glycerides present in the fat fraction of the cheeses. Therefore, it seems possible that mono and diglycerides are the main direct precursors of the FFA in these cheese types, rather than triglycerides.

Table 3.2. Free fatty acid (FFA) compositions[a] in mole percentage, in four Gouda cheeses during ripening[b]

		age (weeks)															
		0		2		6		15		23		29		40		96	
	C6-C9	2,1	*(0,10)*	2,1	*(0,00)*	3,1	*(0,02)*	5,3	*(0,03)*	8,4	*(0,35)*	8,2	*(0,22)*	7,9	*(0,13)*	12,0	*(0,26)*
A	C10-C15	29,0	*(1,08)*	27,0	*(1,13)*	30,6	*(0,63)*	35,1	*(0,38)*	37,4	*(1,07)*	35,6	*(0,47)*	34,9	*(0,20)*	36,0	*(0,64)*
	C16-C18	68,9	*(7,72)*	71,0	*(3,57)*	66,3	*(2,26)*	59,7	*(0,45)*	54,2	*(3,54)*	56,1	*(1,91)*	57,2	*(1,56)*	52,0	*(3,29)*
	C6-C9	2,0	*(0,06)*	2,3	*(0,07)*	3,1	*(0,06)*	5,2	*(0,03)*	6,0	*(0,17)*	6,5	*(0,32)*	7,2	*(0,22)*	12,5	*(0,04)*
B	C10-C15	34,6	*(0,22)*	30,9	*(0,38)*	34,6	*(0,55)*	37,1	*(0,96)*	38,1	*(0,29)*	37,2	*(0,81)*	35,8	*(0,61)*	35,4	*(0,21)*
	C16-C18	63,4	*(6,21)*	66,7	*(2,82)*	62,3	*(2,11)*	57,7	*(5,17)*	55,9	*(1,38)*	56,3	*(0,48)*	57,0	*(1,90)*	52,1	*(0,31)*
	C6-C9	3,6	*(0,20)*	3,4	*(0,03)*	4,1	*(0,08)*	7,0	*(0,06)*	8,2	*(0,19)*	9,9	*(0,10)*	11,4	*(0,11)*	18,0	*(0,07)*
C	C10-C15	45,3	*(0,70)*	35,9	*(0,22)*	34,6	*(0,75)*	37,7	*(1,50)*	38,1	*(0,95)*	39,1	*(0,23)*	39,0	*(0,43)*	40,0	*(0,42)*
	C16-C18	51,1	*(1,58)*	60,7	*(1,84)*	61,3	*(3,07)*	55,2	*(3,89)*	53,6	*(2,88)*	51,0	*(0,31)*	49,6	*(1,31)*	42,0	*(1,59)*
	C6-C9	2,5	*(0,04)*	3,0	*(0,04)*	3,4	*(0,03)*	5,8	*(0,04)*	7,1	*(0,10)*	8,2	*(0,02)*	9,7	*(0,16)*	13,4	*(0,23)*
D	C10-C15	37,2	*(0,19)*	39,1	*(1,06)*	39,9	*(0,29)*	39,8	*(0,37)*	39,1	*(0,22)*	38,7	*(0,08)*	38,0	*(0,19)*	34,9	*(0,21)*
	C16-C18	60,3	*(2,65)*	57,9	*(2,43)*	56,7	*(1,25)*	54,4	*(0,38)*	53,8	*(1,09)*	53,1	*(0,30)*	52,3	*(0,60)*	51,6	*(1,51)*

[a]All saturated, unsaturated, and branched free fatty acids were arbitrarily classified into short, intermediate and long chain FFA according to their carbon chain length and given as their mole percentage. Values in parenthesis represent the standard deviations.

[b]Literature values of fatty acid compositions are given for comparison reasons. Milk fat triglycerides, on average, contain 7.4, 23.7 and 68.9 mole percentage of short, intermediate and long chain fatty acid residues, respectively (Walstra et al., 1999a). Mole percentages of FFA in milk are 9.8, 19.9 and 70.3 FFA (de Jong et al., 1990).

While the FFA levels increased at an average rate of about 6 mg kg^{-1} dry matter $week^{-1}$, Fig. 3.1 also shows some non-linearities during ripening time. Generally, the rate of lipolysis in

the first two weeks was higher than average. Especially cheeses A and B showed a rapid increase in FFA in the first weeks, as shown in Fig. 3.1. This has been observed before in cheese made from ewes' milk (Macedo et al., 1996; Partidário et al., 1999) and Emmental cheese (Chamba et al., 2002). Table 3.2 shows that the initial increase was largely due to the formation of long chain FFA. Probably a lipase with a preference for long-chain fatty acids was already present in curd, and was active in the initial stage of cheese ripening. This might be either the native milk LPL (Geurts, Lettink & Wouters, 2003), a lipase present as a contamination in milk (psychrotrophs), or a lipase present as a contamination in the rennet (Collins et al., 2003a). However, no measurements on specific enzyme activities have been carried out to confirm this. It seemed that this enzyme lost its activity after two weeks, since relatively little formation of long chain FFA was observed after the first two weeks.

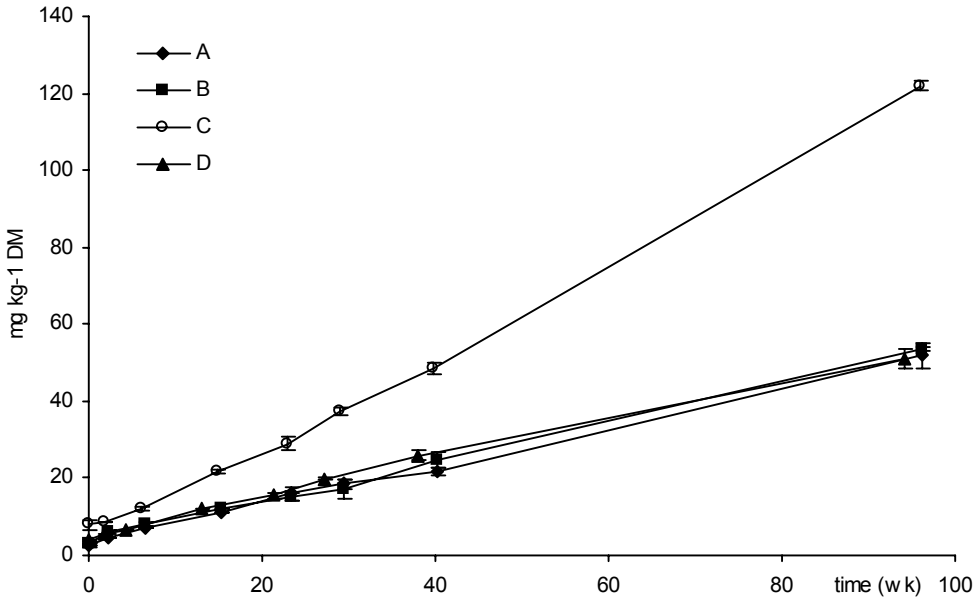

Fig. 3.2. Short chain fatty acid concentrations during the ripening of 4 Gouda type cheeses, in mg kg^{-1} dry matter. Values are a summation of C6:0, C7:0, C8:0 and C9:0 free fatty acids. Error bars represent standard deviations. Butyric acid (C4:0) was not included since it is also produced from amino acids and was formed in a different way than the short chain FFA.

Between 4 and 14 to 22 weeks, a period of decreased FFA production was observed in cheeses C and D, whereas in cheeses A and B the concentration of FFA declined. During this period, the proportion of short and intermediate chain FFA gradually increased at the cost of long chain FFA (Table 3.2). The sharpest decrease of long chain FFA proportion coincided with a decrease in total FFA levels (Fig. 3.1). Downey (1980) has postulated that an enzyme is present in cheese that can re-esterify FFA to a glycerol moiety, but it is also known that some lactic acid bacteria can take up FFA from their growth medium (Partanen, Marttinen &

Alatossava, 2001). No decrease of short chain FFA was observed (Fig. 3.2). It is not known which mechanism caused this decrease in long chain FFA.

3.2. Lactones

During cheese ripening, the amount of lactones increased from 3 to about 50 mg kg^{-1} and from 0.2 to about 5 mg kg^{-1} for δ- and γ-lactones, respectively. The level of δ-lactones in cheese increased very rapidly, and seemed to come to a standstill after 20 weeks (Fig. 3.3). The γ-lactones seemed to reach such a plateau as well, but required a longer ripening time to reach their maximum concentration (Fig 3.4). A similar lactone formation profile based on quantitative data was found in Cheddar cheese by (Wong, Ellis & LaCroix, 1975), but another study on Cheddar cheese did not confirm this profile (Urbach, 1993). Within the group of δ- and γ-isomers, there was no apparent chain length effect on their formation. It is thought that lactones are formed from hydroxy fatty acids (McSweeney & Sousa, 2000). Rehman et al. (2000) reported that lactone formation during cheese ripening depended on both ripening temperature and the presence of NSLAB. However, the mechanism responsible for the formation of hydroxy acids, the subsequent formation of lactones and the influences of temperature and occurrence of NSLAB are not known. The formation of δ-lactones seemed to cease at approximately the same time in every cheese investigated (Fig. 3.3), so lack of either precursor or enzyme activity seemed to prohibit further lactone formation. Since δ and γ lactones are chemically closely related, it is likely that they are formed by the same mechanism. Still, the current data showed that γ-lactone production continued for weeks after δ-lactone formation had ceased, which suggests that exhaustion of a precursor rather than loss of enzymatic activity prevented further lactone formation after the plateau is reached. The formation of almost equal quantities of lactones in four different cheeses suggests that equal amounts of precursor molecules were present in the milks used, despite the fact that different levels of FFA were present (Fig. 3.1). No correlation between levels of FFA and lactones were found, since lactone concentrations increased more than 10 times, while FFA levels increased about 3 fold during ripening. The exact formation mechanism of lactones cannot be concluded from the data presented in this paper, but further experiments are carried out to determine the mechanism of lactone formation. These will be reported in a forthcoming paper.

The current concentrations of the individual lactones are close to, or exceeded, the previously reported aroma thresholds both in water and in oil systems (Siek et al., 1971). Therefore, it is assumed that lactones participate in the overall cheese flavour.

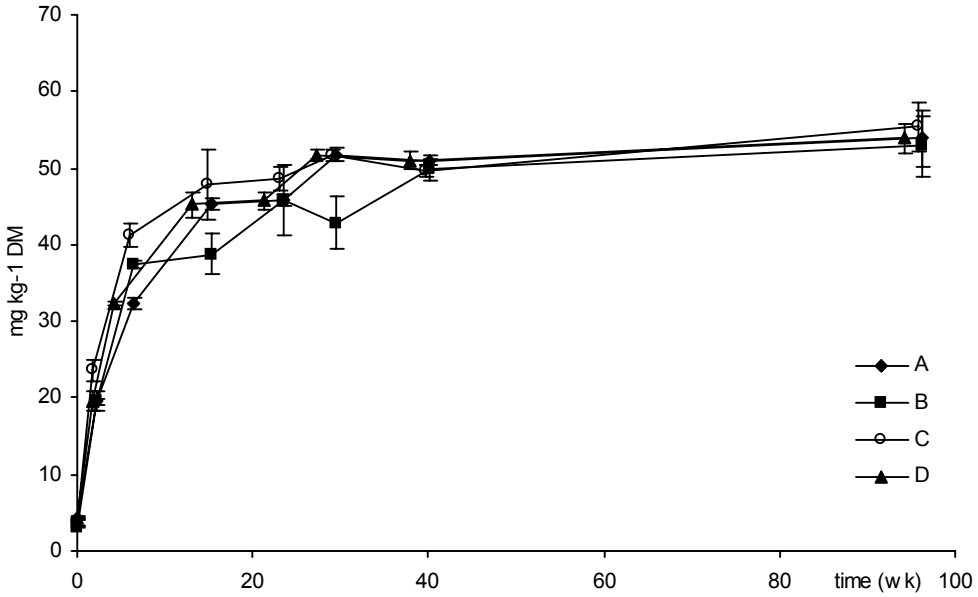

Fig. 3.3. Sum of δ-lactone concentrations during the ripening of 4 Gouda type cheeses, in mg kg^{-1} dry matter. Values are a summation of C6, C8, C10, C11, C12, C13, C14, C16 and C18 saturated δ-lactones. Error bars represent standard deviations.

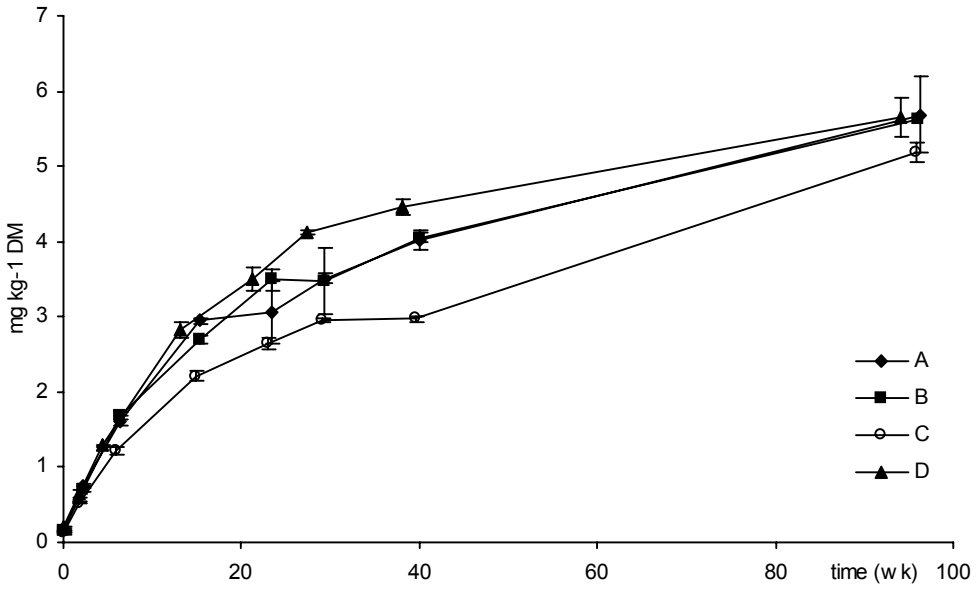

Fig. 3.4. Sum of γ-lactone concentrations during the ripening of 4 Gouda type cheeses, in mg kg^{-1} dry matter. Values are a summation of C10, C12, C12:1, C14, C16 and C18 γ-lactones. Error bars represent standard deviations.

3.3. Ketones

The formation of methyl ketones (or 2-ketones) was very slow, but rather constant in the first 40 weeks (Fig. 3.5). The total concentration doubled from 1 to about 2 mg kg^{-1}. No influence of chain length was observed in relation to formation rate. Furthermore, little difference was observed between the four cheeses investigated. Methyl ketones are important flavour compounds in blue-mould type cheeses (Molimard et al., 1996). Moulds (eg. *Penicilium roqueforti*) are responsible for the conversion of FFA into methyl ketones in these cheese types (Gehrig et al., 1963). The concentrations found in the current cheeses were much lower than those in Blue type cheeses, presumably due to the absence of the moulds. LAB are unlikely to be capable of performing ß-oxidation, which is the key step in the production of methyl ketones by moulds. The formation that occurred in the current Gouda cheeses could be due to a slow enzymatic or chemical reaction that is not yet described.

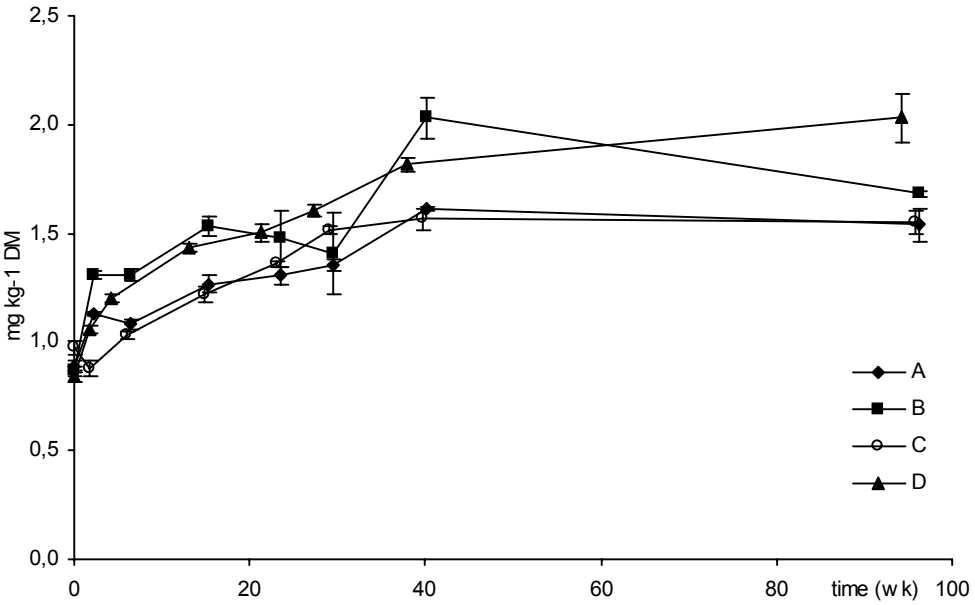

Fig. 3.5. Sum of methyl ketone concentrations during the ripening of 4 Gouda type cheeses, in mg kg^{-1} dry matter. Values are a summation of C7, C9, C11, C13 and C15 methyl ketones. Error bars represent standard deviations.

As shown in Fig. 3.5, the concentration of the sum of all methyl ketones was in the 1 to 2 mg kg^{-1} dry matter range, and some individual ketones exceeded their aroma thresholds in pure water. However, all ketones were present in concentrations substantially below their aroma thresholds in fatty systems (Siek, Albin, Sather & Lindsay, 1969). Because of the presence of fat in cheese and the lipophilic nature of these compounds, it is not expected that methyl ketones play a substantial role in flavour of Gouda cheese.

3.4. Esters

The majority of esters present in the Gouda cheeses were ethyl esters of long chain fatty acids, as reported earlier (Rehman et al., 2000; Alewijn et al., 2003), probably because ethanol is the most predominant alcohol in cheese. Due to the low concentrations, no distribution coefficients for the extraction (Alewijn et al., 2003), and hence no quantities in cheese could be reported. Therefore the values in Fig. 3.6 are expressed in mg mL^{-1} extract. The cheese made from raw milk (cheese C) showed a very strong and rather constant ester formation throughout ripening, while the ester concentrations in the other cheeses (A, B and D) increased only slightly during ripening (Fig. 3.6). It is not clear whether this difference was caused by higher levels of ethanol in cheese C, due to different chemical circumstances, or due to presence of different enzymes in this cheese. To establish whether the higher levels in cheese C were a coincidence, ester concentrations of eight commercially available raw-milk cheeses of different origins and ripening times were determined, and shown in Table 3.3 (cheeses 1-8). The ester concentrations of cheeses made from pasteurised milk (B and D) and raw milk (C) from the current study, at 40 weeks of ripening time, are included in this Table. The ester levels of cheeses 1-4 were similar to those of the pasteurised milk cheeses in Fig. 3.6, while other samples (5-8) contained more or much more esters than the raw-milk cheese of the present study. This showed that ester content in Gouda cheese varied dramatically between different individual cheeses.

Table 3.3. Concentrations of ethyl esters[a] in eight commercially available raw-milk cheeses of different origins versus their (approximate) ripening time.

cheese	age (weeks)	sum ethyl esters (µg ml^{-1})	
1	8	0.02	*(0.0004)*
2	24	0.11	*(0.10)*
3	32	0.26	*(0.052)*
4	32	0.37	*(0.043)*
5	16	1.02	*(0.20)*
6	200	1.53	*(0.18)*
7	40	1.76	*(0.12)*
8	48	10.3	*(0.51)*
B	40	0.04	*(0.004)*
D	40	0.19	*(0.011)*
C	40	1.03	*(0.065)*

[a]Summation of C6, C8, C10, C12, C14 and C16 ethyl esters, concentrations are in µg ml^{-1} cheese extract, as described in the text. Values in parenthesis represent the standard deviations.

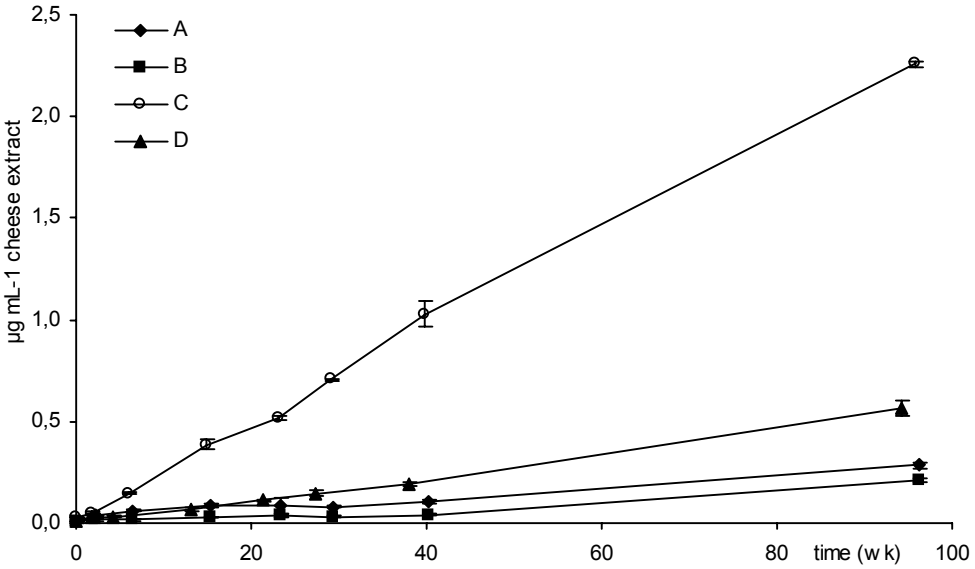

Fig. 3.6. Sum of ethyl ester concentrations during the ripening time of 4 Gouda type cheeses, in µg mL^{-1} cheese extract. Values are a summation of C6, C8, C10, C12, C14 and C16 ethyl esters. The divergent unit is explained in the text. Error bars represent standard deviations.

The similarity of Figs. 3.2 and 3.6 suggested that the formation of short chain FFA and ethyl esters were correlated. It is possible that the esterase that is responsible for hydrolysis of short chain FFA is also capable of esterification of a free fatty acid to an ethanol molecule, producing esters. Liu, Holland and Crow (2003) have indentified an esterase from *Streptococcus thermophilus* that can perform both hydolysis from a glyceride and production of esters. It has been shown that some dairy bacteria produce esterases that are able to catalyse ester formation in cheese environments (Liu, Holland & Crow, 1998). Also, higher ester levels in the eight commercial cheese samples (Table 3.3) correlated well with higher levels of short chain FFA (data not shown). Therefore, it can be assumed that in some of the raw milk cheeses a high esterase activity was produced. The rise of certain bacteria (NSLAB) depends on pre-contamination of the milk, pasteurisation, and competing micro-flora during ripening. Probably some of these bacteria are able to produce a highly active esterase, but more research should be carried out to determine the exact nature of this enzyme, and the bacteria that produce it.

4. Conclusions

Free fatty acids (FFA), lactones, esters and to a lesser extent, ketones, were formed during Gouda cheese ripening. A substantial part of the FFA was already present before cheese ripening started, and therefore the level of FFA in the milk will influence the FFA level of the resulting cheese. Short chain FFA were formed at a fairly constant rate throughout ripening, which showed that both enzyme and substrate were available in the cheeses for 96 weeks. Short chain FFA, and intermediate chain FFA to a lesser extend, were produced proportionally faster than long chain FFA, except for the first two weeks where long chain FFA were preferentially produced. Therefore, LPL and esterases from (non-starter) lactic acid bacteria were assumed to play a role in fat hydrolysis during various stages in cheese preparation and ripening. This resulted in an overall preference for short- and intermediate chain FFA lipolysis during ripening. Lactones were quickly formed in the initial stages of cheese ripening, and their formation was remarkably similar in all cheeses investigated. δ-Lactones were produced more quickly than γ-lactones, but the formation of both lactone isomers seemed to be independent of the formation of FFA. It is thought that exhaustion of the precursors, probably hydroxy acids, prevented further formation of lactones. Ketones were produced only very slightly and rather similarly in the four cheeses investigated, and they do probably not substantially contribute to the flavour of the Gouda-type cheeses. Ethyl esters were the predominant esters found, their contents varied widely in commercial raw-milk cheeses, but were only slightly in the Gouda-type cheeses made from pasteurised milk investigated in the current study. Their formation profiles showed similarities with the formation of short chain FFA, indicating that the same enzyme may be responsible for their formation in this type of cheese.

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Production of free fatty acids, lactones, esters and ketones during the ripening of Gouda cheese: the influences of foil wrapping

Abstract

The influence of conventional coating and foil-wrapping on the development of fat-derived aroma compounds during ripening was investigated. From one batch of milk, Gouda cheeses with three different initial water contents were prepared, and ripened for 14 weeks at 13°C for conventionally and at 5°C for foil-wrapped cheese. In agreement with earlier findings the amount of long chain fatty acids and δ-lactones increased rapidly in the initial ripening period, reaching a plateau-value as ripening time increased, whereas short chain fatty acids and γ-lactones were formed more gradually over the ripening time considered. All the above-mentioned compounds were formed more strongly in conventionally coated than in foil-wrapped cheeses. Long and intermediate chain fatty acids were the only compounds for which the production rate increased with water content. In the relatively short ripening time considered, the production of ketones and esters was too low to draw conclusions. For all fat-derived compounds, most differences observed can probably be attributed to the difference in ripening temperature applied, rather than to changes in the type of packaging.

Keywords

Gouda cheese, foil-wrapping, cheese ripening, free fatty acids, lactones, ketones, esters.

Introduction

Foil-wrapped cheese was introduced in the 1960s as a convenient way of packaging consumer portions of cheese. Later, cheeses were wrapped in foil immediately after brining, and ripening proceeded within this foil. This way of ripening has a number of technological advantages, compared to their conventionally coated counterparts. In this report, conventionally ripened cheeses are regarded as cheeses that are coated with a gel-like, rather porous plastic soon after being pressed. Foil-wrapped cheeses are packed and ripened in a closely fitting almost impermeable plastic film. Compared to conventionally coated cheese, the loss of water during ripening of foil-wrapped cheese is greatly reduced, and the resulting product has no rind that has to be removed before consumption. Soon after production, easy to handle sturdy units of cheese are being ripened, and because of the strong barrier between cheese and outer atmosphere, the ripening conditions require less control, which makes the process easier. Also because of the quite impermeable foil, the possibility of mould contamination is reduced. These advantages may also lead to a decrease in production costs.

The way of packaging could have influence on the processes during ripening. The exchange of volatile compounds between the cheese and its outer atmosphere is limited. Oxygen transport into the cheese will be hindered, as well as the outlet of carbon dioxide. Since water can barely pass the foil, water losses of the cheese during ripening will be limited. To reach equal water contents at consumption, the curd of foil-wrapped cheeses has to be somewhat dryer than curd of their conventionally coated counterparts. Ripening of foil-wrapped cheese is commonly done at 5°C to avoid excessive formation of bitter peptides which occurs in foil-wrapped cheese ripened at 13°C (DOC cheese, personal communication). This might also be the cause of major differences between the ripening processes of both types of cheeses.

Although foil-wrapped cheese production is widely applied, little scientific data are available about the ripening differences between conventionally coated and foil-wrapped cheeses. A study by (Hough, Martinez, Barbieri, Contarini & Vega, 1994) showed that foil-wrapped Reggianito cheese was different from conventionally coated cheese, especially texture-wise, but there was also an influence on aroma and flavour. It was considered likely that ripening processes were altered because of the different gas composition, ripening temperature and water content (Bouzas, Kantt, Bodyfelt & Torres, 1993; Hough et al., 1994; Lombardi, Bevilacqua & Califano, 1994; Romani, Sacchetti, Pittia, Pinnavaia & Dalla Rosa, 2002).

Cheese flavour formation occurs mainly during ripening, and consists of many known and unknown processes, involving the breakdown of proteins, lactose and fat into flavour compounds (Collins, McSweeney & Wilkinson, 2003; McSweeney & Sousa, 2000; Molimard &

Spinnler, 1996). These reactions can be enzymatic, catalysed by enzymes from milk or (non-starter) lactic acid bacteria, or non-enzymatic. Both types of reactions can be influenced by temperature, gas composition within the cheese, redox potential, pH and water activity. These parameters are possibly different if foil ripening is used rather than conventional ripening, and therefore presumably also the flavour forming reactions.
Within the process of flavour formation, the degradation of fat into fatty acids and secondary aroma compounds plays a role in the overall cheese aroma (Collins et al., 2003). While the formation of these compounds during the ripening of conventionally coated Gouda cheeses has recently been described (Alewijn, Sliwinski & Wouters, 2005), little is known about the influence of foil-wrapping on the development of fat-derived flavour. The foil-wrapped cheeses are ripened at lower temperatures, and are produced with lower initial water content, which is then rather constant throughout ripening. Amongst others, these differences may influence the formation of fat-derived flavour compounds during ripening. Therefore, the aim of this research is to gain more knowledge on the comparison of the development of fat-derived aroma compounds in foil-wrapped and conventionally coated Gouda cheeses under industrially applied ripening conditions.

Material and methods

From the same batch of milk, six different batches of Gouda cheese were prepared, according to the general recipe described earlier (Walstra, Noomen & Geurts, 1999), and pressed as rectangular blocks, approximately 15 kg each. A mesophilic mixed-strain starter was used in all batches, and consisted of mixtures of different strains of *Lactococcus lactis,* subsp. *lactis* and *cremoris*, *L. lactis* subsp. *lactis* biovar *diacetylactis*, and *Leuconostoc* sp., and was obtained from CSK Food Enrichment, Ede, The Netherlands. Three cheeses were coated with a gel-like semi-permeable plastic, or "conventionally coated", coded "C". Three other cheeses were wrapped tightly in clear plastic foil, and were coded with "F". For each covering method, curd production was aimed at three different water contents: low, normal and high, coded by L, n, and H, directly after the covering indicator. Samples from the six curds were taken and stored at –20°C before examination. As in industrial practice, coated cheeses were ripened at 13°C, foil-wrapped cheeses at 5°C. Both groups were stored at 80% relative humidity, and turned regularly. Cheeses were sampled at 8 days and 6 and 14 weeks by taking six portions at different positions, at least 5 cm from the cheese edge. For each time point, one single cheese from each particular batch was sampled. The samples were ground and independent duplicates (15g each) were extracted with 4 mL of acetonitrile at 40°C for 10 minutes, with occasional shaking. After a short centrifugation run (5s at 2500*xg*), a sample of the organic top layer was transferred to a GC-vial. The extract was analysed for fat-derived compounds by GC/MS, consisting of a 8000^{top} GC (CE

Instruments, Milan, Italy), equipped with a FFAP GC column (J&W Scientific, Palo Alto, CA, USA) and a Finnigan Automass II (Finnigan, Bremen, Germany). Myristylbromide (Sigma, St. Louis, MO. USA) was used as an internal standard, and extraction coefficients were used to correct for extraction losses, as described earlier (Alewijn, Sliwinski & Wouters, 2003).

Results

Cheeses

The ripening conditions and water contents of the cheeses prepared are listed in Table 4.1. The initial differences in water contents persisted throughout the ripening time, as expected. The initial water contents in the foil-wrapped cheeses were lower than in the conventionally coated cheeses, and were fairly stable during ripening. The conventionally ripened cheeses, initially containing more water, quickly lost water to reach levels of 3 to 5% below the foil-wrapped cheeses after 14 weeks of ripening.

Table 4.1. Covering mode, water contents, ripening temperature and coding of the cheeses used in this study.

Cheese coding	Cover	Water content (%)				Ripening temperature
		0 days	8 days	6 weeks	14 weeks	(°C)
FL	Foil	40.0	39.6	39.4	39.4	5
Fn	Foil	41.3	40.1	40.5	40.7	5
FH	Foil	41.8	41.3	41.4	41.2	5
CL	Coating	42.8	40.8	37.7	35.7	13
Cn	Coating	44.0	41.3	38.7	36.1	13
CH	Coating	44.8	43.3	41.2	38.2	13

Free fatty acids

Already 200 to 250 mg per kg dry matter of free fatty acids (FFA) was present in the curd of the current cheeses, an amount similar to data presented before (Alewijn et al., 2005). The amount in curd was quite significant compared to the amount of FFA that was formed during ripening, indicating that the FFA of curd were significantly contributing to the FFA levels in the resulting cheese. Most FFA present in milk will be transferred into the curd, but a part of the more hydrophilic FFA will be lost with the whey. A previous study reported that the proportions of short chain FFA in curd were much lower than in milk or in milk fat triglycerides (Alewijn et al., 2005). The FFA profiles in the current curds confirmed that finding. Across the six curd samples studied, however, no significant differences were found.

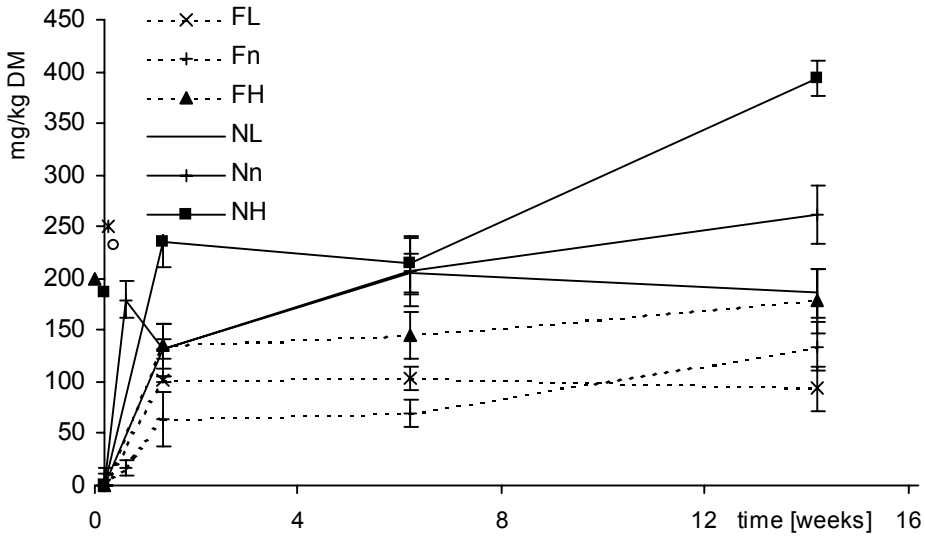

Fig. 4.1. Production of total free fatty acids in mg/kg dry matter, during the ripening period of six different Gouda cheeses from the same batch of milk, conventionally coated or foil-wrapped, with different initial water contents. The initial levels in the curds are given as separate points at t=0. Values are a summation of even- and odd numbered saturated FFA from C6:0 till C18:0, and C10:1, C12:1, C14:1, C16:1, C18:1 and C18:2 free fatty acids. Error bars represent standard deviations of duplicate measurements.

The production of FFA in the current cheeses (FFA levels of curd subtracted) was approximately 100 to 180 and 200 to 400 mg kg^{-1} dry matter during 14 weeks of ripening time for foil-wrapped and conventionally coated cheeses, respectively (Fig. 4.1). In all cheeses studied, the major increase in total FFA concentration, however, occurred in the first 8 days of ripening. This initial increase was largely due to the production of long chain FFA. It probably can be attributed to an enzymatic process that prefers the release of long chain fatty acids from its substrate and of which the enzyme seemed to lose its activity very early in the ripening time. Therefore, it is very likely that the enzyme responsible for the production of the FFA in this stage of the ripening was native milk lipoprotein lipase (LPL), since this enzyme has a preference for intermediate and long chain fatty acids (Jensen & Pitas, 1976). Also, it has been shown that the activity of this enzyme decreases rapidly in cheese conditions (Geurts, Lettink & Wouters, 2003).

In the first 8 days of cheese ripening, the production of FFA was higher in the conventionally coated cheeses than in the foil-wrapped cheeses (Fig. 4.1). This could be the effect of the higher temperature at which the natural ripened cheeses were kept, since the optimum temperature of LPL is closer to 13°C than to 5°C, at which the foil-wrapped cheeses were ripened. When regarding the different water contents of the cheeses, clear differences in FFA levels were observed. Throughout ripening, the production of total FFA

seemed higher when the water content was higher (Fig. 4.1). The slightly higher water content in these cheeses appeared to enhance the action of LPL. This might be due to the increased mobility of LPL and/or its substrate and/or its products in the cheeses with higher water content. If so, it might be argued that mobility of the enzyme/substrate is a bottleneck for the action of this enzyme during cheese ripening.

After 8 days of ripening the production of the long-chain FFA had ceased and a stable concentration or even a decrease in the concentration of long chain FFA was observed. Probably at this time, no strong lipolytic activity was left, although obviously the substrate in the form of triglycerides was still present. The relatively small decrease in the long chain FFA concentration may be explained by the conversion of these molecules into other compounds. Quantitatively, this decrease of long chain FFA cannot be explained by the formation of lactones, ketones or esters, or any other compounds that could be detected by the analytical method used (Alewijn et al., 2003). Therefore, it is most likely that the long chain FFA were re-esterified to a glycerol, or to a mono- or diglyceride, but the exact nature of this process, and the enzyme that might be responsible, is not known.

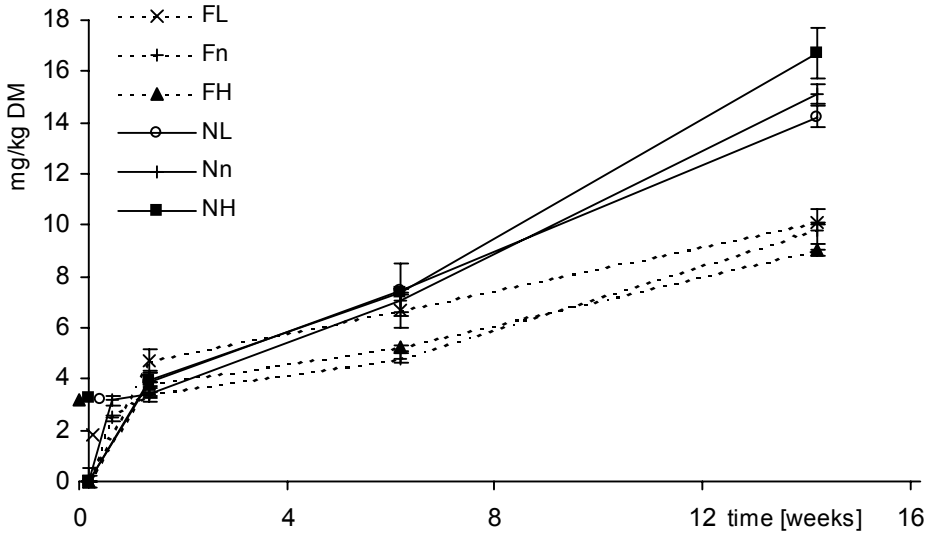

Fig. 4.2. Production of short chain free fatty acids in mg/kg dry matter, during the ripening period of six different Gouda cheeses from the same batch of milk, conventionally coated or foil-wrapped, with different initial water contents. The initial levels in the curds are given as separate points at t=0. Values are a summation of C6:0, C7:0, C8:0 and C9:0 free fatty acids. Error bars represent standard deviations of duplicate measurements.*

In contrast to the long chain FFA, the short chain FFA showed a clear increase throughout the ripening period (Fig. 4.2). This is in good agreement with the previous study on Gouda cheeses (Alewijn et al., 2005). However, similar to the long chain FFA, a difference between foil-wrapped and conventionally coated cheeses was observed, as latter cheeses showed a

higher production of short chain FFA during ripening. The water content did not appear to influence the formation of the short chain FFA, which was also in contrast to the long and intermediate chain FFA. Since the formation of FFA is most likely to be enzymatic, the higher temperature used for the ripening of the conventionally plastic-coated cheeses was probably more important than the difference in coating.

Lactones

The production of lactones is shown in fig. 4.3 (δ-lactones) and 4.4 (γ-lactones). The curds of all cheeses contained only small amounts of lactones. During the initial ripening period, the amount of δ-lactones increased rapidly in all cheeses, but the production rate dropped as the ripening time increased. The γ-lactones were formed more slowly, but the rate of production dropped more slowly than that for the δ-lactones. These profiles of the formation of γ- and δ-lactones are similar to those found in previously followed ripening cheeses (Alewijn et al., 2005). With prolonged ripening, lactones seem to reach a certain plateau value after about 20 weeks, probably due to the exhaustion of the precursors. Since the current cheeses were prepared from the same batch of milk, they should form the same amount of lactones if the conditions permit. In the cases of the conventionally coated cheeses, up to 14 weeks the lactone levels increased faster than in foil-wrapped cheeses. The difference between the two ripening modes was greater for γ-lactones.

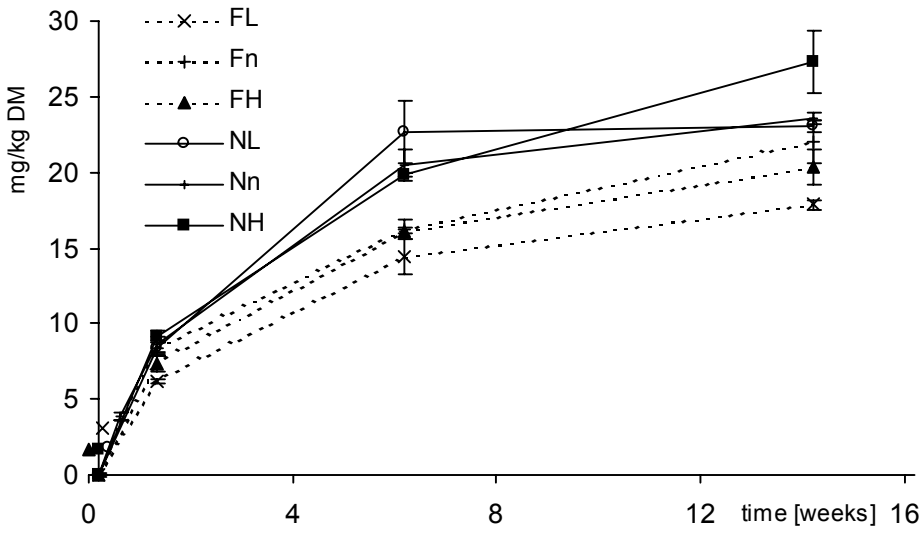

Fig. 4.3. Production of δ-lactones in mg/kg dry matter, during the ripening period of six different Gouda cheeses from the same batch of milk, conventionally coated or foil-wrapped, with different initial water contents. The initial levels in the curds are given as separate points at t=0. Values are a summation of C6, C8, C10, C11, C12, C13, C14, C16 and C18 saturated δ-lactones. Error bars represent standard deviations of duplicate measurements.*

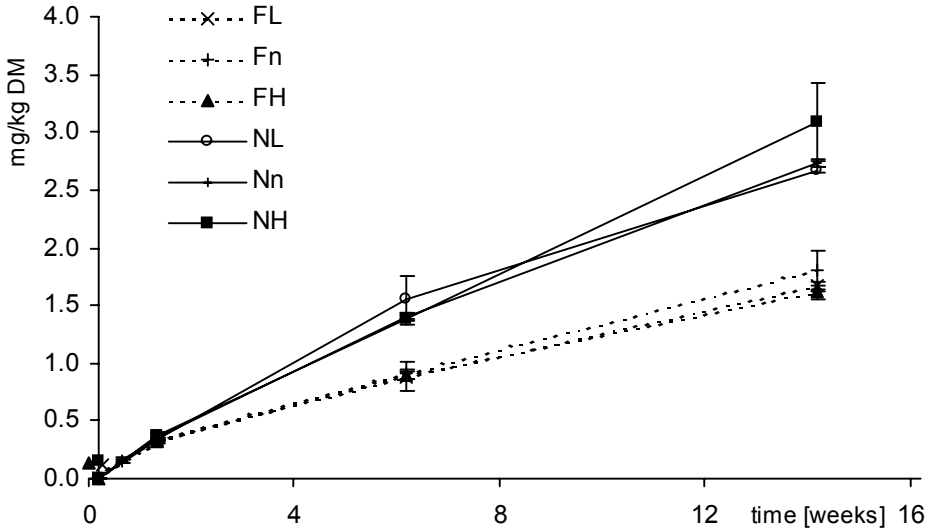

Fig. 4.4. Production of γ-lactones in mg/kg dry matter, during the ripening period of six different Gouda cheeses from the same batch of milk, conventionally coated or foil-wrapped, with different initial water contents. The initial levels in the curds are given as separate points at t=0. Values are a summation of C10, C12, C12:1, C14, C16 and C18 γ-lactones. Error bars represent standard deviations of duplicate measurements.

The differences in water content did not influence the formation of lactones. It has been described that the presence of water is necessary for lactone formation (Urbach & Stark, 1978), but the relatively small differences in water content in the current study (table 4.1) are probably not of influence on the lactone formation.
Apart from the difference between covering and water content, the ripening temperature is the major difference between the two cheese types studied. The water content seemed to be trivial on lactone formation, but from the results presented here, it was not entirely clear whether the covering or the temperature contributed most to the decreased formation of lactones in foil-wrapped cheeses.

Esters and ketones

Apart from free fatty acids and the lactones, also ketones, esters, aldehydes and alcohols have been mentioned as fat-derived compounds that occur in cheese (Collins et al., 2003). Although FFA and lactones are the major fat-derived compounds in Gouda cheese, in a previous study also esters and ketones have been found in these cheeses, albeit in relatively low concentrations (Alewijn et al., 2005). Aldehydes and alcohols have not been found in this cheese type, using the method described earlier (Alewijn et al., 2003).
Metyl ketones were present in the cheese curds at 0.4 to 0.7 mg/kg dry matter, which is comparable to values previously found in curd of other Gouda cheeses (Alewijn et al.,

2005). A slight increase in 2-ketone concentration was found during the ripening period studied, both for foil-wrapped and conventionally coated cheeses. Such a slow formation was observed previously (Alewijn et al., 2005). The ripening time was this time, however, too short to find significant differences between the amounts of 2-ketones formed in foil-wrapped and conventionally coated cheeses. Low levels of methyl and ethyl esters were found in the curds, and as for the ketones, the formation in 14 weeks was slow, in both groups of cheeses studied here. This slow formation of esters was found earlier (Alewijn et al., 2005). Because of the limited formation in the ripening time studied, no significant differences in formation could be observed.

Conclusions

The development of fat-derived flavour compounds is different in foil-wrapped and conventionally plastic-coated cheeses. All FFA and lactones, but especially long chain FFA and γ-lactones, are formed in higher concentrations in conventionally coated cheeses than in foil-wrapped cheeses. This might be due to a number of altered ripening conditions, but especially the higher temperature used for conventionally coated cheese ripening seems to be important. The production of ketones and esters was too low to draw conclusions in the relative short ripening time considered.

Foil-wrapped cheeses generally have lower initial water contents than conventionally coated cheeses. It was shown that higher water content seemed to increase the production of especially long chain FFA in the current cheeses, both in conventionally coated and in foil-wrapped cheeses. However, the water content of the cheeses did not influence the production of small chain fatty acids and lactones.

Therefore, it can be concluded that the production of fat-derived flavour compounds is influenced by the circumstances of the cheeses during ripening, and especially FFA and lactone levels will be lower in foil-ripened cheese. Generally, the industrially applied process of foil-wrapped cheese ripening will produce less fat-derived compounds, possibly leading to a more bland taste. In case of the long chain free fatty acids, the lower initial water content may contribute to the lower production, but for the short chain FFA and the lactones, the differences in water content applied are not of influence. For all fat-derived compounds, most differences observed can probably be attributed to the difference in temperature applied, rather than changes in packaging and the resulting differences in cheese.

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The formation mechanism of lactones in Gouda cheese

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Abstract

Lactones are fat-derived aroma compounds, but the formation mechanism of these compounds during ripening of Gouda cheese is unknown. Both enzymatic and chemical formation pathways were investigated in this study. Lactone formation from milk triglycerides or free fatty acids by lactic acid bacteria enzymatic activity was not observed. Instead, the mechanism of the lactone formation in cheese was a one-step, non-enzymatic reaction, where a hydroxy fatty acid, esterified in a triglyceride undergoes trans-esterification to release the lactone directly. The lactone reaction potential was determined for all major γ- and δ-lactones by controlled heating of the fat. The chemical formation of lactones was temperature-dependent and the Arrhenius parameters of the formation reaction were estimated for each of the lactones. The kinetics of the reaction were first-order, and could be used to explain the formation of lactones in ripening Gouda cheese up to about 40 weeks.

Keywords

Lactones, cheese, milk fat, chemical formation, reaction mechanism.

1. Introduction

Lactones are potent fat-derived flavour compounds that play a role in the overall flavour of cheese (Dufosse, Latrasse & Spinnler, 1994; Moio, Etievant, Langlois, Dekimpe & Addeo, 1994; Urbach 1997; Curioni & Bosset 2002; Singh, Drake & Cadwallader, 2003). Lactones are found in Cheddar (Wong, Ellis & LaCroix, 1975; Chin, Bernhard & Rosenberg, 1996; Rehman, Banks, Brechany, Muir, McSweeney & Fox, 2000), Gouda (Dirinck & de Winne, 1999; Alewijn, Sliwinski & Wouters, 2003; Alewijn, Sliwinski & Wouters, 2005), Gruyère (Bosset & Liardon, 1985), ewes' milk cheeses (Larrayoz, Addis, Gauch & Bosset, 2001), Parmesan (Barbieri et al., 1994) and Blue type cheeses (Gallois & Langlois, 1990). The sensory properties of lactones in dairy products were recognised long ago (Keeney & Patton, 1956), and the aroma thresholds of the individual lactones have been established (Siek, Albin, Sather & Lindsay, 1971), which can be as low as 1 ppm in oil systems, and even lower in water.

It has been known for several decades that heating of milk fat results in the formation of lactones (Keeney et al., 1956; Boldingh & Taylor, 1962). Urbach and Stark (1978) defined the lactone potential of milk as the amount of lactones released when the fat fraction is heated at 180°C in the presence of water and in the absence of air. Hydroxy fatty acids, esterified in glycerides are thought to be the precursors of the lactones formed after heating (Mattick, Patton & Keeney, 1959; Jurriens & Oele, 1965; Wyatt, Pereira & Day, 1967). These precursors are produced in the cow, and their production depends upon the feeding regime and stage of lactation (Mattick, et al., 1959; Parliment, Nawar & Fagerson, 1966; Walker, Patton & Dimick, 1968; Urbach et al., 1978; Urbach, 1982). When milk fat is heated, esterified hydroxy acids are hydrolysed from the glyceride to form a free hydroxy acid, which may then spontaneously lactonize, as described by Mattick et al. (1959). Although this mechanism is accepted for the formation of lactones under quite extreme temperatures (180°C), the formation mechanism of lactones during cheese ripening has not been studied in detail. It is generally assumed that during cheese ripening, lactones are formed by a mechanism starting from esterified hydroxy acids with free hydroxy acids as intermediates (Molimard & Spinnler, 1996; McSweeney & Sousa, 2000; Collins, McSweeney & Wilkinson, 2003; Marilley & Casey 2004), although an alternative mechanism was proposed, where lactic acid bacteria transform unsaturated free fatty acids to free hydroxy acids and lactones (Wanikawa, Hosoi & Kato, 2000; Wanikawa, Shoji, Hosoi & Nakagawa, 2002). It was recently shown that the release of aliphatic free fatty acids and formation of lactones followed a different time pattern during the ripening of Gouda cheese (Alewijn et al., 2005). This study showed that the formation rate of lactones was considerably higher than that of free fatty acids during cheese ripening. If lactones would have to be formed from free

hydroxy acids, or from unsaturated acids produced by lipolysis, a very specific release of these acids from the triglycerides would then be required, which is not very likely. Therefore, it is presumed that the formation mechanism of lactones is somewhat different than suggested to date.

The major aim of this research was to clarify the mechanism of lactone formation during the normal (13°C) ripening of Gouda cheese, and to determine whether this formation is an enzymatic process or resembles the mechanism that occurs during heating. Since the method of determining lactone potentials has not been comprehensively reported (Urbach et al., 1978), and the published lactone potentials do not include all individual major lactones (Stark, Urbach, Cook & Ashes, 1978), potentials of individual lactones in different milk fats were established. The proposed mechanism and the validity of its kinetic properties was verified by comparing the mechanistically predicted values of lactone concentration with the real data in a ripening (Gouda) cheese.

2. Materials and Methods

2.1. Samples

Six commercially available milk samples were obtained from local supermarkets, and an additional six milk samples, ready for Gouda cheese production, were collected from several cheese factories. All milk was fresh (pasteurised), full fat, obtained between February 2001 and October 2002, and stored at –25°C prior to analysis. Full-fat Gouda-type cheese was prepared and ripened as described previously (Alewijn et al., 2005). The dry matter content was calculated from the weight difference of a sample mixed with clean dry sand, before and after 2 hours of drying in a 103°C hot air stove.

2.2. Microbial formation of lactones

Three strains of *Lactococcus lactis* subsp. *cremoris*, B697, B1492 and B1157, were obtained from the NIZO culture collection (NIZO Food Research, Ede, The Netherlands), and a common Gouda cheese starter mixture (BK2) was obtained from CSK Food Enrichment (Ede, The Netherlands). The strains were cultured at 30°C in sterilised (15 min at 121°C) M17 broth (Difco, Detroit, MI, USA) with lactose. Cells in their late exponential growth phase or in the late stationary phase were incubated with 10-undecenoic acid (C10:1), cis-5-dodecenoic acid (C12:1), cis-9-tetradecenoic acid (C14:1), cis-10-heptanoic acid (C17:1), oleic acid (C18:1Δ9), linoleic acid (C18:2Δ9,12) or ricinoleic acid (12-hydroxy-C18:1Δ9), all obtained from Sigma-Aldrich (St. Louis, MO, USA), and minimally 97% pure, or with water-washed milk fat. To the bacterial culture (10 mL), a 10% (w/w) solution of fatty material in 70% ethanol (50 µL) was added. The same incubations were also carried out with cell-free

extracts of the cultures, obtained after cell disruption using 0.1 mm zirconia/silica beads (Biospec, Bartlesville, OK, USA). Incubations were carried out at 13 or 30°C for 12 to 96 hours, and were shaken or left at rest in the presence or absence of oxygen (GasPack, BBL Microbiology, Kansas City, MO, USA). The aqueous broth was extracted three times with ether/heptane, and an aliquot was analysed for lactones on the GC/MS system as described below.

2.3. Fat collection and lactone determination

Fresh milk (100 mL) was mixed 1:1 (v/v) with 96% ethanol, and extracted with 10 mL of ether/heptane (1:1, v/v) at 40°C. After centrifugation at 700*g* for 5 min, the top layer was collected, and the extraction was repeated twice to obtain a large part of the milk triglycerides. The ether/heptane from the combined organic layers was evaporated at low temperature (<20°C). Cheese fat was isolated in a similar fashion.

Independent duplicates of milk fat (0.4 g) and water (20 µL) were kept frozen (-20°C) until analysis. Lactones were extracted with acetonitrile (1 mL) at 30°C. The acetonitrile-layer was analysed directly by GC/MS, consisting of a 8000^{top} GC (CE Instruments, Milan, Italy) equipped with a FFAP GC column (J&W Scientific, Palo Alto, CA, USA) and a Finnigan Automass II (Finnigan, Bremen, Germany). Myristylbromide (18 µg mL^{-1}) was used as an internal standard. To correct for extraction losses that occurred with the acetonitrile extraction of the fat, the distribution coefficients of all individual lactones in this extraction were determined by re-extracting the residue of the first extraction with another 1 mL of acetonitrile. Using the distribution coefficient, the lactone concentration in the fat was calculated, as described earlier (Alewijn et al., 2003).

2.4. Lactone potentials and temperature dependency

To determine the lactone potentials (LP), samples of fat were kept in closed tubes at 160°C for 5 to 220 min and analysed for lactones. All data points (five to nine independent duplicates per milk fat) were used and fitted to eq. 3, minimising the sum of squares. A 95% confidence interval $LP_{95\%CI}$ was calculated assuming normally distributed errors using the following equation

$$LP_{95\%CI} = (LP \pm 1.96 \cdot \sigma), \qquad \textit{(eq. 1)}$$

with a standard deviation of $\sigma = \sqrt{\frac{1}{n-1}\sum_{i=1}^{n}(lac_i - lac_{fit,i})^2}$

where n is the number of observations, and lac_i and $lac_{fit,i}$ the respective observed and fitted lactone concentrations. To determine the temperature dependency of lactone formation, three different milk fat samples, in duplicate, were similarly kept at 20, 40, 60 and 140°C.

Heating times (5 min to 174 hours) were chosen to obtain well-distributed data points over the particular formation curve at each temperature, and the free lactone concentrations were determined for each fat sample. The resulting 42 data points seemed to be described by the Arrhenius equation (4). The Arrhenius parameters and 95% confidence intervals were estimated after linear transformation of equation (5), minimizing the sum of squares, using the Microsoft Excel Analysis ToolPak add-in (Microsoft, Redmond, WA, USA).

3. Results and discussion

3.1. Microbial formation of lactones

Lactones can be produced from unsaturated free fatty acids by lactic acid bacteria (Wanikawa et al., 2002). In the case of a ripening Gouda cheese, the starter lactic acid bacteria could be candidates to perform such a conversion. To check the likelihood of this conversion, three strains of *Lactococcus lactis* subsp. *cremoris*, representative of the starter for Gouda cheese, the mixed strain starter culture itself, and cell-free extracts from these strains were tested for lactone formation with a number of free unsaturated fatty acids, as described above. However, under the *in vitro* conditions tested, no significant amounts of lactones from the various fatty acids were produced. It should be noted that the *in vitro* circumstances were certainly different from those in cheese, and that the number of strains and conditions tested were not exhaustive. Nevertheless, the experiments showed that the conversion of unsaturated fatty acids into lactones by lactic acid bacteria is a reaction that does not occur readily.

3.2. Lactone potentials

Lactone potentials were determined by heating the fat at 160°C, rather than at 180°C as suggested by Stark et al. (1978), to be able to follow the formation of lactones during heating more closely. The concentrations of δ-lactones during heating time are shown in Fig. 5.1.

Lactone formation was fast in the beginning, but then slowed down quickly and lead to an almost constant concentration after 45 min of heating at 160°C. It is presumed that the precursors of the lactones became exhausted. Obviously, under these circumstances the reaction responsible for the conversion of precursor molecules to free lactones is a chemical reaction, not catalysed by enzymes.

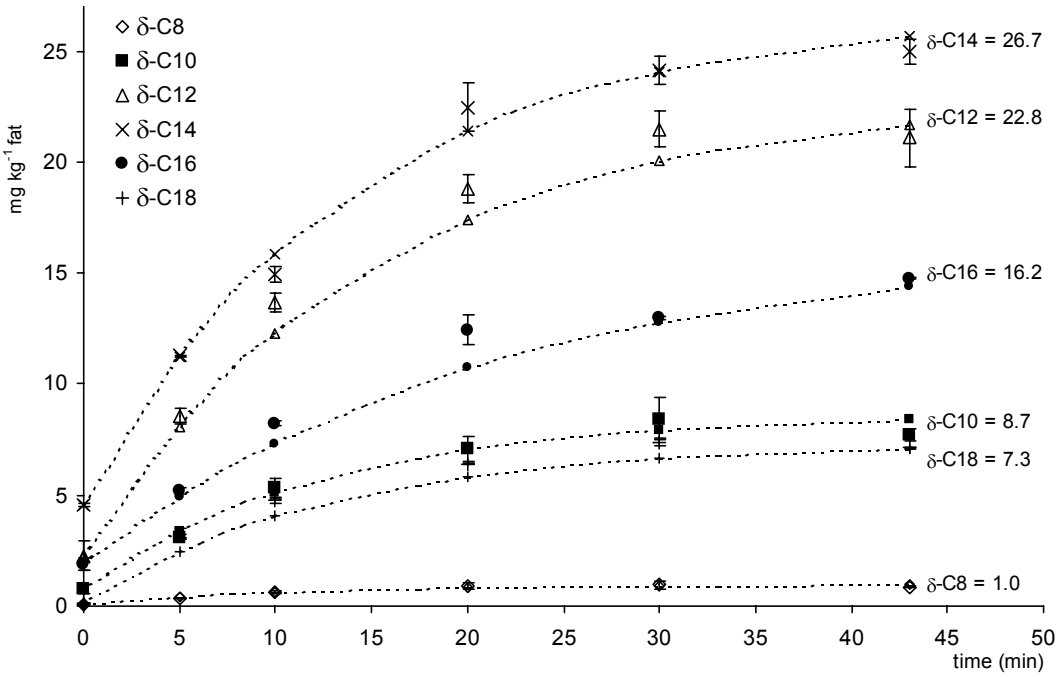

Fig. 5.1. Formation of δ-lactones (mg kg^{-1}) in milk fat B, heated at 160°C. Symbols are experimentally obtained values, dotted lines are calculated trends, as explained in the text. The values on the right hand side are the predicted lactone potentials in mg kg^{-1} fat. Error bars represent standard deviation of duplicates.

The formation of lactones showed kinetics that resembled a first-order reaction mechanism. Generally, a first order reaction can be described by eq. 2,

(eq. 2) $$\ln\left(\frac{[C]}{[C_0]}\right) = -k \times t$$

where C_0 is the initial concentration, C the concentration at time (t), and k is the rate constant. In the current experiment, $[C_0]$ is the lactone potential (LP), and $[C]$ is the remaining concentration of lactone precursor at a given time. The products of the reaction, free lactones (FL), were measured over time, and with

$$LP = [C] + [FL]$$

the current reaction could be described by eq. 3.

(eq. 3) $$\ln\left(\frac{LP - [FL]}{LP}\right) = -k \times t$$

The formation of all δ-lactones (C8, C10, C12, C14, C16) in heated milk fat can clearly be explained by a first order reaction (Fig. 5.1), which was also true for all major γ-lactones (C12, C12:1, C14, C16) (data not shown).

To determine the lactone potential, the maximum lactone concentration after a particular heating time has been used (Stark et al., 1978). However, since the concentration of free

lactones approached the lactone potential in an asymptotic way, it was difficult to determine when the heating time was sufficient to estimate the lactone potential. Therefore, a mathematical approach was used. All time and free lactone concentration combinations, five to nine independent duplicates for each sample of milk fat, were used to fit *LP* and *k* in equation (3), minimizing the sum of squared differences between observed and predicted data. The obtained lactone potentials of individual lactones in different milk fats are given in Table 5.1, showing considerable differences between different milk fats.

Table 5.1. Lactone potentials (in mg kg^{-1}) of milk fats from twelve different full fat milk samples.

	A[a]	B	C	D	E	F	G	H	I	J	K	L	*average*
γ-C12	7.4 *(±0.94)*[b]	8.8 *(±0.37)*	8.0 (±1.56)	7.7 *(±0.80)*	10.0 *(±0.78)*	7.8 *(±1.11)*	8.7 *(±1.06)*	5.8 *(±0.50)*	10.5 *(±0.51)*	9.5 *(±1.47)*	11.0 *(±1.30)*	9.9 *(±0.87)*	*8.8*
γ-C12:1	2.2 *(±0.42)*	2.3 *(±0.09)*	1.6 *(±0.32)*	2.1 *(±0.11)*	2.4 *(±0.21)*	1.9 *(±0.23)*	1.8 *(±0.16)*	1.4 *(±0.17)*	2.3 *(±0.18)*	2.3 *(±0.41)*	2.6 *(±0.37)*	2.4 *(±0.23)*	*2.1*
γ-C14	4.4 *(±0.23)*	4.8 *(±0.34)*	2.6 *(±0.28)*	*n.q.*[c]	*n.q.*	*n.q.*	*n.q.*	*n.q.*	*n.q.*	*n.q.*	*n.q.*	*n.q.*	*3.9*
γ-C16	8.5 *(±2.51)*	1.8 *(±0.15)*	4.5 *(±0.70)*	*n.q.*	4.1 *(±0.41)*	5.6 *(±0.69)*	6.3 *(±0.69)*	6.2 *(±0.97)*	6.9 *(±0.67)*	7.5 *(±0.92)*	6.7 *(±1.00)*	4.9 *(±0.68)*	*5.7*
sum γ	**22**	**18**	**17**	**10**	**16**	**15**	**17**	**13**	**20**	**19**	**20**	**17**	***17***
δ-C8	1.9 *(±0.17)*	1.0 *(±0.14)*	1.1 *(±0.19)*	0.9 *(±0.09)*	1.3 *(±0.16)*	1.6 *(±0.14)*	1.4 *(±0.14)*	1.8 *(±0.21)*	1.8 *(±0.14)*	1.8 *(±0.20)*	1.7 *(±0.13)*	1.6 *(±0.18)*	*1.5*
δ-C10	20 *(±3.96)*	9 *(±0.84)*	8 *(±1.74)*	10 *(±1.04)*	12 *(±1.81)*	13 *(±1.38)*	13 *(±1.27)*	15 *(±1.43)*	14 *(±1.42)*	13 *(±1.43)*	15 *(±2.17)*	14 *(±1.79)*	*13.1*
δ-C12	50 *(±9.05)*	23 *(±1.16)*	21 *(±4.49)*	24 *(±2.96)*	28 *(±2.40)*	31 *(±3.06)*	31 *(±2.12)*	35 *(±3.04)*	33 *(±2.87)*	29 *(±2.48)*	42 *(±7.04)*	34 *(±4.01)*	*31.7*
δ-C14	66 *(±10.3)*	27 *(±1.38)*	26 *(±6.61)*	30 *(±2.92)*	40 *(±3.87)*	48 *(±5.57)*	47 *(±6.69)*	57 *(±6.43)*	51 *(±5.79)*	45 *(±5.73)*	58 *(±6.53)*	53 *(±7.88)*	*45.7*
δ-C16	31 *(±7.12)*	16 *(±0.97)*	35 *(±7.16)*	16 *(±2.34)*	26 *(±3.96)*	26 *(±4.35)*	27 *(±3.49)*	31 *(±3.42)*	31 *(±3.26)*	29 *(±3.19)*	24 *(±3.01)*	37 *(±3.31)*	*27.5*
δ-C18	14.1 *(±1.70)*	7.3 *(±0.37)*	5.8 *(±0.87)*	*n.q.*	10 *(±1.17)*	10 *(±1.55)*	12 *(±1.62)*	13 *(±1.57)*	12 *(±0.90)*	10 *(±1.51)*	12 *(±1.20)*	11 *(±0.90)*	*10.7*
sum δ	**170**	**75**	**92**	**82**	**107**	**120**	**120**	**139**	**131**	**117**	**141**	**140**	***119***
γ/δ ratio (%)	13	23	18	12	15	13	14	10	15	16	14	12	14
total lactone[d]	193	93	108	92	123	135	136	152	151	137	162	157	*137*

[a] A through L represent the different milk samples studied.
[b] The values in parentheses represent a 95% confidence interval of the lactone potential
[c] *n.q.* = not quantified. Reliable quantification was not possible due to co-elution with a fatty acid.
[d] Excludes γ-C6, C7, C8, and C10 lactones, for which a lactone potential could not be determined, as described in the text.

The exact origin of these milk samples was not always known, and these differences might have been due to seasonal variations and different feeding regimes of the cows (Stark et al., 1978; Urbach, 1982). Despite the differences in concentrations, the ratios of individual lactones varied only slightly in the samples investigated. The different lactone potentials can influence the final aroma of cheeses prepared from these milk sources, or the flavour (defect) when this milk is extensively heated.

3.3. Short chain γ-lactones

Short chain γ-lactones (γ-C6, γ-C8 and γ-C10) clearly showed a different formation pattern than the δ-lactones described above. When the milk fat was heated, their formation rate

was slow in the beginning, and increased gradually for 2 hours (Fig. 5.2). Also, in contrast to the other γ- and δ-lactones, the formation of short chain γ-lactones was stimulated by water amounts up to 20% (v/v) (data not shown). The short chain γ-lactones also behaved differently from the other lactones in cheese ripening, as described below. In the current experiment, no lactone potentials could be established for the short chain γ-lactones, and a formation mechanism different from that of the longer chain γ-lactones and the δ-lactones must therefore be considered. This remains to be investigated.

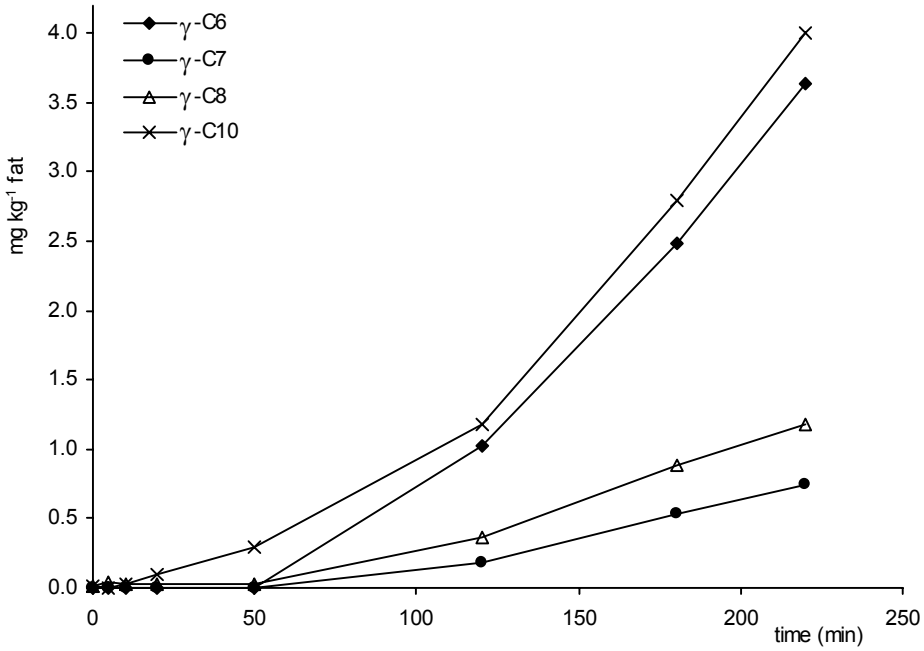

Fig. 5.2. Formation of short chain γ-lactones (mg kg^{-1}) in milk fat B, during heating at 160°C. Error bars represent standard deviations of duplicates.

3.4. Lactone formation mechanism

Lactones are thought to originate from hydroxy fatty acids (Jurriens et al, 1965; Wyatt et al., 1967; McSweeney et al., 2000). These are present in milk fat, incorporated in the milk fat triglycerides, but are not present in their free form. Free hydroxy acids might be produced by hydrolysis of the hydroxy acid from the triglyceride, as originally suggested by Mattick et al. (1959). However, whereas milk fat consists of about 99% of triglycerides esterified with aliphatic fatty acids (Walstra, Geurts, Noomen, Jellema & van Boekel, 1999), less than 1% of the available aliphatic fatty acids were hydrolysed from the triglycerides after heating milk fat at 140°C for 220 min. In contrast, the amount of lactones produced reached almost the maximal potential of the fat at that time. It is therefore unlikely that the formation mechanism of lactones includes a hydrolysis of the hydroxy fatty acid from the triglyceride. Addition of diluted acid (HCl) and diluted NaOH (both up to 300 μmol g^{-1} fat)

had no substantial effect on the formation of the major lactones, whereas the hydrolysis of aliphatic free fatty acids was greatly enhanced (results not shown). This also indicated that the formation of lactones does not include a hydrolysis step followed by lactonization, but is a direct lactonization instead. Based on these observations, a reaction mechanism for δ- and long chain γ-lactones is proposed (Fig. 5.3).

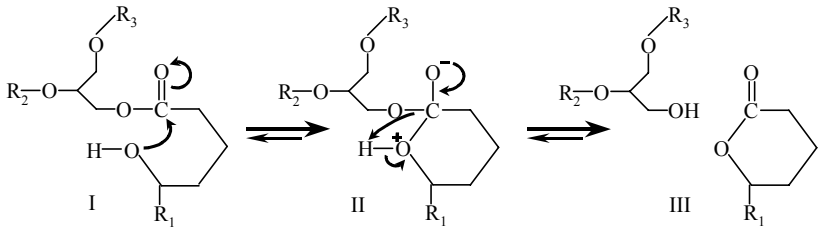

Fig. 5.3. Proposed formation mechanism of δ-lactones from esterified hydroxy fatty acids in milk fat. Long chain γ-lactones form 5-membered rings analogously, with the hydroxy group on C4 rather than the depicted C5 for δ-lactones. R_1 = $(CH_2)_n$-CH_3; R_2 and R_3 = CO-$(CH_2)_n$-CH_3.

When the starting molecule (I in Fig. 5.3) is in the correct conformation, the reaction starts by a nucleophilic attack of the hydroxy group on the carboxyl C-atom. A negative charge is temporary stored on the neighbouring oxygen atom. The reaction proceeds slowly without water addition prior to heating. Addition of 5% (v/v) water enhances the formation rate substantially, whereas adding more water had no additional effect (data not shown). This indicates that water plays a catalytic role in the reaction, probably by shielding the charge of the intermediate structure (II) from its non-polar environment. In the next step, the reaction terminates by splitting off the lactone, leaving a glycerolate anion, which will readily become a (di)glyceride after addition of the proton from the former hydroxy group. The unimolecular nature of the proposed reaction agrees well with the observation that 1st order reaction kinetics can be used to describe the reaction.

3.5. Temperature dependency

Temperature dependency of chemical reaction rates can be described by the Arrhenius equation (eq. 4).

(eq. 4) $$k = A \cdot e^{(-E_a/R \cdot T)}$$

The formation of individual lactones in pure milk fat was determined at 20, 40, 60 and 140°C, and used to calculate the relevant Arrhenius parameters. It was reported earlier that the Arrhenius parameters E_a and A are strongly correlated when using a relatively narrow temperature range, so the following reparameterisation was used (Brands, 2002):

(eq. 5) $$k = -\frac{1}{t}\left(\frac{LP - [FL]}{LP}\right) = X \cdot e^{(-Y \cdot E_a)}, \text{ with}$$

$$X = k_0 \cdot e^{(-E_a/R \cdot T_{av})}$$

$$Y = \frac{1}{R}\left(\frac{1}{T} - \frac{1}{T_{av}}\right)$$

$$T_{av} = \frac{\sum T}{n}$$

In these equations, E_a is the activation energy (kJ mol^{-1}), R the gas constant (8.31 J mol^{-1} K^{-1}), k_0 a dimensionless pre-exponential factor, and the rate constant k was substituted as shown in equation (3).

The *LP* was calculated (section 3.2 of this chapter) and corrected for the initial lactone concentration. All the data (42 measurements) for milk fat heated at 20, 40, 60 and 140°C at the different time points were used simultaneously to fit X and Y in equation (5), minimising the sum of squares. This yielded the activation energies and pre-exponential factors for the major lactones in cheese (Table 5.2).

Table 5.2. Kinetic parameters[a] for the formation of the major lactones in milk fat.

	E_a (kJ mol^{-1})		A (x10^6 h^{-1})		k at 13°C (x10^{-3} h^{-1})	k at 160°C (x10^{-3} h^{-1})
γ-C12	62.1	*(±4.0)*	192	*(±8.4)*	0.87	34.7
γ-C12:1	62.6	*(±4.1)*	187	*(±3.7)*	0.69	28.4
γ-C14	63.4	*(±8.8)*	115	*(±1.2)*	0.31	13.3
γ-C16	61.2	*(±4.5)*	104	*(±2.6)*	0.69	26.0
δ-C8	56.1	*(±3.0)*	32.6	*(±2.5)*	1.82	51.0
δ-C10	56.8	*(±2.9)*	34.4	*(±2.8)*	1.43	41.7
δ-C12	56.9	*(±3.0)*	29.4	*(±2.6)*	1.18	34.7
δ-C14	56.8	*(±2.9)*	30.5	*(±2.6)*	1.29	37.6
δ-C16	54.1	*(±3.0)*	9.6	*(±1.0)*	1.27	31.5
δ-C18	51.8	*(±7.2)*	8.4	*(±0.9)*	2.86	62.0

[a] E_a (energy of activation) and A (the pre-exponential factor) are the terms calculated for the Arrhenius-equation (eq. 4) with R = 8.31 J K^{-1} mol^{-1} and T = absolute temperature (K). The values in parentheses represent the 95% confidence intervals for E_a and A. The reaction rate constant, k, is calculated as an example for 13°C and 160°C.

3.6. Fit with experimental cheese data

To check the model data for the formation of lactones, a typical Gouda cheese was prepared and the formation of lactones was followed during ripening. The ripening of this cheese was previously described in more detail (Alewijn et al., 2005). The potentials of the individual lactones are listed (Table 5.1), with milk fat *'K'* being a sample from the milk used to prepare this cheese. Together with the kinetic data on the formation of lactones (Table 5.2) and the fat content in dry cheese matter (data not shown), the formation of

individual lactones during ripening of this cheese at 13°C was predicted. The formation of the individual δ-lactones in the actual cheese environment could be accurately predicted for up to 40 weeks of ripening time (Fig. 5.4).

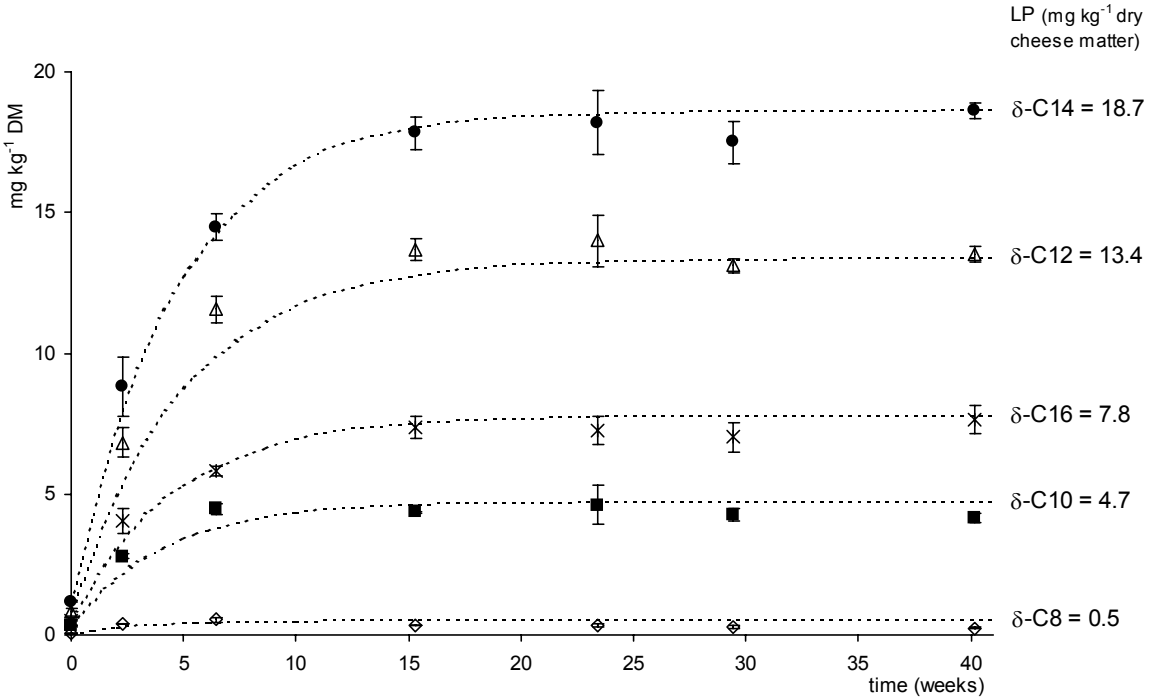

Fig. 5.4. Concentrations of major δ-lactones (mg kg^{-1} dry cheese matter) during the ripening of a typical Gouda cheese. Symbols represent lactone concentrations (independent duplicates) determined in cheese samples as described in the text, with error bars representing standard deviations. Dotted lines represent calculated lactone concentration trends based on the E_a and A values in Table 5.2, and the lactone potential of this particular cheese is expressed in mg kg^{-1} cheese dry matter (DM).

The formation of the major γ-lactones (C12, C12:1 and C14) could be predicted in a similar way (data not shown). Only δ-octalactone (δ-C8) showed a clear (and unpredicted) decrease after 8 weeks of ripening time, which was observed in other cheeses as well. For this deviant behaviour no clear explanation could be found. Moreover, the short-chain γ-lactones consistently showed a formation pattern in ripening cheese resembling the curves obtained at 140°C (Fig. 5.2), although the formation rate was much lower at the ripening temperature. This reaction cannot be described by a first-order reaction as for the formation of the other γ- and δ-lactones. The mechanism of the short-chain γ-lactones is not yet understood and the formation in cheese ripening could therefore not be predicted.

Similar to the cheese described above, the formation of long-chain γ-lactones and δ-lactones could also be predicted for several other cheeses that were followed during ripening (Alewijn, et al., 2005). This suggests that the mechanism presented above is the

predominant mechanism in most Gouda cheeses, and probably in most other cheese types as well. Since the formation of lactones is a non-enzymatic process, only limited control of lactone formation is possible in ripening cheese with a given lactone potential, as the key influencing factors, temperature and water activity have rather strict practical limits.

4. Conclusions

The presented method describes a relatively fast and easy way to determine the lactone potential of milk fat. With this lactone potential, which varies for different batches of milk, the calculated kinetic parameters can be used to predict the concentration of the major individual lactones for Gouda cheese ripened at 13°C for up to 40 weeks. This enables an accurate estimation of the formation of individual lactones in all cheeses where no additional microbial activity contributes to the lactone formation. Such a microbial activity was never convincingly observed with the Gouda-related strains that were used in this study. However, the mechanism of small chain γ-lactones proved to be different from that of the other lactones, and their formation could not be predicted with the data presented.

The results presented here indicate that the originally proposed two-step formation mechanism (hydrolysis of the hydroxy fatty acid followed by ring-closure) of the δ-lactones and major γ-lactones at 140°C is not very likely, and that the formation of lactones is a one-step intramolecular trans-esterification reaction. It was also shown that the same (chemical) reaction occurs under cheese ripening conditions.

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The formation kinetics of 2-ketones in milk fat

relevance for milk processing and cheese ripening

Chapter 6

An edited version of this chapter has been submitted for publication as: Alewijn, M., Sliwinski, E. L. & Wouters, J.T.M. Formation kinetics of 2-ketones in milk fat - relevance for dairy products.

Abstract

Methyl ketones are well-known fat-derived flavour compounds, occurring in high levels in heated milk products and blue mould type cheeses.

The non-enzymatic formation of these compounds has been studied, and appeared to be a water-catalysed release of an esterified ß-keto acid. The initial precursor concentration, or ketone potential, has been determined for the odd-numbered ketones C9-C15 in twelve different milk samples, and averaged 14.7, 18.1, 40.1, and 81.2 mg kg-1 fat, respectively. The amount of ketones formed depended on the concentration of the ketone precursor, and the formation followed first-order kinetics. The temperature dependency of the reaction complied with the Arrhenius equation, and the reaction parameters involved have been determined.

With these data, ketone formation in milk fat containing products could be calculated in time/temperature combinations that are often applied in dairy processing. Although for most common processing and storage conditions the resulting amount of ketones is probably not of major sensorial importance, their chemical formation will occur in all products containing milk fat. The data presented enable the prediction of the concentration of individual ketones in these products throughout production and storage.

Keywords

2-Ketones; Formation kinetics; Chemical formation; Gouda cheese; Ketone potential

1. Introduction

Milk fat is present in most dairy products. Apart from being a nutritionally significant ingredient and an important structural compound, it can be partly responsible for the product flavour. Flavour is a key quality factor, and during processing and storage, both wanted and unwanted changes may occur that result in a different aroma profile of the product. Apart from many possible enzymatic reactions that lead to changes in the aroma profile, non-enzymatic reactions may occur as well. For example, hydroxy fatty acids in milk triglycerides may spontaneously react to form free lactones, as reported earlier (Alewijn, Smit, Sliwinski, & Wouters, 2006). As lactones are flavour-active, this shows that also non-enzymatic reactions may play a role in flavour development. Another class of possible flavour-active fat-derived compounds are the methyl ketones (2-ketones), which may be produced both enzymatically and non-enzymatically.

Methyl ketones are the key flavour compounds in blue mould type cheeses, and their flavour detection threshold is quite low (Siek, Albin, Sather, & Lindsay, 1969; Kinsella, 1975). In blue mould cheeses, free fatty acids produced by lipases are intracellularly toxic for the mould cell. Blue moulds produce enzymes that are able to ß-oxidise and decarboxylate these fatty acids to produce 2-ketones (Gehrig & Knight, 1963; King & Clegg, 1979; Okumura & Kinsella, 1985; Cerning, Gripon, Lamberet, & Lenoir, 1987; Larroche, Besson, & Gros, 1996). Ketone levels in blue type cheeses of up to 48 mg kg^{-1} fatty matter (Gonzalez de Llano, Ramos, Polo, Sanz, & Martinez-Castro, 1990), 35 mg kg^{-1} cheese (Gallois & Langlois, 1990), or 80 mg kg^{-1} fatty matter in Camembert cheese (Molimard & Spinnler, 1996) can be reached. Curioni and Bosset (2002) showed that ketones are sensorially very important in these cheese types.

Ketones are also known to be produced by heating of milk or milk fat. The exact mechanism is unknown, but extensive heating of milk is known to produce a flavour-defect in milk (Walstra & Jenness, 1984), which can be attributed to the formation of 2-ketones (Forss, 1979). Other studies confirmed the temperature-dependent formation of ketones in milk and milk fat (Jeon, Thomas, & Reineccius, 1978; Calvo & de la Hoz, 1992; Valero, Villamiel, Miralles, Sanz, & Martinez-Castro, 2001; Contarini, Povolo, Leardi, & Toppino, 1997). Formation of ketones is also observed during storage of sterilised milk (Arnold & Lindsay, 1969; Loney, 1970). Hall, Andersson, Lignert, and Olofsson (1985) studied the development of ketones in milk powder and reported zero-order formation kinetics for this reaction. The term "ketone potential", referring to the amount of ketones that is released upon heating of milk fat at 180°C, was introduced in 1978 (Stark, Urbach, Cook, & Ashes, 1978). Ketones are also found to be suitable markers for heat-treatment of milk (Contarini & Povolo, 2002), however, no mechanism of the formation of ketones was formulated, nor has this heat-

induced utilisation of the ketone potential been related to the formation of ketones during cheese ripening or other low-temperature long term storage.

In contrast to Blue type cheese, moulds and their enzymes are absent in Gouda-type cheeses, and consequently, lipolysis in general and the formation of ketones is substantially lower in these cheese types (McSweeney & Sousa, 2000). Ketones were, however, detected and quantified in Gouda cheese (Alewijn, Sliwinski, & Wouters, 2003), and it was shown that the concentration of ketones increased from about 1 to 2 mg kg^{-1} in 40 weeks of ripening of this cheese type. This increase was not likely to be due to the activity of mould enzymes. Therefore, the mechanism of ketone formation during ripening of non-mould cheeses might be similar to the heat induced formation of ketones. Experimental work for a previous study on the formation of lactones during the ripening of Gouda cheese (Alewijn et al., 2006) indicated that ketones could also be formed non-enzymatically. This study aims to characterise the formation of 2-ketones from milk fat, and to indicate the implications for the processing and ripening of dairy products containing milk fat.

2. Materials and Methods

2.1 Samples

The same milk samples that were used to determine the lactone potentials in milk (Alewijn et al., 2006) were used in this study, and consisted of commercially available milk samples obtained from local supermarkets (coded A-F), and milk samples ready for cheese production, obtained from several cheese factories (coded G-L). The Gouda cheese sample considered was described before (Alewijn, Sliwinski, & Wouters, 2005).

2.2 Fat collection and extraction of ketones

The sample preparation and the extraction of 2-ketones was carried out simultaneously with the extraction of lactones, as described earlier (Alewijn et al., 2006). The 2-ketones in the GC chromatogram were identified using their mass spectrum, their retention times were checked using authentic compounds. Due to chromatographic coelution with the solvent, 2-heptanone and smaller 2-ketones could not be reliably quantified. Quantification of the ketone concentration in milk fat was calculated as described earlier (Alewijn et al., 2003).

2.3 Ketone potentials and temperature dependency

For the determination of the ketone potentials (KP), milk fat samples were heated as described earlier (Alewijn et al., 2006). For each ketone potential, at least 5 time points (independent replicates) were used (Table 6.1), and fitted by minimising the sum of squares. Confidence intervals were given as explained in the text. Using the KPs obtained,

three different milk fat samples (A, B, and C), kept at 20°C (fats A and B, *n*=10), 40 (fats A and B, *n*=12), 60 (fats A, B, and C, *n*=15) and 140°C (fat C, *n*=5) were used to estimate the temperature dependency, using a reparameterised Arrhenius equation, as used earlier (Alewijn et al., 2006). For every ketone, 42 individual data points were used and fitted simultaneously. Cheese and milk storage data from previous studies (Arnold et al., 1969; Alewijn et al., 2003) were used to judge the model values obtained.

3. Results and discussion

3.1 Ketone potentials

When milk fat was heated at 160°C, ketone levels increased rapidly, and then levelled off in time to a stable plateau concentration (Fig. 6.1). Already after 20 to 30 minutes, the further increase in the ketone concentrations observed became small. At 160°C, the formation of ketones is a non-enzymatic reaction, and the levelling off in time was assumed to be caused by the depletion of their precursors. The concentration of these precursors in fresh milk fat is considered to be the ketone potential (KP), which might be an important characteristic for

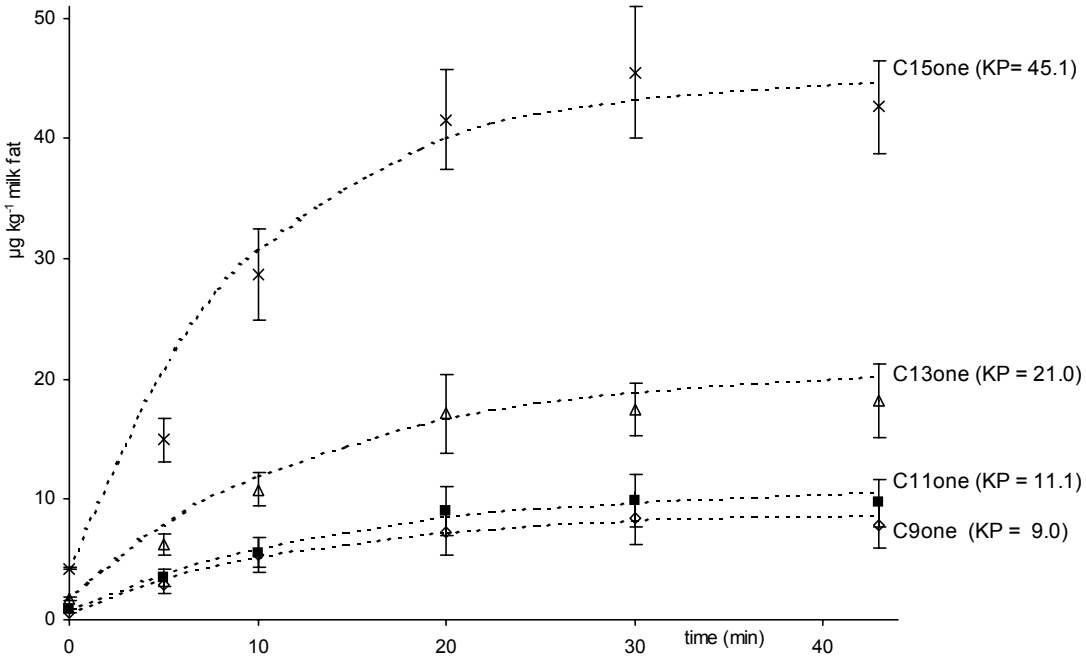

Fig. 6.1. Formation of 2-ketones in mg kg^{-1} milk fat at 160°C in the presence of water. The marker points represent experimentally obtained data, dotted lines represent the model fitted to the data, as described in the text. On the right hand side the ketone potential (KP) for 4 individual ketones in this particular fat (fat 'B' from Table 1) is given, as calculated by the model. Error bars represent the standard deviations.

milk fat. A comparison can be made with the heat-induced formation of lactones from milk fat, as described earlier (Alewijn et al., 2006). The formation of ketones at 160°C was faster than that of lactones, but the profile and the kinetics of both formations seemed similar, and therefore the KP could be determined as had been done before for the lactone potentials (Alewijn et al., 2006).

The formation of all quantified ketones (C9, C11, C13, C15, C17) in heated milk fat showed kinetics that could be well explained by a first order reaction. Therefore, eq. 1 was used to fit the data for the ketone formation during heating, for all milk fats A through L (Alewijn et al., 2006).

(eq.1) $$\ln \frac{\left([KP]-[free\ ketone]\right)}{[KP]} = -kt$$

where *KP* is the ketone potential (µg g^{-1} fat), *k* the reaction rate (h^{-1}) and *t* the time (h).

For each fat, at least 5 independent replicates were used to minimise the sum of squares. A 95% confidence interval was calculated using eq. 2.

(eq. 2) $$KP_{95\%CI} = (KP \pm 1.96 \cdot \sigma)$$

with $$\sigma = \sqrt{\frac{1}{n-1}\sum_{i=1}^{n}(free\ ketone_i - free\ ketone_{fit,i})^2}$$

Table 6.1. Ketone potentials (mg kg^{-1}) of different milk fats A-L.

sample	n[b)]	C9one		C11one		C13one		C15one		C17one		sum ketones
A	9	15.5	*(±1.33)* [a)]	25.3	*(±2.93)*	56.4	*(±6.22)*	105.4	*(±10.99)*	30.2	*(±2.64)*	232.9
B	6	9	*(±0.88)*	11.1	*(±1.01)*	21	*(±2.14)*	45.1	*(±6.50)*	*n.q.* [c)]		86.2
C	5	10.5	*(±1.30)*	12.9	*(±1.53)*	26.1	*(±3.53)*	50.6	*(±8.87)*	*n.q.*		100.1
D	5	14.7	*(±1.12)*	18	*(±1.76)*	41.1	*(±5.09)*	74.6	*(±9.55)*	*n.q.*		148.3
E	5	12.6	*(±1.41)*	16.5	*(±1.68)*	38.3	*(±3.41)*	76.1	*(±8.79)*	*n.q.*		143.4
F	5	14.9	*(±1.49)*	18.6	*(±1.34)*	41.4	*(±4.55)*	86	*(±8.39)*	*n.q.*		160.9
G	5	15.7	*(±1.50)*	18.2	*(±1.74)*	41.8	*(±5.06)*	84.5	*(±12.00)*	*n.q.*		160.3
H	5	14.4	*(±1.27)*	16.5	*(±1.85)*	37.2	*(±3.83)*	80.1	*(±9.53)*	*n.q.*		148.1
I	5	17.6	*(±1.80)*	20.5	*(±2.38)*	45.9	*(±3.88)*	94.9	*(±9.68)*	*n.q.*		179
J	5	19.2	*(±1.46)*	20.4	*(±2.89)*	43.5	*(±4.87)*	87.4	*(±11.54)*	*n.q.*		170.5
K	5	17.6	*(±1.62)*	20.6	*(±2.14)*	45.5	*(±4.40)*	92.6	*(±11.02)*	*n.q.*		176.3
L	5	15.1	*(±1.29)*	18.8	*(±2.07)*	43.4	*(±4.30)*	90.9	*(±9.59)*	*n.q.*		168.1
average		14.7		18.1		40.1		80.7		30.2		156.2

[a)]Values in parentheses represent the 95% confidence interval of the ketone potential.
[b)]*n* represents the number of independent duplicated used for the ketone potentials in the fat.
[c)]2-heptanone (C17on) could not be quantified reliably due to co-elution with decanoic acid.

The resulting ketone potentials are listed in Table 6.1. The data in this Table showed that these milk fats had a considerable ketone potential and that especially the C13 and C15 methyl ketones were produced in significant levels. There was, however, substantial

variation in the ketone potentials of the different milk fats. It is thought that the ketone potentials are determined by the diet of the cows (Urbach & Stark, 1978), and therefore these differences in the ketone potentials were expected. However, the proportions of the individual ketones, roughly 10, 12, 26 and 52% of the total ketones of 2-nonanone to 2-pentadecanone, respectively, were quite similar in all milk fats investigated. Smaller ketones were very likely also present in the milk fats, but were not detected due to the GC-method used. The ketone potentials presented here are slightly higher than the lactone potentials of the same fat, as reported earlier (Alewijn et al., 2006).

3.2 Reaction mechanism

The ketone potentials were determined by heating the milk fats at 160°C up to 43 minutes. This caused only very slight hydrolytic release of free fatty acids (less than 1% of the total available fatty acids), but near-complete formation of the 2-ketones. Consequently, hydrolysis of the ß-keto acid from the triglyceride is not likely the first step of the formation mechanism, and the formation of ketones probably occurs through a thermodynamically more favourable path. Since the formation of ketones can be described by a first-order reaction (see Fig. 6.1), the following reaction mechanism as depicted in Fig. 6.2 is proposed.

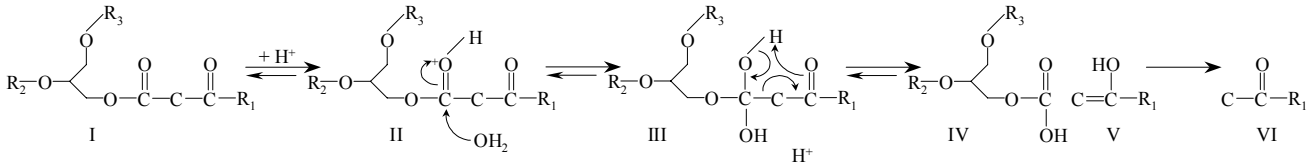

Fig. 6.2. Proposed reaction mechanism for the chemical formation of 2-ketones from milk fat triglycerides. R_1 = -$(CH_2)_n$-CH_3, R_2 and R_3 = -CO-$(CH_2)_n$-CH_3. The structures I – VI are mentioned in the text.

It is thought that a molecule of water carries out a nucleophilic attack at the C1 of the ß-keto acid residue, which is the most likely candidate because of its lowest electron density. This attack will be faster if the oxygen is protonated (structure **II**). The necessary split of the carbon chain is facilitated by a cyclic intermediate (structure **III**), which results in the glyceride residue (**IV**) which will probably readily decarboxylate into a diglyceride, and the enol molecule (**V**). This structure shows keto-enol tautomery, and the equilibrium for simple ketones (**VI**) in watery systems is almost completely on the keto-side (Carey & Sundberg, 1990). This mechanism requires a second-order reaction, since two molecules (triglyceride with ß-keto acid residue and water) are necessary for the reaction. But since water is present in large excess on a molecular basis, the reaction can still be considered to be a pseudo-first order reaction. It must be noted that this mechanism is somewhat speculative, and none of the intermediates have been identified.

Addition of water was necessary for the reaction, without water addition the reaction proceeded only very slowly. The addition of 10 µL water to the reaction vessel with 0.4g milk fat allowed the reaction to proceed at about 50% of the maximum reaction rate. This maximum rate was observed when 20 µL of water was added, in which case water was present at 1000x the ketone precursor concentration, on a molecular basis. The presence of more water did not lead to higher reaction rates (results not shown).

3.3 Temperature dependency

The temperature dependence of ketone formation was determined similarly as described for the lactones from milk fat (Alewijn et al., 2006). Again, Arrhenius-like depencency was assumed, and the available data from all time points, temperatures and different milk fats were fitted in one single step, by minimizing the sum of squared differences between observed and calculated data. The reparameterised Arrhenius formula (eq. 3) was used (Brands, 2002), as described earlier (Alewijn et al., 2006).

(eq. 3) $$k = -\frac{1}{t}\left(\frac{KP - [free\ \ ketone]}{KP}\right) = X \cdot e^{(-Y \cdot E_a)}$$

with $$X = k_0 \cdot e^{(-E_a / R \cdot T_{av})}$$

$$Y = \frac{1}{R}\left(\frac{1}{T} - \frac{1}{T_{av}}\right)$$

$$T_{av} = \frac{\sum T}{n}$$

In these equations, X and Y are the parameters to be estimated by fitting the experimental data. T_{av} is the average temperature of all observations. After fitting, the 'traditional' Arrhenius parameters E_a, the activation energy in kJ mol^{-1}, and A, the pre-exponential factor (h^{-1}), could be calculated. These parameters are reported in Table 6.2.

Table 6.2. Energy of activation E_a*, pre-exponential factor* A*, and calculated rate constants* k *at 13°C and 121°C for the formation of 2-ketones in milk fat.*

	E_a (kJ mol^{-1})	A (x10^{12} h^{-1})	$k_{13°C}$ (x10^{-6} h^{-1})	$k_{121°C}$ (x10^{-3} h^{-1})
C9one	102.8 *(±5.7)*	12.1 *(±0.41)*	2.06	288
C11one	102.6 *(±5.4)*	9.88 *(±0.26)*	1.78	245
C13one	101.1 *(±5.4)*	7.11 *(±0.12)*	2.46	282
C15one	99.3 *(±5.6)*	5.98 *(±0.51)*	4.36	409
C17one	97.6 *(±14.1)*	2.72 *(±1.14)*	3.99	309

Both the energy of activation E_a and the pre-exponential factor A for the formation of ketones are rather similar within the group of ketones. Due to relatively high Ea and A values, ketone formation rates at low and ambient temperatures (i.e. 13-20°C) are rather low, while formation rates are about five orders of magnitude higher at sterilisation (121°C) conditions. The E_a and A values differ considerably from the values found for lactone formation from milk fat, as described earlier (Alewijn et al., 2006). For example, at 13°C, the formation rate constant of lactones from milk fat is about 500 times higher. However, at 121°C the formation rate constant of ketones is about 2 times higher than that of the δ-lactones. These differences can obviously influence the relative amounts of fat-derived flavour compounds from both groups in products containing milk fat.

3.4 Fit with experimental storage data

Fig. 6.1 already showed that the formation of ketones at 160°C could be satisfactorily described by assuming first-order kinetics. Now, the Arrhenius parameters presented in Table 6.2 will be tested using the formation of ketones in a ripening cheese (13°C) as an example.

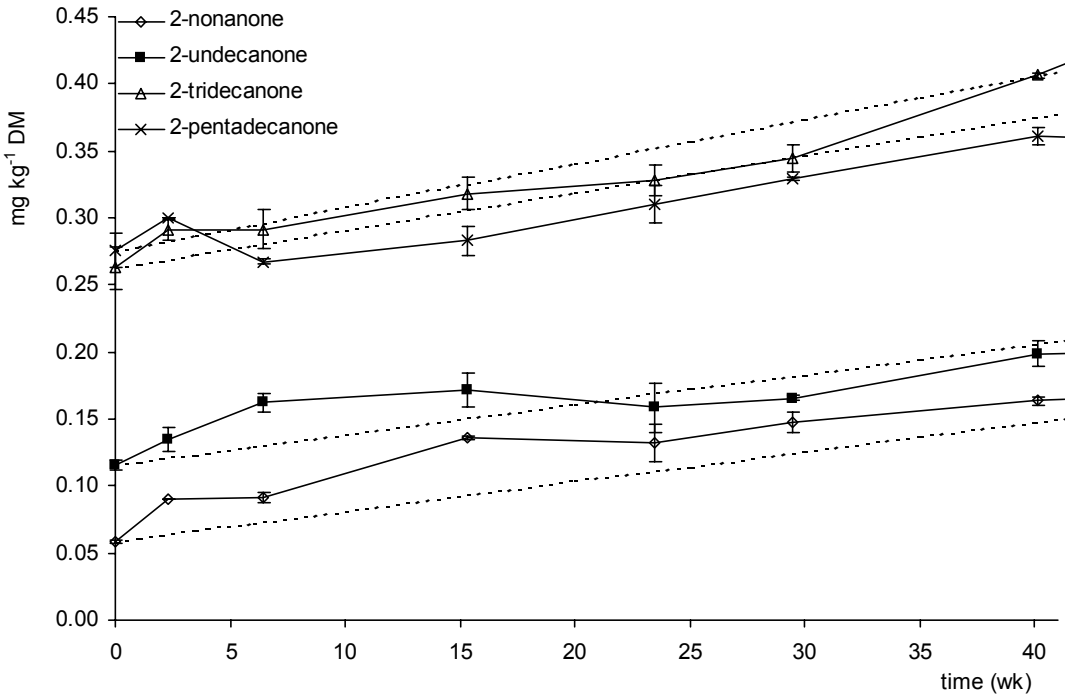

Fig. 6.3. Formation of 2-ketones during ripening of Gouda-type cheese, in mg kg^{-1} dry matter, from Alewijn et al. (2005). Marker points represent experimentally obtained data, error bars represent standard deviations. Dotted lines represent concentrations of 2-ketones as calculated by the model explained in the text. The ketone potential from milk fat "H" was used, as the cheese was prepared from that particular batch of milk.

Fig. 6.3 showed the experimentally determined concentrations of individual ketones during 40 weeks of the ripening of a Gouda cheese (Alewijn et al., 2005). The dotted lines represent the calculated ketone concentrations with the data presented here (Table 6.2 and fat "H" in Table 6.1). Perhaps due to the measuring error of the low levels of ketones in cheese, the correlation with the absolute levels of ketones leaves some room to be improved, but the calculation shows that the rate of formation and its trend was quite accurately predicted for the four ketones under consideration. These results support the hypothesis that the formation of ketones in Gouda cheese is a non-enzymatic process, and there is no need to suspect traces of enzymatic activity from either lactic acid bacteria or minor presence of (blue) moulds to explain the formation of ketones in these cheese types. This also implicates that control of ketone formation is only possible by influencing the concentration of precursor in the milk fat, and by adjusting the temperature.

Another example can be found in Table 6.3, where the development of ketones during storage of concentrated milk at 27°C is reported (Arnold & Lindsay, 1969). Again the ketone potentials and kinetic data presented in this paper were used to calculate the formation of these ketones. From the data in this Table it is clear that the formation of these ketones in milk can be described reasonably well using the parameters presented in Table 6.2. Another study on the development of ketones in milk fat during storage was reported by Tamsma, Kontson, and Kurtz (1974). However, the data presented did not correspond with the

Table 6.3. Comparison between experimentally determined amount of 2-ketones formed during storage of concentrated milk (Arnold et al., 1969), and calculated ketone concentrations based on values reported in this paper.

		methyl ketones (ppm)			
milk sample[a]	storage at 27°C (weeks)	C9[a]	C9 calculated[c]	C11[a]	C11 calculated[c]
A	0	0.05	0.05	0.11	0.11
	6	0.10	0.13	0.24	0.19
	13	0.31	0.21	0.28	0.28
	26	0.37	0.37	0.38	0.45
A[b]	104	0.16	0.08	0.20	0.14
B	0	0.03	0.03	0.08	0.08
	6	0.12	0.12	0.14	0.18
	13	0.22	0.20	0.24	0.27
	26	0.47	0.34	0.34	0.42

[a)] Data from Arnold et al. (1969), 2 commercially available concentrated milk samples (A and B) were stored at 27°C and 1°C, the ketone levels were determined spectrophotometerically, as their 2,4-dinitrophenylhydrazine derivatives, expressed in mg kg^{-1} (ppm) concentrated milk.

[b)] Sample stored at 1°C.

[c)] Ketone levels calculated using the kinetic data from Table 6.2, the ketone potential of an 'average' milk fat (Table 6.1) and assuming 20% milk fat in the concentrated milk used by Arnold et al. (1969).

predicted ketone formation characteristics presented in this paper, as they resulted from an incubation in water-free systems, which has a clear influence on the reaction rate, as explained in section 3.3.

The examples with ripening cheese and concentrated milk storage above show that using first-order kinetics fit reasonably well to real experimental data obtained at relatively low temperatures. However, due to the low concentration of ketones formed, especially as compared to the much higher ketone potential, these systems are quite sensitive to measuring errors, evaporation effects and high-temperature sample preparation. Hall et al. (1985) suggested the occurrence of zero-order kinetics for the formation of ketones at low temperature. Our predicted data for the ketone formation in cheese at 13°C (Fig. 6.3) show also an apparent straight line, indicative for zero-order kinetics. However, zero-order kinetics would imply a straight line throughout the complete non-enzymatic ketone formation. This was not observed for the high temperature ketone formation (Fig. 6.1). Therefore, it is justified to conclude that the non-enzymatic ketone formation from milk fat proceeds with first-order kinetics, also at low temperatures.

3.5 Consequences for dairy products

The Arrhenius parameters and ketone potentials presented enable the calculation of the non-enzymatic (or spontaneous) formation of ketones from milk fat in any time/temperature combination. Table 6.4 gives an example of the –hypothetical– formation of 2-ketones in typical time/temperature combinations found in dairy processing and storage. This Table shows that, in most of the typical cases probably only a few percent of the total ketone precursors would develop into free ketones. Fresh milk is low in ketones, and pasteurised milk fat in cheese curd contains about 0.01 to 0.04 mg kg^{-1} fat, for 2-nonanone and 2-pentadecanone, respectively (Alewijn et al., 2005). The amounts of ketones formed during pasteurisation or 5 days storage of butter at room temperature are calculated to be below these levels, and should then contribute only slightly to the ketone level present in milk. The other conditions mentioned, however, might substantially affect the amount of methyl ketones in the product, compared to the levels already present in untreated milk fat.

In terms of sensorial relevance, the flavour threshold is the most practical parameter. For 2-nonanone, for example, the flavour threshold is about 11 ppm in butter (Kinsella, 1975). This corresponds with 75% of the ketone potential of an 'average' milk fat (Table 6.1). In watery systems, the flavour threshold of 2-nonanone is about 0.2 ppm (Siek, Albin, Sather & Lindsay, 1971), which would correspond with about 19% of the ketone potential of milk, assuming 3.5% 'average' fat (Table 6.1). Based on these numbers, and disregarding any

Table 6.4. Calculated increase of individual 2-ketones at different time/temperature combinations[a], in mg kg^{-1} fatty matter.

condition	time	T (°C)	2-ketone (mg kg^{-1} fat) C9	C11	C13	C15	C17
average untreated milk fat[b]			0.14	0.21	0.59	0.64	0.12
young cheese	60 days	13	0.044	0.046	0.14	0.51	0.17
mature cheese	6 months	13	0.13	0.14	0.42	1.5	0.52
old cheese	1 year	13	0.26	0.28	0.85	3.0	1.0
butter 5 days at room temperature	5 days	20	0.010	0.011	0.033	0.11	0.039
half year storage of sterilised milk	6 months	20	0.36	0.39	1.2	4.0	1.4
pasteurisation	15 sec	73	0.0002	0.0002	0.0007	0.0020	0.0006
sterilisation	10 min	121	0.69	0.72	1.8	5.3	1.5
ultra sterilised milk	1 h	121	3.7	3.9	9.9	27	8.0

[a]Calculated increase in fat with an "average" ketone potential (Table 6.1) and the kinetic data from Table 6.2, excluding the initial or blank ketone level.
[b]Average blank value from the untreated milk fats A-L in Table 6.1.

possible synergistic effects of the ketone homologues, no major influence in sensorial terms is to be expected originating from non-enzymatic ketone development under conditions normally used in dairy processing. Assuming 'average' ketone potentials (Table 6.1), only after very extensive heating individual ketones could exceed their flavour thresholds. Further sensorial considerations are beyond the scope of this paper.

4. Conclusions

The formation of 2-ketones from milk fat proceeds directly from ß-keto-acids that are esterified in the triglycerides. This reaction proceeds in the presence of water, and shows pseudo-first order kinetics. The amounts of precursors in different milk fats have been determined (the ketone-potentials), and with these data the formation kinetics of individual 2-ketones were obtained from experiments in model systems.

With these kinetic data, it was possible to calculate the non-enzymatic formation rates at different temperatures, and hence to calculate the formation of ketones in a number of typical dairy related situations. As an example, the formation of ketones in Gouda-type cheeses could be reasonably predicted for a ripening period of about three quarters of a year, using the ketone-potential of the particular milk fat. While the possibility that ketones in Gouda-type cheese are formed by some enzymatic action is still present, it was shown that there is always a non-enzymatic reaction present that forms 2-ketones directly from esterified ß-keto acids. Also, the ketones formed during storage of concentrated milk, data

which were obtained using different techniques and in a different laboratory, could be predicted satisfactorily using the kinetic data presented.

The amounts of 2-ketones produced in most common time/temperature combinations are in the order of a few percent of their potential. These amounts, generally not more than 2-3 mg per kg fat, are well under the previously established flavour thresholds, and are therefore not likely to play a major role for the flavour formation in the processing conditions for the products considered. However, non-enzymatic formation of 2-ketones will occur in all products containing milk fat, and the potentials and kinetics data presented here may therefore help to predict the non-enzymatic formation of ketones at different production stages and temperatures for these products.

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7. Discussion and concluding remarks

7.1. Introduction

The ripening of cheese is a complex event, where many (bio)chemical changes occur in the starting material. The main constituents in the curd, notably protein, carbohydrates and fatty material, all change to some extent during ripening, without many options for the cheese producer to directly control these processes. After the cheese has been pressed, brined and coated, the ripening process and the resulting aroma formation occurs in a 'yellow box'. The work presented in this thesis started with the goal to investigate what aroma compounds are formed in the ripening phase, thereby specifically focussing on the fat-derived aroma compounds. As no straightforward analytical method was available, such a method has been developed, as described in Chapter 2. This tool was used to follow the formation of these compounds during ripening of several cheeses, and with this roadmap of the formation of fat-derived compounds, including differences between two often-used packaging and ripening conditions, it was tried to get more insight into the formation mechanism of some specific compounds. This was done with the intention to find the factors that may lead to more steering possibilities for the cheese manufacturer in terms of speed, efficiency and control of the balance of flavour formation, at least for the aroma compounds investigated. The current chapter will bring those results together, and discuss how they fit into the goal of enhanced control of the ripening of (Gouda) cheese.

7.2. The analytical method

The method that was used to investigate fat-derived compounds has been described in Chapter 2. When examining the suitability of a method, several factors should be considered, such as the range of detectable compounds, sensitivity, repeatability and accuracy, robustness and ease of use. Since there was no specific group of fat-derived compounds selected at the start of the research, the goal was to find a suitable method to detect a wide range of compounds. The range of compounds that was detected with the method of choice was rather broad, with a large distribution in volatility and hydrophobicity, and the use of mass spectroscopy led to rapid identification of the compounds found throughout the research. There was, however, a very strict volatility-limit in this method: all compounds that eluted in the gas chromatographic method used before or together with the solvent acetonitrile were undetectable. This was certainly the case for 2-heptanone and shorter chain ketones and for the shorter chain esters. These compounds, a part of which were surely fat-derived compounds, could not be included in this work.
The sensitivity of the method was certainly sufficient for the qualitative and quantitative analysis of free fatty acids (FFA) and lactones in cheese. The signal-to-noise ratio was for most of these compounds many times greater than 10, although there were some

exceptions for branched or odd-numbered acids or lactones. Ketones and esters were readily detectable and quantifiable as well, although their concentrations in the cheese samples were closer to the detection limit, which was especially true for the compounds with chain lengths below 8 and above 16 carbon atoms. The minor members of the groups of esters and ketones, such as esters with an odd-numbered, and ketones with an even-numbered chain length, were probably not detected. A detection problem that was encountered in chapters 5 and 6 of this thesis was due to the phenomenon of co-elution. Both esters and ketones occasionally suffered from co-elution with FFA, despite the use of a mass-sensitive detector, quantification remained a problem as the much larger FFA peaks heavily interfered with the signal of interest. FFA are notorious in chromatography for their tendency for tailing, which increases with column life. There were instances that this prevented the quantification of some individual minor compounds.

7.3. Formation of compounds

According to the literature, the formation of fat-derived (aroma) compounds in cheese can be represented by Fig. 7.1. To conclude this thesis, the formation routes in this scheme will be checked with the outcome of the results on the formation of various compounds, as reported in the previous chapters, and a new scheme for their formation will be presented. This new scheme will describe the formation of these compounds for Gouda type cheeses specifically.

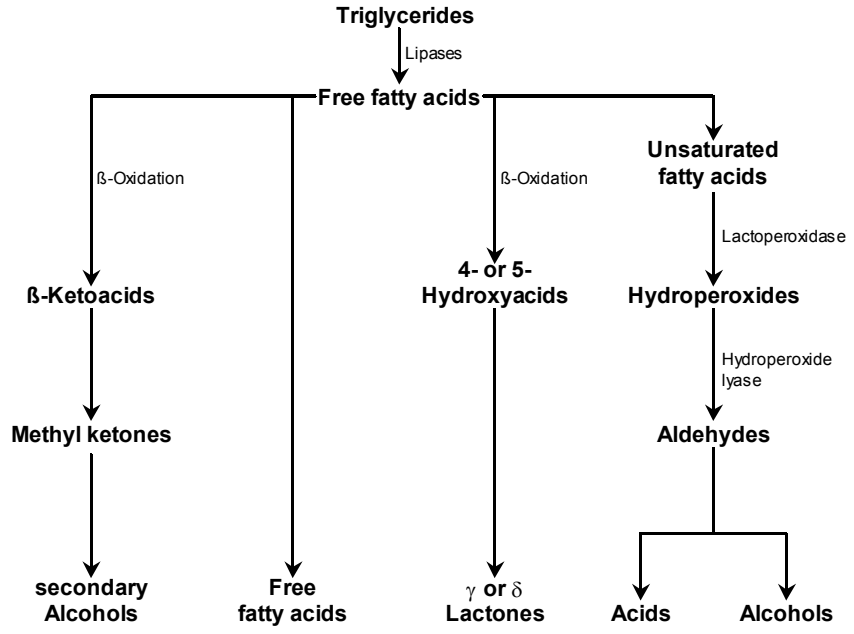

Fig. 7.1. Possible formation routes of fat-derived flavour compounds. From (Molimard & Spinnler, 1996).

7.3.1. free fatty acids

It is evident from this thesis that by quantity, the FFA are the major fat-derived compounds in cheese. Fig. 7.1 suggests that they are mainly formed by hydrolysis from milk fat triglycerides. The results described both in chapter 3 and 4 showed that relatively high levels were already present in curd. It should therefore be noted that a quite significant part of FFA in cheese was already present in milk and curd, presumably formed by the action the native milk lipase LPL. The distribution within these fatty acids in cheese was skewed towards the longer chain fatty acids as compared to that in milk, which was explained as the result of preferential loss of the short chain fatty acids into the whey.

Also during ripening, the formation of the long chain FFA followed a pattern quite different from that of the short ones, as observed in chapters 3 and 4. In proportion, the long chain FFA were the main compounds formed throughout ripening and they seemed to get a boost in formation in the initial stage of the cheese ripening, whereas only small amounts were liberated afterwards. This seemed to be due to the action of native milk lipase, an activity that is lost early in the ripening phase. The foil-ripening described in chapter 4 showed that less long chain fatty acids were liberated during the first 8 days of ripening compared to conventional ripening. Also, cheeses with lower water contents formed less long chain FFA in this initial ripening period. These characteristics could be attributed to the action of native LPL, which was, like in milk and curd, very probably the major contributor to the formation of long chain FFA until very early in the ripening phase.

In contrast to the long chain fatty acids, the short chain fatty acids were liberated in a more continuous way throughout ripening, as shown in chapters 3 and 4. The short chain fatty acid formation was slower in foil-wrapped cheeses as compared to that in conventionally prepared cheeses. Their formation was, however, fastest in the cheese prepared from non-pasteurised milk. These differences indicated an enzymatic mechanism to liberate these short chain FFA, which was, most probably, not the action of LPL, but the combined action of the esterases produced by the bacterial strains present in the cheese. As known from literature, all common cheese lactic acid bacteria, starters as well as non-starters, produce these enzymes, but with strongly varying activities and some different characteristics (Holland et al., 2005; Liu, Holland & Crow, 2004).

From the above, it seems not justified to consider the formation of FFA in Gouda cheeses as one single event, and therefore a subdivision is proposed in the formation route of FFA, as presented in Fig. 7.2 at the end of this chapter.

In order to control the levels of FFA in cheese, the formation of both groups of fatty acids should be considered. First of all, the initial level – adjustable by controlling the action of milk LPL in milk – is of great importance, although a large part of the more flavour-active short chain FFA is lost with the whey and does not end up in cheese, whereas the long

chain FFA do. Active pre-LPL hydrolysis of fat may also produce extra mono- and diglycerides, which are included in the cheese and subsequently more accessible than triglycerides for the esterases that are active during ripening. In the initial ripening stage, LPL is not very stable, and the control of its activity during ripening is probably not very relevant anymore. Secondly, as some non-starter lactic acid bacteria possess very active esterases, the natural variation of these strains may be a good source to enhance the formation of short chain FFA. However, the esterases will probably also have an effect on the formation of another class of fat-derived flavour compounds, the esters.

7.3.2. esters

Esters appeared to be the most variable class of compounds among the cheeses reported in this thesis. The cheeses studied in chapters 3 and 4 showed that indeed their levels can be different by two orders of magnitude. The formation of esters and short chain FFA were correlated, which is not surprising if one assumes that the same enzymes catalyse their formation. As mentioned in the references of chapter 3, esterases from some lactic acid bacteria possess both (de-)esterification and trans-esterification capabilities, thus being able to synthesise (volatile) esters from FFA as well as from glycerides. The work presented in chapter 3 seems to support the wide range in enzymatic ester-forming activities in different cheeses, made with different starter strains.

This wide variety of ester forming potential offers great opportunities to influence, and perhaps control, ester formation in Gouda-like cheese types. Unfortunately, there was not enough time in this period of thesis work to study ester formation in more detail. More work needs to be done in this field to be able to understand the influences on these two reaction types, the exact enzyme subtypes involved and the micro-organisms that produce them. Especially the latter factor seems to be the key control point for the production of esters in Gouda-like cheese types.

7.3.3. lactones

The formation of lactones was found in all cheeses studied. During the long ripening of Gouda cheese, a rather smooth formation curve was found, with ever-decreasing formation rate. The same profile was found for both γ- and δ-lactones, but γ-lactones developed at a slightly slower rate. In the experiments described in chapter 4, a clear difference in quantity of lactones formed was found between the foil-wrapped and the conventionally ripened cheeses. From the data presented, it was impossible to conclude whether foil-wrapping itself, or the lower temperature applied during foil-wrapped ripening was the cause of the slower formation of lactones in the cheeses ripened in foil. It was presumably not due to the difference in initial water content in these differently ripened cheeses. From the kinetic data presented in chapter 5, however, it could be calculated that the difference in ripening

temperature applied, 5°C and 13°C for foil-wrapped and conventionally ripened cheeses, respectively, resulted in an approximately 2 times lower reaction rate constant for the lactone formation at the lower temperature. The fact that these reaction rates could be calculated and correctly be predicted using the data presented in chapter 5, offered additional support that in the case of Gouda cheese the formation of lactones is primarily a non-enzymatic event.

This fact limits the possibilities of influencing the lactone formation. Based on the kinetics found, the ripening temperature is an important factor that drives lactone formation. However, when ripening a fairly traditional product as Gouda cheese, there is little or no room to adjust the temperature without affecting seriously the many other parts of the ripening process. Another key factor that determines the amount of lactones formed might be the lactone potential, which is the amount of lactone precursors (esterified hydroxy fatty acids) in the milk. As mentioned in the introduction of chapter 5, this potential is influenced by the feeding regime of the cows, the cow's breed, and the season/lactation stage considerably. If the ripening temperature of a cheese should remain unaltered, adjusting the lactone potential appears the only route to influence the lactone level that will develop in cheeses. On the more speculative side, it may still be possible to find micro-organisms that have the possibility of transforming (un)saturated fatty acids into lactones. As mentioned, there are articles that report this mechanism (Wanikawa, Hosoi & Kato, 2000; Wanikawa, Shoji, Hosoi & Nakagawa, 2002). However, as the mechanism presented uses both lactobacilli and yeast, it is unlikely that this mechanism can be immediately used to prepare traditional Gouda cheese. However, recent work describes an analytical method that scans large numbers of strains on the presence or absence of certain specific flavour compounds when grown with a suitable precursor (Smit, Engels, Bruinsma, Hylckama Vlieg, Wouters & Smit, 2004). This method would enable the screening of a large number of naturally available lactobacilli for their capabilities to form lactones.

One other option of influencing the lactone level in milk fat might be heat pre-treatment. Enhancement of lactones in very young cheese may be desired. On industrial scale, milk cream is usually (partly) separated and treated, which may allow differentiated heat treatment. The kinetics presented in chapter 5 may give an indication at which time and temperature the milk fat should be kept, in order to generate the lactone level required. However, apart from the formation of lactones, other changes may happen to the milk fat. One example of that is the formation of methyl ketones.

7.3.4. ketones

Quantitatively, in this study ketones were found to be present in relatively low amounts compared to most other fat-derived compounds in Gouda-like cheeses. However, their individual aroma thresholds are rather low, and methyl ketones are key aroma compounds

in blue cheese types and other dairy products. Due to the analytical method used, not all ketones could be quantified reliably and this complicated the estimation of sensory relevance of the ketones. It was shown that ketones were present in all curds of the cheeses studied and that during cheese ripening the amounts increased only slightly. No major differences were observed between ketone levels of any of the cheese types studied. Chapter 6 describes the non-enzymatic mechanism through which the ketones in Gouda-like cheeses are probably formed, including their formation kinetics. From the quantitative data obtained and subsequent application of non-enzymatic kinetics, the very slow formation of methyl ketones could be calculated and successfully predicted for different ripened cheeses. There is not much to do to control this non-enzymatic formation of ketones, as ripening temperatures are not flexible enough to allow greatly enhanced ketone formation.

The ketone levels were already quite significant in curds, and as they are formed by applying heat, a similar pre-treatment as suggested for the lactones will enhance ketone levels in a cheese. At higher temperatures, ketones will form even faster than lactones, and milk fat will unleash its near-complete ketone potential in several minutes. This would result in ketone levels that are likely to be sensorially significant. Apart from other changes in the fat, the lactone level will be influenced as well. If a heat-treatment is applied to cream, fat or milk, the combined data of chapters 5 and 6 allow calculating the balance between the two groups of compounds.

Furthermore, the ketone potential of a certain milk fat may also be influenced. As mentioned in the introduction of chapter 6, factors as the cow's breed, season, stage of lactation and certainly the feeding regime may significantly influence the ketone potential of a milk fat. The conditions used to process the milk determine largely the proportion of this potential that will be converted into free methyl ketones.

One other possibility to influence or enhance the ketone formation is using the microbiological route. Some moulds are very effective in converting FFA into methyl ketones, which is well known for blue-veined cheeses (Molimard et al., 1996). Although these eukaryotic microorganisms use a rather complex enzyme system, it should be possible, now or in the near future, to transfer parts of this system into a prokaryotic starter organism to be used in Gouda-type cheeses. It is, however, doubtful if such an approach will be practically accepted any time soon. Another approach is to scan naturally ocurring lactic acid bacteria for their ketone-forming potential, as suggested for the lactones (section 7.3.3).

The formation of ketones turned out not to be very significant in Gouda-like cheese ripening. Therefore, the application of the kinetics presented in chapter 6, although interesting for this type of cheese, will be more of interest for milk fat containing products that are intensively heat-treated during production or that will be stored at ambient temperatures for extensive periods of time. These products will develop ketones, whether

this is desirable or not, and the kinetics presented will help in predicting their formation, as has been demonstrated clearly for some products currently on the market.

7.3.5. other compounds

Apart from FFA, esters, lactones and ketones, which all covered to some extent in the various chapters of this thesis, Fig. 7.1 also mentions (secondary) alcohols and aldehydes, which are not discussed so far. This is mainly due to the fact that they were not detected by the analytical method used, simply because they were either not formed, or only formed in quantities below the detection levels. There was some indication on the detection levels of these fat-derived alcohols and aldehydes from non-Gouda cheeses in the analysis method used, as presented in chapter 2. Based on this information it could be concluded that if these compounds were formed or present in Gouda cheeses, their levels were very low. Although their contribution in the flavour could not be assured with certainty, their formation will quantitatively clearly be of minor importance, and these compounds are therefore not considered as major fat-derived compounds in Gouda-like cheese types.

The aldehydes in Fig. 7.1 are formed from hydroperoxides, which in turn are derived from unsaturated fatty acids. Whether this reaction is a non-enzymatic photo- or auto-oxidation, or catalysed by peroxidases, both reaction types rely on oxygen, which is certainly not abundant in Gouda-type cheeses. It seems therefore justified to assume that the conversion of unsaturated fatty acids to aldehydes will – quantitatively – not be a significant reaction.

The alcohols in Fig. 7.1 are formed either from ketones via a reductive step yielding 2-alcohols, or from aldehydes, yielding 1-alcohols. In the environment of a ripening Gouda cheese, reduction is a much more likely event than oxidation. Since the precursors for the alcohols (ketones and aldehydes) are not produced to a great extent (see above), alcohols themselves constitute probably also not a major portion of the fat-derived compounds in cheese. Both reaction pathways leading to alcohols can therefore safely be assumed as minor events in a ripening cheese.

7.4. Relevance of compounds

With the data presented in this thesis, some clarity is obtained about the formation of the fat-derived compounds, which may lead to ways to control their formation in real-life cheeses. However, the question whether this would result in an altered aroma of the resulting cheese has not been answered, let alone if this changed aroma is considered desirable. Although the topic has been mentioned throughout the thesis, no practical work on sensory relevance of these compounds has been presented. Only previously reported aroma thresholds of individual compounds (Hall & Andersson, 1983; Rothe, Woelm, Tunger & Siebert, 1972; 1971; Urbach, Stark & Forss, 1972) could be compared with the concentrations found and predicted in this study, indicating that some FFA and lactones

could exceed their reported thresholds. It is unclear if these compounds, in the concentrations found, actually contribute to the flavour in cheese to the average consumer. This depends, among other things, on the cheese matrix, the interaction with other cheese aroma compounds, and possible synergistic effects (Siek, Albin, Sather & Lindsay, 1969) that fat-derived homologous compounds may have. To study these effects properly, specialist knowledge and a dedicated, trained sensory panel is required, which was not available it the course of this thesis work. Therefore, it can be concluded that this thesis offers some technical knowledge about the formation of potentially flavour-active compounds, which may help the cheese producer to steer the process in a desired direction.

7.5. Conclusion

After reviewing in brief the formation of the fat derived compounds in Gouda cheese in section 7.3, we propose the following simplified scheme (Fig. 7.2).

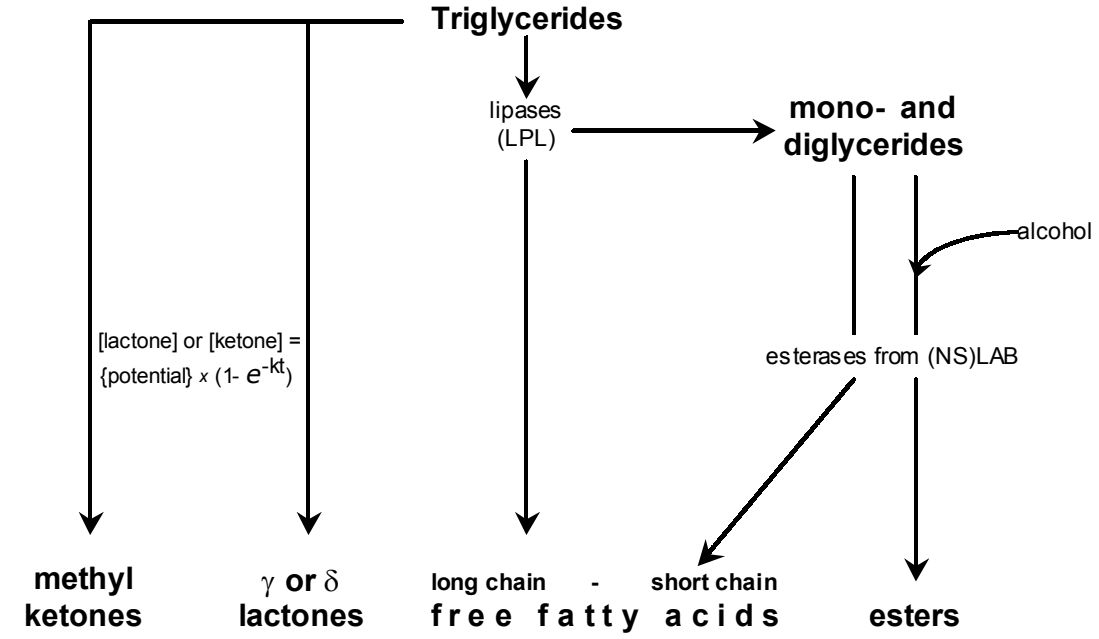

Fig. 7.2. Main pathways for the formation of fat-derived aroma compounds in Gouda cheese.

This scheme shows that in Gouda-type cheese, methyl ketones and lactones are formed in a non-enzymatic way, and follow well-known first-order kinetics. In contrast, the FFA and esters are enzymatically produced. In terms of control, enzymatic reactions are usually easier to influence, using other organisms, for example. The non-enzymatic reactions, on the other hand, are not so easy to influence, but are easier to predict quantitatively. As stated above, the interrelation between the enzymatic and non-enzymatic reactions should not be neglected when trying to influence the formation of one group of compounds, as anything done for that purpose would affect the formation of the other group of flavour compounds.

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Summary

Cheese is a traditional product, which is appreciated in its many different forms by consumers around the globe. One important characteristic is its flavour, which is built up from many different organic flavour compounds that act together to form the flavour perceived. Most of this flavour is formed while the cheese is ripening, which is a stage were the cheese producer has only little control over the processes occurring. This means that the chemical, physical and microbiological composition of the curd will largely determine the process of flavour formation in the ripening period that follows. Many flavour-forming processes are not completely understood.
Cheese flavour that is formed during ripening may originate from carbohydrates, protein and fat. Although not yet completely understood, the formation of flavour compounds from proteins and carbohydrates has been extensively described in the scientific literature. In contrast, the processes that lead to the formation of flavour molecules starting from milk fat, have received little scientific attention. Therefore, this thesis aims to study the formation, and possible control, of the fat-derived flavour compounds.

It is known from literature that fat-derived flavour compounds include free fatty acids, lactones, ketones, aldehydes, esters, and alcohols, varying in chain length from 4 to over 18 carbon atoms. As no method was capable of measuring all these compounds simultaneously, a method was developed that could. It was found that a citrate-assisted solvent extraction method with acetonitrile was capable of extracting all these compounds, with satisfying reproducibility. The use of gas chromatography and mass spectroscopy assured detection with sufficient sensitivity and accuracy, combined with easy identification of compounds. This method is described in chapter 2, and was tested on a Gouda, a Cheddar and a Danish Blue cheese to get an overview of the different quantities of fat-derived compounds in these different cheeses. It was observed that most individual compounds were present in all cheeses, but with very different quantities and ratios.

With this detection tool available, chapter 3 describes the formation of fat-derived compounds during the ripening of four different batches of Gouda cheese. It was observed that the concentrations of all compounds detected, free fatty acids, lactones, esters and ketones, increased during ripening. Other possible groups of fat-derived compounds, aldehydes and alcohols, were not detected in these cheeses. The free fatty acids were the most abundant group, and already present in the curds. Their amounts increased throughout ripening, quite linearly in the ripening time under consideration, but the quantity of short and intermediate chain length compounds increased faster, which increased their

proportions significantly as the cheeses got more mature. Also the ester levels, mainly ethyl esters, increased throughout ripening, but large differences were observed across the four cheeses, with the cheese made from unpasteurised milk showing by far the most ester formation. This particular cheese also showed the greatest formation of short and intermediate chain free fatty acids. The extreme variability of ester levels was confirmed by checking eight commercially available Gouda cheese samples, which showed more than two orders of magnitude difference in ester concentrations. Lactone concentrations increased fast in the beginning of the ripening phase, but their formation rates fell, reaching plateau levels after 4 to 5 months of ripening. There was a small but distinct difference between the formation of γ- and δ-lactones, but within these groups the behaviours of the individual lactones were very similar. The concentration of the methyl ketones increased throughout ripening, but the amount formed during ripening was low compared to the amount already present in curd. Their formation was very similar across the ripening of the four different cheese batches studied.

Chapter 4 describes a new ripening study, concerning six batches of cheese from exactly the same batch of milk, ripened both as conventionally coated and foil-wrapped cheeses. As the latter category loses less water during ripening, the curds are generally prepared with less water, and therefore the influence of high, intermediate and low water content was studied within each ripening condition. This study confirmed that long chain free fatty acids are mainly formed in the initial ripening phase, and that shorter free fatty acids are produced more gradually during the ripening. All fatty acids were more strongly produced in the conventionally coated than in the foil-wrapped cheeses. The water content was of smaller influence, and of influence on the production of long chain fatty acids only. The ripening conditions were certainly important for the formation of lactones, and again the conventionally coated cheeses showed higher production. In case of the lactones, the differences in water content did not influence the lactone formation, and as for the free fatty acids, the formation profiles confirmed the curves in the chapter 3. In the cheeses of this ripening study, however, ester and ketone levels were too low to draw definite conclusions.

The observations above showed formation profiles of the major groups of fat-derived aroma compounds during the ripening of Gouda cheese. The main information about formation mechanisms, obtained from the observations above, was the link between the formation of short chain free fatty acids and esters, and it was hypothesised that these are formed by the same or similar enzymes; esterases from starter-, and more frequently from non-starter lactic acid bacteria. The fast initial formation of long chain fatty acids was attributed to the

action of a lipase that loses its activity very early in the ripening phase, which is likely to be native milk lipoprotein lipase.

Further work was done on the formation of lactones, and chapter 5 describes that lactones were, in Gouda cheese, most likely formed via a non-enzymatic pathway. Their precursors were 4- and 5 hydroxy fatty acid moieties, esterified in the milk fat triglycerides, that spontaneously lactonised to form a free lactone and a glyceride with one more free alcohol function. The reaction seemed to obey first-order kinetics, and thus was dependent on the precursor concentration. With sufficient time, all of the precursor would be converted into free lactones, and the initial level of precursor was therefore called the lactone potential. The lactone potentials of many different milk fats have been determined, and showed a wide variation. The formation of free lactones was temperature-dependent, and the first-order kinetic parameters could be estimated using an Arrhenius-type approximation. Using these kinetics and the lactone potentials, the formation of all major individual γ- and δ-lactones was accurately explained for the ripening of a Gouda cheese up to about 40 weeks.

Also the formation of methyl-ketones was unlikely to be an enzymatic event in Gouda cheese, and experiments in model systems led to a hypothesis on the formation of ketones in milk fat, as explained in chapter 6. It is likely that these compounds were formed from ß-keto fatty acid moieties that are esterified in the milk fat, which split off from the glyceride in a water-dependent reaction. Again, the initial quantity of these compounds in milk determined the amount of ketones that can be formed, and was called the ketone potential, which was quite variable in the milk fats considered. In a similar manner as for the lactones, the kinetic properties of ketone formation were studied, and it was found that ketones obey (pseudo-) first order kinetics. These kinetics were temperature-dependent in an Arrhenius-like relationship. The kinetic parameters for the formation of the major 2-ketones are reported, and explained the very slow formation in ripening cheese quite well. Although the formation at cheese-ripening temperatures was very low, their temperature-dependency was such that with increasing temperatures the formation increases fast. At high temperatures, the formation was much faster than the formation of lactones, which, in contrast, showed already significant formation at cheese ripening temperatures. Therefore, the non-enzymatic formation of ketones is likely to be important for milk-fat containing products that receive an extensive heat-treatment, or that are stored at (supra-)ambient temperatures for extensive periods of time. Several hypothetical examples are given in the text.
As a conclusion, in chapter 7 a scheme has been set up for the development of fat-derived compounds in Gouda cheese. This does not contain aldehydes and alcohols, but showed the non-enzymatic formation of lactones and 2-ketones, the formation of long chain free fatty

acids, mainly by milk lipoprotein lipase, and the formation of intermediate and short chain free fatty acids and of esters by esterases from (non-)starter lactic acid bacteria. In terms of control of fat-derived flavour compound development, most benefit could be obtained by paying more attention to the application of bacterial cultures with desired types of esterases, as this would influence the development of the more flavour active free fatty acids and esters. Furthermore, selection of milk fat with enhanced lactone and ketone potentials, whether or not accompanied by a heat (pre-)treatment, would be a tool to influence the development of fat-derived flavour compounds.

Samenvatting

De vorming van vet-afgeleide aromacomponenten tijdens de rijping van Goudse kazen

Kaas is een traditioneel product dat in vele verschillende vormen en smaken over de hele wereld wordt gemaakt en gegeten. Een belangrijk aspect van kaas is het aroma, de optelsom van smaak en geur, die op hun beurt weer worden gevormd door een groot aantal chemische verbindingen. Een groot deel van deze verbindingen wordt pas tijdens de kaasrijping gevormd, en juist tijdens rijping heeft de kaasproducent maar weinig directe invloed op de processen die verlopen. Daarom bepaalt de chemische, fysische and microbiologische samenstelling van de wrongel in hoge mate de aromavorming tijdens de rijping. Van de aromavormende processen die optreden tijdens de rijping zijn nog niet alle eigenschappen bekend.
Tijdens de kaasrijping worden er aromaverbindingen gevormd uit de bouwstenen van de wrongel, dit zijn koolhydraten, eiwitten en vetten. De aromavorming uit koolhydraten en met name uit eiwitten is een zeer complexe verzameling van reacties, en vanwege het vele wetenschappelijke onderzoek dat al gedaan is en nog steeds loopt worden de grote lijnen van deze reacties steeds duidelijker. Aan de andere kant is aan vorming van aromaverbindingen vanuit melkvet slechts weinig wetenschappelijk onderzoek gedaan. Het doel van dit proefschrift is dan ook om de vorming, en mogelijk de sturing van vet-afgeleide aromaverbindingen te onderzoeken.

Uit de wetenschappelijke literatuur is bekend dat vet-afgeleide aromacomponenten bestaan uit vrije vetzuren, lactonen, ketonen, aldehyden, esters en alcoholen, met een ketenlengte van 4 tot meer dan 18 koolstof atomen. Al deze verbindingen hebben individueel een bepaalde smaak en/of geur, die mee kan spelen bij het uiteindelijke kaasaroma. Omdat aan het begin van dit onderzoek geen goede methode bestond om al deze uiteenlopende verbindingen te kunnen meten, werd deze ontwikkeld. Het bleek dat al deze componenten na een bepaalde vloeistof-extractie met acetonitril analytisch gemeten konden worden, met voldoende reproduceerbaarheid. Het gebruik van gaschromatografie in combinatie met massaspectroscopie zorgde ervoor dat de detectie gevoelig en precies genoeg was, en dat bovendien de gedetecteerde verbindingen relatief eenvoudig geïdentificeerd konden worden. Deze meetmethode is beschreven in hoofdstuk 2, waar de methode ook getest was met 3 verschillende kazen. De verschillende hoeveelheden vet-afgeleide componenten in Gouda, Cheddar en Danish Blue kazen lieten zien dat bijna alle verbindingen aanwezig

waren in alle kazen, maar in zeer verschillende hoeveelheden en verschillende onderlinge verhoudingen.

Nu de analytische methode beschikbaar was kon in hoofdstuk 3 de vorming van vet-afgeleide verbindingen tijdens de rijping van vier verschillende soorten Goudse kaas beschreven worden. Het bleek dat alle gevonden klassen van componenten, de vrije vetzuren, lactonen, esters en ketonen, in concentratie toenamen tijdens de rijping. Andere, uit de literatuur bekende klassen van verbindingen, de aldehyden en alcoholen, werden in deze kazen niet gevonden. De vrije vetzuren waren wat concentratie betreft de grootste groep, en deze waren ook al aanwezig in de wrongel van de kazen. Hun concentratie nam vrijwel lineair toe met de rijpingstijd, maar de korte en middellange vetzuren werden relatief sneller gevormd dan de lange vetzuren, zodat het aandeel van deze –over het algemeen wat doordringender ruikende- verbindingen hoger werd in oudere kaas. Ook de hoeveelheden van de esters, voornamelijk ethyl esters, namen toe tijdens de rijpingstijd. Er werden bijzonder grote verschillen waargenomen tussen de vier verschillende soorten Goudse kaas, waarbij de boerenkaas, gemaakt uit rauwe melk, met afstand de grootste hoeveelheid esters vormde. Deze kaas had ook wat meer korte en middellange vrije vetzuren dan de andere kazen, maar dat verschil was minder groot. Het verschil in esterconcentraties was opvallend zodat besloten werd om de estergehaltes van een aantal kazen uit de handel te meten. In tegenstelling tot de andere klassen van componenten bleek dat de esterhoeveelheden in deze kazen inderdaad met een factor 100 kon verschillen. In de vier bestudeerde kazen bleken de lactonen vrijwel op dezelfde manier gevormd te worden. De lactonconcentraties namen in het begin snel toe, maar stabiliseerden zich na 4 tot 5 maanden na het begin van de rijping. Er werd wel een klein maar consequent verschil gevonden tussen de vorming van γ- en δ-lactonen. Ook de laatste klasse van vet-afgeleide verbindingen, de methyl-ketonen, werd gevormd tijdens de rijping, maar de hoeveelheid die gevormd werd was klein ten opzichte van de hoeveelheid die al in wrongel aanwezig was. Ook was het verschil tussen de vier Goudse kazen gering.

Hoofdstuk 4 beschrijft een nieuwe rijpingsproef, waarbij zes verschillende kazen uit één hoeveelheid melk werden geproduceerd. Drie daarvan werden geplastificeerd, de meest bekende vorm, en drie andere werden vacuum-verpakt. Omdat de vacuum-verpakte kazen tijdens de rijping minder water verliezen werden er per verpakking een kaas met laag, middel en hoog watergehalte gemaakt. Er werd vervolgens gekeken naar de invloed van verpakking en watergehalte op de vorming van de vet-afgeleide verbindingen tijdens de rijping. Dit toonde aan dat alle vrije vetzuren sterker werden gevormd in geplastificeerde dan in vacuum-verpakte kazen. Ook bleek dat lange vetzuren voornamelijk in het begin van de rijping gevormd werden, terwijl de concentratie van kortere vetzuren veel constanter en

gedurende de gehele rijping toeneemt. De verpakking had ook duidelijke invloed op de vorming van de lactonen, en weer werden er meer aromaverbindingen gevormd in de geplastificeerde kazen. De invloed van het watergehalte was gering, en alleen van invloed op de lange vetzuren. Wel werden voor de vrije vetzuren en de lactonen de vormingsprofielen uit hoofdstuk 3 bevestigd. In de kazen uit dit hoofdstuk waren de niveaus van de ketonen en esters echter te laag om goede conclusies te kunnen trekken over de vorming van deze verbindingen.

De waargenomen vormingsprofielen van de belangrijkste klassen van vet-afgeleide aromacomponenten uit de voorgaande hoofdstukken laten zien dat er een verband bestaat tussen de vorming van korte vrije vetzuren en esters. Er werd daarom voorgesteld dat beide klassen aromaverbindingen gevormd worden door dezelfde of verwante enzymen, en dat deze enzymen uit zuurselbacteriën, of meer waarschijnlijk uit secundaire – toevallige-melkzuurbacteriën afkomstig zijn. De snelle initiële vorming van de lange vrije vetzuren werd toegeschreven aan de activiteit van een lipase dat zijn activiteit zeer snel na het begin van de rijping verliest, wat zeer waarschijnlijk het melk-eigen lipoprotein-lipase is.

Naar aanleiding van de eerdere hoofdstukken is besloten meer onderzoek te doen naar de vorming van lactonen. Hoofdstuk 5 beschrijft dat deze verbindingen in Goudse kaas zeer waarschijnlijk niet-enzymatisch gevormd worden. Zij worden gevormd uit in melkvet veresterde 4- en 5 hydroxy vetzuren, die spontaan lactoniseren en in één stap een lacton en een glyceride met een extra vrije alcohol-groep achterlaten. Deze reactie leek volgens 1e-orde kinetiek te verlopen, en was daarom afhankelijk van de bronmoleculen, de veresterde 4- en 5 hydroxy vetzuren. Voldoende rijpingstijd zou ervoor zorgen dat al deze bronmoleculen omgezet worden in lactonen, en daarom wordt de initiële concentratie bronmoleculen de lactonpotentiaal genoemd. De lactonpotentialen van verschillende melkvetten werden bepaald, wat liet zien dat er een behoorlijke natuurlijke variatie bestaat, die mogelijkheden biedt om te sturen in de uiteindelijke lactonconcentraties in een gerijpte kaas. De vorming van lactonen was temperatuur-afhankelijk, een benadering van de vormingssnelheid kon worden gegeven door gebruik te maken van een variant van de Arrhenius-kinetiek. Door gebruik te maken van deze kinetiek en het eerder bepaalde lactonpotentiaal van een melkvet kon de vorming van alle belangrijke individuele γ- en δ-lactonen nauwkeurig voorspeld worden voor de eerste 40 rijpingsweken van een eerder gevolgde Goudse kaas.

Waarschijnlijk was de vorming van methyl-ketonen in Goudse kaas niet toe te schrijven aan de werking van een bepaald enzym. Experimenten in een modelsysteem hebben geleid tot een hypothese voor de vorming van ketonen in melkvet, wat wordt beschreven in hoofdstuk

6. Ketonen werden waarschijnlijk gevormd uit ß-keto-vetzuren die veresterd zijn in melkvet. Deze splitsen zich af van het glyceride in een water-afhankelijke reactie. Ook voor de ketonen gold dat de initiële hoeveelheid van deze molecule bepalend was voor de hoeveelheid ketonen dat gevormd kan worden, en deze hoeveelheid wordt de ketonpotentiaal genoemd. Ook deze ketonpotentiaal liet een behoorlijke natuurlijke variatie zien bij verschillende melkvetten. Vergelijkbaar met de lactonen werd de vormingskinetiek bestudeerd, en een (pseudo) 1e-orde kinetiek werd gevonden. De temperatuur-afhankelijkheid van de vormingssnelheid kon weer goed worden benaderd met de Arrhenius-vergelijking. De grootheden die nodig zijn om de vorming van de belangrijkste ketonen te voorspellen zijn gerapporteerd, en deze verklaarden de in eerdere hoofdstukken gevonden
langzame vorming tijdens de kaasrijping. Hoewel deze verbindingen dus langzaam gevormd worden tijdens de kaasrijping, gaat de vormingssnelheid bij hogere temperaturen zeer snel omhoog, veel sneller dan voor de lactonen. Er werd daarom aangenomen dat de spontane vorming van ketonen van groot belang kan zijn voor producten die melkvet bevatten en een intensieve hittebehandeling krijgen, of voor langere tijd bij kamertemperatuur worden bewaard. Van de ketonvorming in enkele mogelijke praktijksituaties worden in de tekst van dit hoofdstuk voorbeelden gegeven.

Ter conclusie wordt in hoofdstuk 7 een schema gegeven voor de vorming van vet-afgeleide componenten in Goudse kaas en verwante kaassoorten. In tegenstelling tot het algemeen aangenomen schema in de literatuur laat dit schema geen vorming zien van aldehyden en alcoholen, maar wel de nieuw gevonden spontane vorming van lactonen en ketonen. De vorming van vetzuren wordt opgesplitst, waarbij de vorming van lange vetzuren vooral wordt toegeschreven aan melk-eigen lipoprotein lipase, en kortere vetzuren vooral aan esterases van zuursel- en andere melkzuurbacteriën, die ook de vorming van esters voor hun rekening nemen.
Wat de sturing van de vorming van vet-afgeleide verbindingen betreft wordt het meeste verwacht van mogelijk gebruik van bacteriële cultures die beschikken over de gewenste types van esterases. Deze kunnen bijdragen aan de betere ontwikkeling van de aroma-actieve vrije vetzuren en esters. Verder kan er geselecteerd worden op melkvet met hogere lacton- en ketonpotentialen, al dan niet vergezeld van een hitte-(voor)behandeling, wat een vrij eenvoudige en krachtige beïnvloeding van de vorming van vet-afgeleide verbindingen kan zijn.

Nawoord

Ruim zes jaar geleden begon ik als AIO bij de sectie Zuivelkunde, terwijl ik tijdens mijn studie daar nooit één vak had gevolgd. Het volgen van de (aroma)ontwikkeling van de vetfractie in een gecompliceerd biologisch product leek me interessant en uitdagend. Na een inwerkperiode, waarin de nadruk vooral lag op de vorming van vetzuren en lipases leek het nodig om eerst het hele spectrum van vet-afgeleide componenten beter te bekijken. Er bleek meer te bestaan dan alleen de vetzuren, en na het inmiddels gebruikelijke gescherm met aromadrempels werden deze componenten ook zeer relevant geacht. De kaasrijpingsstudies, uitgevoerd bij het Nizo en bij DOC Kaas brachten enig inzicht in het verloop van de vorming tijdens de rijping. De zoektocht naar de biologische routes waarlangs deze componenten worden gevormd werd gestart, zodat deze routes gebruikt konden worden om de vorming een gewenste kant op te kunnen sturen. Na vele experimenten bleek de vorming van lactonen en ketonen toch hoofdzakelijk een chemische reactie te zijn. Een relatief beperkt aantal van de experimenten die aan de universiteit werden uitgevoerd zijn uiteindelijk verwerkt tot dit proefschrift. Het schrijven daarvan heb ik onderschat, en vergde meer tijd en doorzettingsvermogen dan gedacht naast een volledige baan. Gelukkig is er een groot aantal personen die mij direct of indirect hebben bijgestaan bij het werk en de afronding daarvan, en die wil ik op deze plek in het boekje graag bedanken.

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Speciale dank gaat uit naar de sponsors van dit project, in mijn geval DOC Kaas en de Leerdammer Company, en ook naar het Mesdagfonds, die samen het project "Stuurbaar maken van smaakvorming in kaas" zijn gestart. Onze halfjaarlijkse besprekingen leverde een mooie gelegenheid voor mijzelf om alles weer eens op een rijtje te zetten, maar gaf ook voldoende feedback met de praktijk en nuttige tips. Daar wil ik Jacques Stadhouders, Jaap Geerts, Mewis Hettinga, Jannes Oosterveld, Klaas Visser en Robert Rieshuis, en natuurlijk ook Gerrit Smit en Wim Engels van het NIZO graag voor bedanken. Bart, mijn mede-aio, ik

vond het fantastisch om mee te kijken en te discussiëren met jouw onderzoek, en heb jouw commentaar en inzichten over mijn promotie-onderzoek ook enorm gewaardeerd.
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Na een korte tijd achter de oude computer op de labtafel verhuisde ik met de andere aio's naar kamer 211B, en na wat schilderwerk en een "labwarming" was dat al gauw mijn thuis op de vakgroep. Dit kwam vooral door alle collega's, die met soms heel andere onderwerpen een heel andere kijk op mijn onderzoek hadden, en ook mijn gezicht op de wetenschap verbreed hebben. Ondanks het feit dat er met negen personen op de kamer er meestal wel iemand zich moest concentreren was er altijd wel tijd voor een praatje. Tussendoor, heel belangrijk, koffiepauze! "Tingeling" met de koffiemok en het lepeltje, en dan was het tijd voor de –liefst dagelijkse– ontmoeting met de rest van de vakgroep, en natuurlijk de mensen van levensmiddelenfysica.
's Avonds was thuis de afdeling, oftewel haarweg 267. Als aio is het best prettig om tussen de studenten te wonen, voor de gesprekken over de meest uiteenlopende onderwerpen, het eten, en natuurlijk de feestjes. Alle afdelingsgenoten: erg bedankt voor de gezelligheid!
Natuurlijk was het niet alleen werken wat de klok sloeg, een paar keer per week mocht er 's avonds muziek gemaakt worden. Alle muzikanten bij blaaskapel "De Rijnpijpers", studentenorkest "De Ontzetting", maar ook de bandjes "Nozel", en nu nog steeds "The Freaky Fish" mogen in dit dankwoord zeker niet ontbreken, ik heb er met heel veel plezier gespeeld, maar ook met veel plezier onder het genot van een drankje nagepraat, wat altijd een zeer welkome afwisseling was met het werk overdag.
Tenslotte mijn familie, ik ben nu eindelijk echt klaar met "studeren" (maar het was toch een baan, dat aio-schap). Ik hoop dat ik weer een beetje meer tijd voor jullie krijg, en ik bedank jullie voor steun vanaf het allereerste begin.
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Martin

Curriculum vitae

Martin Alewijn werd geboren op 3 september 1975 te Waalwijk. Na het behalen van het VWO diploma aan het OLV-Lyceum te Breda in 1993, werd in datzelfde jaar begonnen met de studie Levensmiddelentechnologie aan de toenmalige Landbouwuniversiteit Wageningen. Tijdens deze studie specialiseerde hij zich in de levensmiddelenchemie, waarbij het eerste afstudeerproject werd uitgevoerd bij de gelijknamige vakgroep, waar de veranderingen van celwandpolysacchariden in uien tijdens bewerking bestudeerd werden. Daarna volgde een toxicologisch afstudeerproject, uitgevoerd bij het Rikilt-DLO, waar met behulp van ^{1}H-NMR-fingerprinting een vergelijking werd gemaakt tussen een genetisch gemodificeerde aardappel en zijn ouderorganisme. Eind 1997 volgde hij een praktijkstage aan de University of New South Wales, Sydney, Australië, waar een methode werd ontwikkeld voor het scheiden van triacylglycerolen uit dierlijke vetten. Terug in Nederland werd nog een laatste afstudeerproject gedaan bij de leerstoelgroep Levensmiddelenmicrobiologie, waar werd gekeken naar de werking van het lactoperoxidasesysteem met jodide op verschillende (pathogene) micro-organismen. Dit alles leverde hem in september 1998 het universitaire diploma Levensmiddelentechnologie op.

Na zijn afstuderen volgde een korte oriëntatie op de arbeidsmarkt, met enkele korte projecten op de Landbouwuniversiteit en op een routinelaboratorium van Nestlé, Nunspeet. In oktober 1999 begon Martin als AIO (Assistent In Opleiding) bij de bij leerstoelgroep Procesontwerpen en Kwaliteitskunde van Wageningen Universtiteit, afdeling Zuivelkunde. Dit promotieonderzoek richtte zich op het ophelderen van aromavorming vanuit vet tijdens de rijping van (Goudse) kaas. Dit werk vormde de basis voor dit proefschrift, getiteld "The formation of fat-derived flavour compounds during the ripening of Gouda-type cheese", waarmee het promotiewerk in mei 2006 werd afgesloten.

Aansluitend aan de AIO-periode is Martin vanaf mei 2004 werkzaam als Scientist bij Heineken Technical Services, waar de aromachemie van bier centraal staat.

Educational activities

Discipline specific activities

Courses

- VLAG course Food Fermentation, December 1999
- Masterclass on lipases: from structure to applications in food and chemical industry, April 2000

Meetings

- Flavour - symposium van het genootschap ter bevordering van melkkunde, November 2000
- NIZO Dairy Conference on Food Microbes, June 2001
- Het panorama van J. Mesdag, May 2002
- 7th Congress on Lactic acid Bacteria, September 2002
- IDF Pre-summit symposium on innovative research in dairy science and technology, September 2003
- 7th Cheese symposium, October 2003
- IDF Symposium on cheese, March 2004

Training

- Methods of Enzymatic BioAnalysis and Food Analysis, Boehringer, November 1999

General courses

- VLAG PhD student trip, 2000
- PhD student week VLAG, 1999

Optional courses and activities

- Preparation PhD research proposal
- Section talk Product Design and Quality Management group, 1999-2004, Wageningen

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