Assessment of the entrapment of free fatty acids in goat milk by β-cyclodextrin and reduction of goaty flavour

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Attestation of Authorship

I declare that the thesis *Assessment of the entrapment of free fatty acids in goat milk by β-cyclodextrin and reduction of goaty flavour* is a true account of my own research. To the best of my knowledge, the work submitted is original and contains no material that has been submitted for a degree at any tertiary education institution.

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Abstract

Goat milk and its products have a significant role in human nutrition in many developing countries and some developed countries. Goat milk feeds more people in developing countries than cow milk. It is also useful as a food for people with cow milk allergies and gastrointestinal disorders. In developed countries, goat milk is often perceived as 'healthier' than cow milk and possibly for this reason has a growing market. However, its characteristic goaty flavour is a barrier to increased consumption. Prior research has shown that β-cyclodextrin, which can trap small hydrophobic molecules, reduces goat milk flavour due to certain free fatty acids (FFAs) when added at low concentrations to goat milk and goat milk products. This thesis explores the chemistry of this phenomenon. The concentration of FFAs responsible for goat milk flavour was increased when a lipase from *Pseudomonas fluorescens* was added. After addition of lipase, β-cyclodextrin was added at various times up to four hours. In all cases, FFA concentrations were increased by β-cyclodextrin, which at first sight is contrary to the idea that β-cyclodextrin reduces goat milk flavour. A chemical model to explain this paradox was developed. It was proposed that β-cyclodextrin increased FFA concentration by a mass action effect, because as a trap for FFAs it was a chemical 'sink' so enhancing lipase activity. The method to measure FFA concentration in these experiments determines total FFAs, trapped or otherwise, so it was of major interest to show whether or not these FFAs remained trapped in β-cyclodextrin, in suspension or solution, and were therefore unavailable for odour/flavour sensing in the headspace above goat milk. This was tested by dynamic headspace analysis of four goat milk treatments: milk; milk plus β-cyclodextrin; milk plus lipase; and milk plus lipase plus β-cyclodextrin. β-Cyclodextrin alone in milk reduced the profile of FFAs in the headspace, particularly of octanoic acid. Lipase greatly increased the headspace profile of FFAs as expected, but when β-cyclodextrin was also present, the profiles were even lower than for milk plus β-cyclodextrin. The reason for this apparent synergism is unknown, but it confirms why β-cyclodextrin is so effective in reducing goat milk odour/flavour in spite of its mass action effect. The remarkable effectiveness of β-cyclodextrin in reducing goat milk odour might also be due to preferential binding of goat-milk characterising branched chain fatty acids, like the potent 4-methyloctanoic acid, but which are present in only low concentrations in goat milk fat. A spectrophotometeric competition experiment with phenolphthalein showed that although branched chain fatty acids were more strongly bound by cyclodextrins including β-cyclodextrin than their straight chain geometric isomers (nonanoic for 4-methyloctanoic acid),

the difference was not marked. It was concluded that the remarkable effectiveness of β-cyclodextrin in reducing goat milk flavour is best explained by its ability to bind nearly all FFAs.

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Chapter 1

Introduction

Background

Milk, whether produced by cow, buffalo, sheep or goat, is either consumed by persons proximate to the milker, sold directly to the neighborhoods, processed into cheeses on farms, or sold to industrial dairies. Cow milk dominates the industrial sector and this is mainly confined to the developed West. The situation is very different for goat milk, since most of the goat milk is auto-consumed (used by households or families) or sold locally through the informal sector. It is difficult to establish the amount of goat milk produced and auto-consumed or sold in the local market because of the lack of world statistical data on goat milk, and generally very little national data. The best available data are probably from the Food and Agricultural Organization in 2001 (FAO, 2001) of the United Nations.

Table 1   Leaders[a] in goat milk production (FAO 1990, 1994, 2001)

Country	Goat milk production (1000 tonne year^{-1})
India	3128
Bangladesh	1327
Sudan	1151
Pakistan	818
France	480
Greece	460
Iran	398
Somalia	390
Spain	350
9 Nthn Mediterranean countries[b]	1840
10 Sthn Mediterranean countries[C]	618

[a]Countries with >300,000MT per year goat milk production.
[b]Portugal, Spain, France, Italy, Yugoslavia, Romania, Macedonia, Bulgaria, Greece, Turkey,
[C]Lebanon, Israel, Jordan, Egypt, Libya, Tunisia, Algeria, Morocco, Syria.

According to the FAO, most of the world population has access to goat milk. World goat milk production reached about 12.5 million tonne in 2001, most of it auto-consumed,

sold to the neighborhoods or used for kid suckling. An informal survey of the countries listed in Table 1 showed that probably less than 5% of the total goat milk would be traded with relatively few situations where it is as organised and controlled as the cow milk industry.

The reasons that goat milk is the dominant milk product in developing countries can be traced to the biology of the animal. In its modern context, goat farming is generally more economical and cost effective than farming of other ruminants. Goats are 2.5 times more economical than sheep on free range grazing under semi-arid conditions. In particular goats can eat and metabolise a wider range of vegetative matter than other ruminants. For this reason goats cope well in arid environments, where diet choices are few. The small body size, the species' agreeable social nature, low housing requirements and managerial problems make goats an attractive farming proposition with low barriers to entry. Goats are prolific breeders and achieve sexual maturity at the age of 10 to 12 month; gestation is short and at the age of 16 to 17 months can begin to give milk. There are no religious taboos against goat slaughter and meat consumption as there are for bovines (Hindu) and porcines (many faiths). Goats create employment opportunities to the rural poor besides effectively utilising unpaid family labour. There is ample scope for establishing cottage industries based on goat meat and milk products and value addition to skin and fibre.

The wild goat (*Capra hircus*), was the chief ancestral stock from which the various breeds of domestic goats have been derived (Zeder and Hesse, 2000). Representatives of this ancestral stock can still be found today in the arid areas Asia Minor. The goat was first domesticated on mountains of Iran, Turkestan and Baluchistan and then, as now, provided milk, meat and clothing for the inhabitants (Randhawa, 1980).

During the last 20 years, the number of goats around the world increased by about 60% not only in the countries with low income (75%) but also in those with high (20%) or intermediate (25%) income (Morand-Fehr and others 2003). Smith and others (2007) claim that goat farming is well placed to develop in New Zealand in the 21st century, building on growth in the past 20 years. In this period there has been a new and growing interest in goat milk and goat milk products in developed Western countries, and has seen the emergence of organised goat milk dairy sectors in the developed European countries (Dubeuf and Jean-Paul, 2005) and elsewhere (for example N.Z.) in the manner of the cow milk industry. There are several reasons for this. Some individuals who have allergies to cow milk protein are unresponsive to goat milk (Lara-Villoslada and others 2004). For example (Meier et al., 2001a) showed that at least 50 percent of all patients sensitive to cow milk protein can tolerate

goat milk protein. Thus there is a base consumer market for goat milk and its products. Another reason is that the nutritional value of goat milk is perceived to be higher than that of cow milk (Chilliard and others 2003). Although the differences between the milks are minor, perception is reality in the marketplace. Finally it is the author's view that goat milk has a healthy food image in the West that cow milk does not necessarily have. Since the publication of the popular eco-study *Silent Spring* (Carson, 1962) there has been a growing distrust of industrialisation in its broadest sense. This includes the food industry, where the cow milk dairy sector can be perceived as a multinational business indifferent to the environment. This contrasts sharply with the perception of goat milk which in the West has a 'healthy food' image 'untainted' by big business. Third, goat milk, with its different physicochemical properties can fill gastronomic requirements of connoisseur consumers which corresponds to a growing market in many developed countries (Haenlein, 2004).

While gourmets may seek the flavour and texture differences that goat milk products offer, the perception of mainstream consumers is that goat milk and goat milk products are unpleasant to consume. The main reason for this is purportedly due to the presence of a particular class of fatty acids present in the storage triacylglycerols, the formal name for what is trivially called fat in this case milk or dairy fat. These fatty acids are methyl- and ethyl-branched on C4, typically on acyl chains with eight and nine carbon atoms (Bruhn, 1999). These will be discussed in more detail later. When milk is stored or converted to cheese, there is a progressive hydrolysis of the triacylglycerols yielding di and mono-acylglycerols, and at the same time liberating free fatty acids. Below chain lengths of about C10, these free fatty acids are volatile to a greater or lesser extent (Attaie and Richter, 1996) and contribute to a 'goaty flavour' sensed by smell. This flavour limits goat milk consumption, and ways of overcoming this problem is the main subject of this thesis.

This research aim was by addition of entrapment agent and formation of inclusion compounds between β-cyclodextrin (β-CD) and short chain free fatty acids (FFAs) to reduce the perception of goaty flavour in milk and its products and increase consumer acceptability of goat milk, to promote its health benefit.

The structure of the thesis follows in Chapter 2 which also summarises the relevant properties of milk, in particular goat milk, the properties of cyclodextrins and the existing state of knowledge about flavour modification of milk with these carbohydrates.

Chapter 2

Goat Milk and Cyclodextrins

Milk from ruminants

To most people in contemporary developed countries, the term milk is synonymous with cow milk. Yet on a worldwide basis, there are more people who drink the milk of goats than from any other animal (Haenlein and Ace, 1992).

Table 2 compares the composition of milk from four ruminants. Although the compositions vary somewhat, the nutrients are qualitatively and quantitvely much the same at the macroscopic level. At the level of amino acid sequences in the caseins and probably other proteins however, there are species differences that translate into different tolerances of humans, especially infants, to the various milks. Thus goat milk is reportedly easier to digest than cow milk (Jenness, 1980; Almaas and others 2006). However, in developing countries that is not the reason that goat milk is favoured. Rather, the species is dry-adapted and can consume a wide range of cellulosic matter (Nastis, 1996).

Table 2   Typical proximate analysis of milk from different ruminants as g per 100 g by weight

Component	Cow	Goat	Sheep	Buffalo
Protein	3.2	3.1	5.4	4.5
Fat	3.9	3.5	6.0	8.0
Carbohydrates	4.8	4.4	5.1	4.9
Energy (kJ)	275	253	396	463
Sugar (lactose)	4.8	4.4	5.1	4.9
Cholesterol	14	10	11	8.0
Calcium (mg)	120	100	170	195
Fatty acids				
Saturated	2.4	2.3	3.8	4.2
Monounsaturated	1.1	0.8	1.5	1.7
Polyunsaturated	0.1	0.1	0.3	0.2

Source of data is McCane and others (2007). Reported in (http://www.buffalomilk.co.uk http://en.wikipedia.org/wiki/Milk - cite_ref-68)

Carbohydrate, protein and micronutrients in goat milk

Lactose

Lactose is the major free carbohydrate that has been identified in milk of the goat as in other milks. The lactose concentration is usually found to be slightly lower than that found in cow milk, but the magnitude of the difference is hard to quantify because of the variation in methods of analysis employed. Milk from farm animals has been considered to be a good natural source of lactose for human nutrition (Martinez-Ferez and others 2009).

Goat milk is an attractive natural source of carbohydrates for applications in human nutrition due to both its composition and concentration. Goat milk shows interesting similarities in comparison with human milk in terms of their oligosaccharides (C2 to C10) composition, which suggests that goat milk carbohydrates could mimic the physiological activities described for human (Martinez-Ferez, 2009).

Proteins in goat milk

Milks in general are a reliable source of high quality proteins, which are well balanced in amino acid profile. Goat milk is no exception. Goat milk proteins are similar to the cow milk proteins, for which most research has been done. The main types of proteins in milk are α-, β-, and κ-caseins, α-lactoalbumin, and β-lactoglobulin, but they differ in genetic polymorphisms and their frequencies in goat populations. Various reports have indicated that goat milk forms a softer curd than cow milk following acidification Jenness (1980) and Haenlein and Ace (1992). This difference in curd structure is attributed to the low levels of alpha-s1-casein in goat milk, compared to cow milk (see next). This is the key reason why goat milk is considered more easily digestible than cow milk (Jasinka, 1995).

α-s-Caseins

Although the overall protein composition of cow and goat milk is similar, major α-s-casein in cow milk is α-s1-casein, but in goat milk, it is the α-s2-casein. These two forms of α-s-casein result in different digestibility for humans and in cheese making properties (Remeuf, 1993). Studies have shown that α-s1-casein is one of the proteins in milk which can induce allergies (Dairy goat cooperative, 2003). This means that the allergenic burden imposed by cow milk is higher than for goat milk (Bruhn, 1999).

β-Casein

β-Casein has several forms depending on the genetic makeup of the animal. The two major forms are called A1 and A2 β-casein. The major difference between A1 and A2 β-casein is a single amino acid at position 67 in a strand of 209 amino acids. A1 β-casein has the amino acid histidine at position 67, while A2 β-casein has a proline (Figure 1). Human milk, goat milk, sheep milk and other species' milk contains β-casein with proline in position 67 and thus are A2 (Elliott and others 1999).   It has also been proposed that because human milk and goat milk are both A2, it is a/the reason that goat milk is more easily digested by humans than cow milk. However, a parallel claim is made for better digestibility being due to lack of α-s1-casein (see previous section).

Protein chain showing amino acids in A1 and A2 beta-casein

One amino acid difference at position 67 in the protein chain
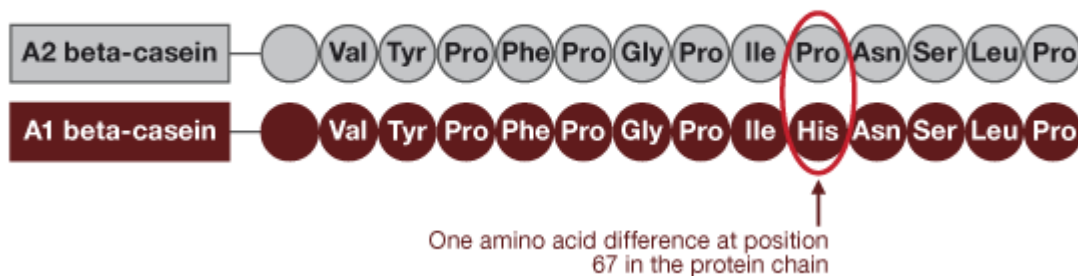
Figure 1 A1 and A2 beta-casein variants showing the single amino acid difference at position 67. Adapted from http://www.betacasein.org

Variants A1 and A2 of β-casein are common among many dairy cattle breeds. A1 is the most frequent form in Holstein-Friesian, Ayrshire and Red cattle. In contrast, a high frequency of A2 is observed in Guernsey and Jersey cattle (Cieslarová, 2006) and as noted above goats milk is exclusively A2. A2 Corporation of Auckland claims that milk from cows that produce only A2 is less likely to contribute to cardiovascular disease and diabetes in humans. According to A2 Corporation, a bioactive peptide (BCM-7) (Tyr-Pro-Phe-Pro-Gly-Pro-Ile) is yielded by the successive gastrointestinal proteolytic digestion of β-casein A1, but not A2. It is derived from residues 60 to 66 of A1 (and related variants) by the combined action of pepsin, pancreatic elastase and leucine amino peptidase. However, proline at 67 prevents the formation of this peptide. A2 Corporation goes on to claim that "BCM-7, or further truncated forms such as BCM-4 and BCM-5, have been shown to have a number of [nasty] effects both in vitro and in vivo. A2 Corporation markets cow milk sourced exclusively from A2 bovines.

BCM-7 is purported to play a role in the aetiology of human diseases (Mulloy, 2010) Epidemiological evidence from New Zealand claims that consumption of β-casein A1 is associated with higher national mortality rates from ischaemic heart disease. It seems that the populations that consume milk containing high levels of β-casein A2 have a lower incidence of cardiovascular disease and type 1 diabetes. BCM-7 has also been suggested as a possible cause of sudden infant death syndrome. In addition, neurological disorders, such as autism and schizophrenia, seem to be associated with milk consumption and a higher level of BCM-7.

Goat milk is A2 milk and is thus perceived to be a healthier food choice than cow milk.

β-Lactoglobulin and α-lactalbumin

β-Lactoglobulin and α-lactalbumin are the major whey proteins of cow milk with concentration of 3 g L^{-1} and 1 g L^{-1}, respectively. β-Lactoglobulin is present in the milk in many other mammalian species a notable exception being humans. Because this protein is not present in human milk, it assumed to be a cause of allergenic response from cow milk, however comparative studies showed no differences between the allergenicity (Haenlein, 1996, Hambling and others 1992).

Goat milk reduces allergic reaction

Resistance to digestion is a key reason why some proteins cause allergic reactions (Lara-Villoslada, 2004). The protein in cow milk that is most resistant to digestion is β-lactoglobulin because it is not present in human milk. Goat milk contains similar levels of β-lactoglobulin to cow milk, but the β-lactoglobulin present in goat milk is digested more efficiently. The reason for this is that goat milk contains high concentration of α-s2-casein and produces a soft, fragile casein curd which helps physically with digestion of milk proteins particularly with β-lactoglobulin. This results in less intact β-lactoglobulin remaining in the intestine. This means that the allergenic burden imposed by β-lactoglobulin from goat milk is less than from cow milk. Researchers in Spain have addressed this issue by comparing allergic symptoms following introduction of goat milk or cow milk to young mice immediately following weaning from their mothers. Eight of 13 mice sensitized with cow milk showed symptoms typical of food allergy, compared with only 1 of 13 mice sensitized with goat milk. The severity of allergic reactions was almost three times greater in mice with allergy to cow milk compared to the single mouse with allergy to goat milk (Lara-Villoslada et al., 2004). Therefore goat milk is less likely to lead to development of allergy to milk proteins than cow milk (Lara-Villoslada et al., 2004).

Taurine as a component of goat milk

Taurine (2-aminoethane sulphonic acid, Fig 2) is derived from methionine and cysteine metabolism. Taurine supports neurological development and helps regulate the level of water and mineral salts in the blood. Taurine is also thought to have antioxidant properties. Taurine is considered a conditionally essential amino acid in the profile of free amino acids although it is not an α-amino acid. Goat milk contained nearly three times higher concentrations of free amino acids than cow milk, and nearly two thirds the level in human milk. Human milk and goat milk both contains naturally high concentration of taurine. Both goat milk and goat milk products are a good source of this amino acid (Domagała, 2003; Prosser and others 2008).
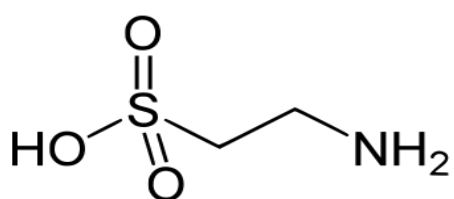
Figure 2   2-Aminoethane sulphonic acid

Micronutrients in goat milk

In common with other milks, goat milk is a source of important vitamins and minerals including calcium, phosphorus and iodine. Similarly goat milk also contains many bioactive compounds such as nucleotides, free amino acids and polyamines that have important functions other than just as nutrients. A number of studies have shown that micronutrients in goat milk can be absorbed more efficiently than from cow milk. Murry and others (1999) showed that young growing pigs fed goat milk had a higher blood concentration of iron, magnesium and phosphorus and bone mineral density than those fed cow milk. They found that goat milk enhanced calcium content of the bones *femur*, *sternum* and the muscle *longissimus dorsi* over cow milk and also found a beneficial effect of goat milk on iron uptake. Likewise, Park and others (1986) and Alferez and others (2006) showed that goat milk had more bio-available iron than cow milk when tested in anaemic rats. A series of studies in animals with symptoms of malabsorption have shown increased absorption of calcium, phosphorus, iron, copper, zinc, magnesium and selenium from goat milk compared to cow milk (Barrionuevo and others 2002).

Milk and goaty flavour

Flavour is a major attribute affecting consumer acceptance of milk and other dairy products. Volatile free fatty acids, derived from conventional fats, contribute to the flavour of milk itself and many dairy products. In some cases they provide desirable characterising or background flavour notes to products, but in other cases they contribute less desirable flavours.

Goats have distinctive smell that unhabituated people find objectionable. Indeed goats can be a source of ridicule or humour in Western cultures. Sexually mature male goats (bucks) produce an odour from their scent glands that act as a sex pheromone. The glands are located at the inner base of the horn and yield a secretion of branched chain fatty acids (Van Lancker and others 2005), which when they occur in milk cause a distinctive flavour. From a milking perspective if a buck is within scent range of milking does, the milk exhibits an enhanced goaty flavour that is due to a certain class of fatty acids (Sugiyama and others 1981) that are part of the fat phase of milk (Table 3).

In the case of meat and milk from sheep and goats, a family of branched chain fatty acids characterises the species' flavours. These fatty acids are typically eight carbon atoms long, with a branch alkyl chain (methyl, ethyl) on carbon four. In the milk fat nearly all fatty acids are esterified in the form of triacylglycerols (the formal name of what is commonly called fat), but some are not esterified and are termed free fatty acids (FFAs). The lower molecular weight FFAs are more volatile than the higher FFAs like oleic acid and where they are odorous they impact the human perception of smell and are a defining character of milk flavour (Van Lancker and others 2004). In the case of goat (and sheep) milk, small fraction of the lower molecular weight FFAs, define a species flavour that is the main subject of this thesis. These fatty acids are branched in their alkyl chain. The next section discusses the fatty acids in milk fat in more details.

Milk fat

Fatty acids are organic compounds consisting of a hydrocarbon chain and a terminal carboxyl group, with representative structure of ROOH where R can range from one carbon (formic acid, HCOOH) to 30 carbon atoms or maybe more. Figure 3 shows the structure of octanoic acid which has eight carbon atoms. Depending on the chain length, they are generally classified as short chain up to C6, medium chain C7 to C12 and long chain, more than C12. Nearly all fatty acids in milk fats are esterified as triacylglycerols, but a fraction exists as FFAs, the form in which they can be available for odour sensing. Fresh cow or goat milk contains about 0.09 g L^{-1} of FFAs, roughly 0.25% by weight of total fat that is around 3.7% the mass of milk (Table 3).
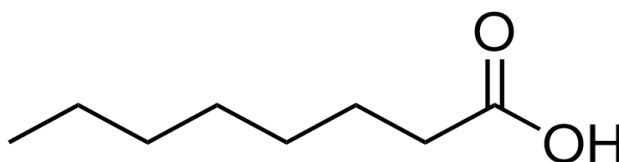
Figure 3   The structure of octanoic acid

Consider all fatty acids as a group, esterified or not, present in cow and goat milk. The data from Maree (2003) show that irrespective of species, milk fat is dominated respectively by palmitic, oleic, myristic and stearic acids (Table 3). In the lower molecular weight range there is a major difference between the fatty acids of cow and goat milk. Goats have a higher proportion of capric (C6, hexanoic), caprylic (C8, octanoic), caproic (C10, decanoic) and lauric (C12, dodecanoic) acids than cow milk. Significantly the trivial names of three of these fatty acids derive from the Latin name from goat, *capra*.

Table 3   The typical fatty acid profile[1] of milk from cow and goat.
Values are percent of total means

Fatty acid	Cow	Goat
Saturated		
Butyric (C4:0)	3.1	2.6
Hexanoic C6:0	1.0	2.3
Octanoic (C8:0)	1.2	2.7
Decanoic (C10:0)	1.2	2.7
Lauric (C12:0)	2.2	4.5
Myristic (C14:0)	10.5	11.1
Palmitic (C16:0)	26.3	28.9
Stearic (C18:0)	13.2	7.8
Arachidonic (C20:0)	1.2	0.4
Unsaturated		
Oleic (C18:1)	32.2	27.0
Linoleic (C18:2)	1.6	2.6
Linolenic (C18:3)	Not detected[2]	Not detected[2]
C20 to C22 Acids	1.0	0.4

[1] From (Maree, 2003) and (Belitz and others 2009)
[2] These animals were presumably not raised on pasture, which always
yields some linolenic acid and conjugated linoleic acid in the profile
(Parodi, 1997; Pariza and others 2000)

Take butyric acid for example. It comprises about 3% of milk fat fatty acids in cow and goats (Table 3) and in other species (not shown). With its low molecular weight and thus inherent volatility, it is a major fatty acid present in the headspace above milk and milk products, probably accounting for its low odour threshold in aqueous solutions (Belitz et al., 2009). The other fatty acids in milk are decreasingly volatile as molecular weight increases to the point that palmitic or oleic acids, the two dominant fatty acids in milk fat, would contribute little to the headspace volatiles but are certainly present in the mix of free fatty acids that accrue when milk is developing hydrolytic rancidity. FFA concentrations in isolated milk fat are typically expressed as palmitic acid equivalent because palmitic acid dominates profile (Table 3).

Branched chain fatty acids contributing to goaty flavour

In a comparison of the fatty acid profile of sheep, goat and cow milk cheeses, Ha and Lindsay (1991) found that 4-methyloctanoic acid (4-MeO) and 4-ethyloctanoic acid (4-EtO) acids (Figures 4 and 5) were relatively abundant in sheep and goat, but not cow milk cheeses. Another study by Brennand and others (1989a) also showed that 4-EtO had an extremely potent odour, with the lowest threshold, 6 ppb, of all the fatty acids tested (Table 4).
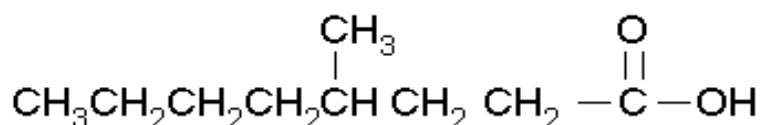
Figure 4   4-Methyloctanoic acid (4-MeO)
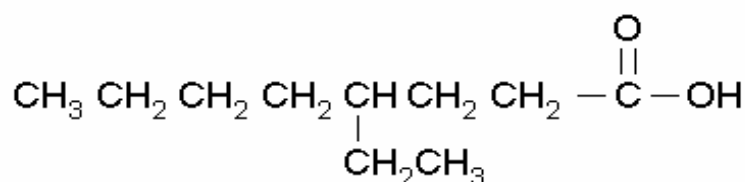
Figure 5   4-Ethyloctanoic acid (4-EtO)

Table 4   Aroma threshold values of short, medium and branched chain
             fatty acids in acidic solution

Fatty acid	Descriptor	Threshold value (ppm)	pH of solution
Butyric (butanoic)	Goat	0.4	3.2
Hexanoic (caproic)	Goat	6.7	3.2
Octanoic (caprylic)	Pungent, goaty	2.2	3.2
Nonanoic	Goat	2.4	2.0
Decanoic (capric)	Goat	1.4	3.2
4-Methyloctanoic	Goaty, muttony	0.02	2.0
4-Ethyloctanoic	Goaty, very goaty	0.006	2.0
4-Methylnonanoic	Muttony, soapy	0.65	2.0
2-Ethyldecanoic	Soapy, waxy	0.02	2.0

Adapted from experimentation (Brennand and other1989a) and by their reporting earlier authors

Fatty acids exhibiting branching at 4C have goaty/mutton/sheep aroma notes and this extends to unsaturated fatty acids. Brennand and others (1989a) also reported that addition of 4-ethyloctenoic acid to milk resulted in a goaty odour. It is important to note that these aroma

data were generated in acidic solution. Under these conditions the fatty acids are nearly fully protonated and have maximum volatility. If present as salt (soaps) they are not volatile.

Volatile FFAs in milk products

Lipases hydrolyse acylglycerols liberating FFAs, and are important in cheeses which are all subject to endogenous lipase activity. Table 5 from (Ha and Lindsay, 1993) shows the headspace composition of FFAs from milk fats of three species subjected to lipase activity. The headspace was dominated by the FFAs that comprise the bulk of the shorter chain and therefore more volatile fatty acids in milk fat (Table 5), with the various more volatile branched chain fatty acids present in very much lower concentrations. Crucially from an odour perspective, those fatty acids branched at 4C (4-MeO and 4-EtO) were very nearly absent or absent in the headspace above cow milk. The headspace concentration above sheep milk was higher, but not as high as in goat.

Table 5 Concentration of volatile branched-chain and other volatile fatty acids released by lipases in cow, sheep and goat milk fats[1] (ppm)

Compound	Cow	Sheep	Goat
Butyric acid	33,000	32,000	30,000
2-Methylbutanoic acid	60	85	120
3-Methylbutanoic acid	11	28	38
Pentanoic acid	200	99	81
3-Methylpentanoic acid	30	10	23
4-Methylpentanoic acid	16	32	104
Hexanoic acid	16,000	22,000	20,000
Heptanoic acid	180	151	283
Octanoic acid	13,000	21,000	20,000
4-Methyloctanoic	3	80	223
Nonanoic acid	53	49	286
4-Ethyloctanoic	Not detected	13	55
4-Methynonanoic acid	3	19	27
Decanoic acid	30,000	65,000	61,000
2-Ethyldecanoic acid	Not detected	Not detected	97
9-Decanoic acid	419	231	303

[1]Data are from Ha and Lindsay (1993)

The compositional differences between goat and cow milk for the fatty acids in Table 5 are based on a single sample of milks. From a statistical perspective the data are very limited but nonetheless show that percent differences between species are much greater for the branched chain fatty acids (e.g. 4-MeO) than for the dominating fatty acids in the headspace. Because the average total fat content in goat milk is similar to that in other ruminant species

like cow and sheep, and if goat milk derives its distinctive flavour properties from its lipid fraction, then the BCFAs are strongly implicated, knowing that the odour thresholds are low (Table 4).

Whereas Table 5 shows the headspace concentration of FFAs above milk fats treated with lipase, Table 6 shows the concentration of volatile free fatty acids, including BCFAs, in goat cheeses adapted from Ha and Lindsay (1993).

The first two data columns show the concentration of volatile FFAs in U.S. goat cheeses 1 and 2, each of which would be capable of being sensed by odour provided the concentration was above the threshold value (Table 4). The third data column shows the equivalent volatile free fatty acids for a French cheese. The fourth data column shows the total volatile fatty acids for the French cheese after the total fat was hydrolysed to FFAs, and the fifth column shows the percentage of the total volatile fatty acids that are free as FFAs in French cheese.

Table 6	Volatile fatty acids in some goat cheeses				
	Two USA cheeses		A French cheese		
	Volatile free fatty acids ([1]ppm)		Volatile free fatty acids (ppm)	Volatile total fatty acids (ppm)	Percentage of free to total volatile fatty acids
FFAs (Straight chain)	Cheese 1	Cheese 2 aged			
Butyric acid	0.72	31.8	3.50	7030	0.05
Pentanoic	Not detect	0.31	0.02	5.33	0.38
Hexanoic	76.0	61.2	11.3	6000	0.19
Heptanoic	0.03	0.9	0.33	21.6	1.53
Octanoic	3.69	70.3	30.9	6000	0.51
Nonanoic	0.06	1.3	0.38	19.6	1.94
Decanoic	21.0	183	88.2	21410	0.41
BCFAs					
4-Methyloctanoic	0.02	0.26	0.09	9.70	0.93
4-Ethyloctanoic	0.01	0.05	0.01	2.84	0.35
4-Methylnonanoic	0.01	0.11	0.05	1.80	2.78
8-Methylnonanoic	Not detect	0.00	0.41	0.63	65.1
2-Ethyldecanoic	Not detect	0.07	Not detect	2.90	Not detect

[1]Parts per million by mass of the original cheese.
Adapted from Ha and Lindsay (1991b); Brennand and others (1989b)

The data in Table 6 for cheese can be compared with data in Table 4 for aroma thresholds in acid solution. At pH 2 the aroma threshold for 4-methyloctanoic acid is 0.02 ppm (Table 4). In the USA cheeses 1 and 2 and the French cheese, the concentrations were 0.02, 0.26, and 0.09 ppm. Because the pH of aged cheeses is just typically around 5 or less, only about half the volatile 4-methyloctanoic acid will be protonated on the basis that the pKa of these BCFAs is also around 5. Nominally these three values should be halved for a valid comparison with Table 4. Even if this adjustment is made, the values remain near or above the 0.02 ppm threshold for 4-methyloctanoic acid and thus could be sensed. Moreover the data in Table 4 were obtained from sniffing while the cheese will be sensed during mastication and swallowing. Importantly, retronasal sensing will apply in this situation. The same argument will apply to others BCFAs. It seems clear that there are enough volatile free fatty acids to characterize the cheeses as coming from goat.

8-Methylnonanoic acid is unusual in that 65 percent in that of it was present as FFA, while all other fatty acids never exceeded 3% (Table 6). Why this was the case is not known.

Ways to prevent goaty flavour

Goat and gourmet among features which allow distinguishing goat milk from milk of other species. The characteristic flavour of goat milk cheeses is important in marketing these cheese to the gourmet market (Morand-Fehr et al., 2003; Chilliard et al., 2003), but to the initiated, the flavour can be considered objectionable. There are several ways that this flavour could be overcome so as to make goat milk and products more popular with mainstream consumers. These are by the physical removal of fat from goat milk, by breeding to lower the fat content, by breeding to minimise BCFA production in the mammary gland, by swamping the goaty flavour with other flavours, or by removal of the BCFAs from the native milk.

Physical removal of fat from goat milk

Cow milk is routinely subjected to continuous centrifugation to separate fat content of milk. By Stokes' Law the separation of fat globules in milk is proportional to the square of the radius of the globule. Therefore, smaller sizes of fat globules are slower to separate than large globules. The mean fat globule radius in goat milk is much smaller (about 10 μm) than in cow milk (about 50 μm) and for this reason goat milk does not cream naturally. Although is technically possible to separate goat milk, the time taken in existing goat milk separators would be 25 times longer in existing cow milk separators ($50^2 \div 10^2 = 25$). Separation of goat cream is simply not done.

Moreover removal of fat from milk would limit its uses in cheese production to lower fat products that may be less attractive to the gourmet market. Nevertheless the typical goat flavour is one quality component of particular importance to the cheese producer. Therefore a lower fat content could be a barrier in goat milk and its products markets.

Selective breeding to lower goat milk fat content

Selective breeding is routinely applied to the cow milk industry. For example, a recent conventional breeding program has generated a genetic line of so-called 'green-top' cows that produce a low fat milk, around 1% with a higher than normal proportion of unsaturated fatty acids. Scientists at the Fonterra-owned research company ViaLactia have identified a cow with a natural gene mutation that caused its milk to be significantly lower in saturated fat (Leake, 2007). Cows bred from this animal express the gene and it is expected that these genetically different cows can meet the demands of health conscious consumers. At the very least the milk from these animals can be used to establish a point of difference that could be used to promote a type of brand of milk. If such an animal were identified in caprines a similar industry progression could develop. And certainly, the goat milk industry could benefit from such a discovery because a skim milk cannot be made from goat milk because the centrifugation requirements would be too expensive.

Given that centrifugation is not an option to reduce fat content, and that no exceptional nanny goats have been identified, conventional breeding to progressively lower fat content over many generations presents another option to reduce goaty flavor. Most of the genetic data available on economic traits pertain to milk and fat yields and fat percentage. They indicate differences between animals within and between breeds that could be utilized for breed improvement (Haenlein and Ace, 2009).

Goats of different breeds produce milk with different fat content (Park and others 2007). The American Dairy Goat Association recognizes six breeds of dairy goats in the U.S.A. The five principal breeds are listed in Table7.

Table 7   Aaverage milk fat and protein content of dairy goat breeds. The five principle breeds in the USA

Breed	Fat (%)	Protein (%)
Alpine	3.56	3.06
LaMancha	3.80	3.29
Nubian	4.61	3.66
Saanen	3.52	3.02
Toggenburg	3.38	3.01

Dairy Herd Improvement Program (1989)

Milks from these breeds have fat contents ranging from 3.38 to 4.61%, the lowest being Toggenburg (Harris and Springer, 2009). Selective breeding within the Toggenburg might help reduce goaty flavour, although this requires that BCFAs reduce in concert with other fatty acids. This is an important point and would require monitoring of BCFA content in milk to help direct a breeding program. Monitoring would be best done by gas chromatography. However BCFAs are present in very low concentrations and are swamped in conventional gas chromatographic analyses of methyl esters. Overall it appears that selective breeding is not practicable for the goat milk industry given the size of the market and the time and money required doing this, typically decades through conventional selective breeding programs.

Masking goaty flavour by other flavours or β-CD

Young and others (2011) reported that addition of vanilla and sugar as flavouring improved liking of yoghurt made from goat milk (Table 8). This result with flavours is hardly surprising but does show that flavours can be very useful in masking unpleasant flavour. However, the primary focus of this experiment was to explore the effects of β-CD addition. On to 1 to 9 liking scale, β-CD improved the liking of yoghurt. CDs are discussed in detail in the next sections.

Table 8	Effect of β-CD on liking of plain and flavoured goat milk yoghurts with 59 consumers	
	Mean liking score ± standard deviation	
Treatment	Plain yoghurt	Vanilla/sugar yoghurt
No β-CD	2.25 ± 1.95	5.63 ± 1.99
β-CD (0.35 %)	3.39 ± 2.05	6.32 ± 1.49
Adapted from Young and others (2011)		

Cyclodextrins

Physiochemical properties of cyclodextrins and their derivatives

CDs are a group of products formed during bacterial digestion of starch (Loftsson, 2009; Menone, 2002). Over the last few years they have found a wide range of applications in food, pharmaceutical, and chemical industries as well as agriculture and environmental engineering (Singh and others 2002). These cyclic oligosaccharides consist of (α-1, 4)-linked α-D-glucopyranose units, otherwise conventionally called glucose. They possess a lipophilic central cavity and a hydrophilic outer surface.

The most notable feature of CDs is their ability to form inclusion complexes, host-guest complexes with a wide range of solid, liquid and gaseous compounds. Complexation occurs in the lipophilic cavity, and the details are discussed in a later section.

Due to the chair conformation of the glucopyranose units, the cyclodextrins are shaped like a truncated cone rather than perfect cylinders. The hydroxy groups are orientated to the cone exterior with the single primary hydroxy groups of the glucose residues on C6 at the narrow edge of the cone, and the two secondary hydroxyl groups (C2 and C3) at the wider edge (Figures 6 and 7).

The central cavity is lined by the skeletal carbons and ethereal oxygens of the glucose residues (between C1 and C5, and between C1 and C4 of the adjacent glucose molecule), which gives the cavity a lipophilic character. The polarity of the cavity has been estimated to be similar to that of an aqueous ethanolic solution (Menone, 2002). α-, β- and γ-Cyclodextrins (α-CD, β-CD and γ-CD) consist of six, seven, and eight glucopyranose units, respectively.

The CDs, in particular β-CD, have limited aqueous solubility (Szejtli, 1998; Loftsson, 2009). The low solubility of the CDs is due to their intermolecular hydrogen bonding involving hydroxy groups. Derivatisation disturbs this bonding and can increase aqueous solubility. CDs can be modified by substitution of hydrogen atom on any of the primary and secondary hydroxy groups, to produce esters or ethers or other derivatives. This is done to increase their aqueous solubility (Szejtli, 1985; Müller-Goymann and others 2006). CD derivatives are characterized by the nature, position or degree of the substituent (Szejtli, 1991) and (Yalkowsky, 1999) . Although it seems counter intuitive, Research shows that there is a correlation between the hydrophobicity of a derivative and its solubilising capacity (Muller and Branus, 1986). The most hydrophobic derivatives are the most soluble ones.

The most common derivatives of β-CD are the partially alkylated e.g. dimethyl and trimethyl-CDs. Methylation of secondary hydroxyl groups increases the diameter of the hydrophobic cavity of CDs and greatly increases solubility of β-CD. Hydroxyalkylation is an alternative modification to alkylation, and also makes β-CDs highly soluble. Some currently available CDs obtained by substitution of OH groups located in the CD rings, are for example, the hydroxylated-CDs and carboxymethyl-CDs.

As a special case of derivatization, CDs can also be covalently immobilized (Han and others 2007). Immobilized β-CD has been used to remove cholesterol from cream and other food products and can be recycled for repeated use.
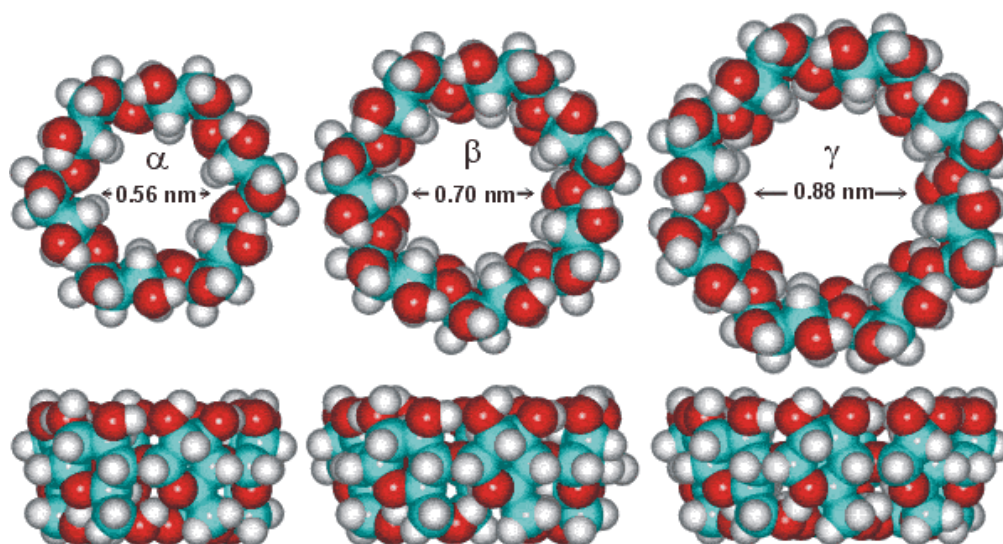
Figure 6   Atomic views of the chemical structures of α-CD, β-CD and γ-CD.
Carbon is blue, hydrogen is white and oxygen is red.
http://www1.lsbu.ac.uk/water/cyclodextrin.html
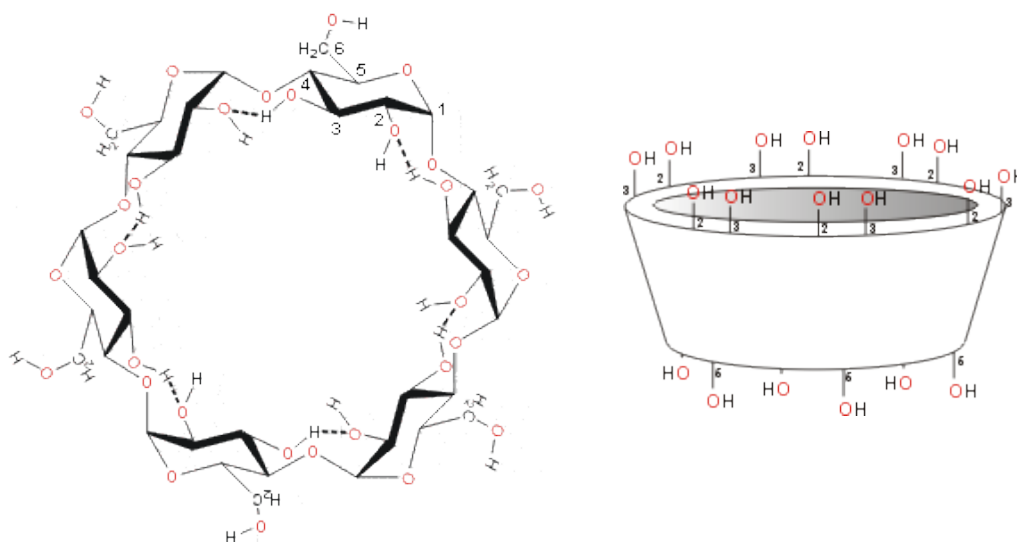
Figure 7   A line view of the chemical structure of α-CD and the 'bottom-less bucket' view. The line view suggests that the hydroxy groups are in the core. While in fact the hydroxy groups are placed at the top and bottom edges of the molecule. The primary hydroxy groups are orientated to the cone exterior (bottom) and two secondary hydroxy groups at the wider top edge of the bucket. The source was http://www1.lsbu.ac.uk/water/cyclodextrin.html

All three native CDs have similar structures in terms of bond lengths and orientations but have different diameters to accommodate different numbers of glucose residues.

Table 9 lists the physiochemical properties of the three native CDs. β-CD is less soluble than the other two, but its cavity size makes it more suitable for inclusion of hydrophobic compounds of commercial interest, and its limited solubility can be overcome by substitution of β-CD hydroxy groups. A literature review of physical characteristic of CDs shows that there are some differences in stated cavity diameter of β-CDs. Its stated diameter varies from 0.6 to 0.7 nm, but the literature is seldom specific on the atoms between which the gap is measured, and the conical shape further complicates this issue. But this variation is of no direct concern in this thesis.

Table 9   Properties of the native cyclodextrins

Cyclodextrin	α-CD	β-CD	γ-CD
Number of glucose molecules	6	7	8
Molecular mass (g mole $^{-1}$)	972	1134	1296
Solubility in water g L^{-1} (M)	145 (0.121)	18.5 (0.016)	232 (0.168)
Cavity diameter (nm)	0.47 to 5.3	0.60 to 0.65	0.75 to 0.83
Molecule diam. at widest point (top) (nm)	1.46	1.54	1.75
Height of torus (nm)	0.79	0.79	0.79
Approximate cavity volume g^{-1} CDs (mL)	0.1	0.14	0.2

Szejtli (1985); Connors and Burnette (1997)

Complex formation and inclusion compounds

The most useful feature of CDs is their ability to form inclusion complexes also known as host-guest complexes, with a wide range of solid, liquid and gaseous compounds. In CDs complexation, the host-guest binding includes hydrophobic interaction, van der Waals interaction, hydrogen binding, and charge and or dipole-dipole interaction. In hydrophobic interaction, the most non-polar portion of guest molecule is enclosed in the CD cavity (Connors and others 2000). Complexation of water insoluble compounds involves the incorporation of guest molecule within core of complexing agent. At the same time the hydrophilic groups of the complexing agent in this case CDs, interact with the water, ensuring that the complex remains soluble (Loftsson and Duche, 2007 and Vries, 1996).
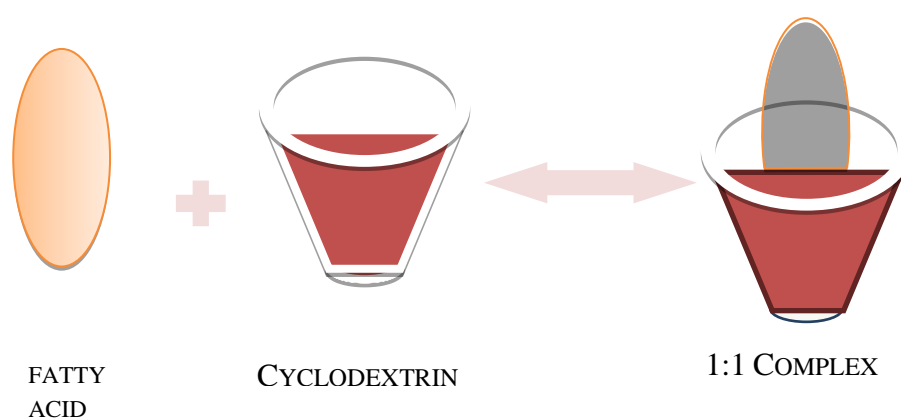

FATTY ACID   CYCLODEXTRIN   1:1 COMPLEX

Figure 8   This scheme illustrates β-CD forming a 1:1 inclusion complex with a fatty acid molecule

In hydrophilic complexes (Figure 8) a guest molecule is held within the cavity of the CD host molecule (Del Valle, 2004). No covalent bonds are broken or formed during

formation of the inclusion complex. The main driving force of complex formation is the release of enthalpy-rich water molecules from the cavity. Water molecules are replaced by hydrophobic guest molecules present in the solution to attain a dipolar association and decrease of CDs ring strain resulting in a stable state (Schneiderman and Stalcup, 2000).

Production of CDs and their regulatory status

CDs are commercially produced from starch by means of enzymatic conversion. The production of CDs involves treatment of ordinary starch with a set of enzymes derived from various microorganisms.

The preparation of CDs involves four major steps; cultivation of microorganisms that produce the enzyme CD glycosyltransferase (CGTase); separation, concentration and purification of CGTase; enzymatic conversion of starch to a mixture of CDs; and separation, purification and crystallization of the resulting CDs. Starch degradation and conversion to cyclic products is accomplished by a combination of α-amylase and CGTase. Starch is solubilised by heating, α-amylase is used to generate oligosaccharides, and the CGTase is added for the enzymatic conversion to cyclic products. CGTases can synthesize all forms of CDs, thus the product of the conversion results in a mixture of the three main types of cyclic molecules. The ratios of products are dependent on the particular enzyme used. Each CGTase has its own characteristic α:β:γ synthesis ratio (Biwer and others 2002).

Purification of the three types of CDs takes advantage of their different water solubilities. Compared with short oligosaccharides, β-CD is poorly water soluble at 18.5 g L^{-1} or 16.3 mM at 25°C, and can be easily isolated through crystallization while the more soluble α- and γ-CDs, 145 and 232 g L^{-1} respectively, remain in solution. They are usually isolated by means of time consuming chromatography techniques that are expensive compared with crystallization.

CDs are nominally non-toxic ingredients and are not absorbed in the upper gastrointestinal tract. However they are metabolised by the colon microflora. In the USA, Europe and Japan, β-CD has been on the GRAS (Generally Rrecognised As Safe) list since 1998 as a flavour carrier (Table 10) and protectant at a maximum level of 2% (Szente and Szejtli, 2004b).

Table 10   Regulatory status of the main type of cyclodextrins

	Food approval status			
Cyclodextrin	USA	Europe	Japan	New Zealand/Aust.
α-CD	[1]GRAS	Planned	Yes	Novel foods
β-CD	GRAS	Food additive	Yes	Processing aid
γ-CD	GRAS	Pending	Yes	Novel foods

[1]GRAS (Generally Regarded As Safe according to the U.S. Food and Drug Administration)

In Japan α-, β- and γ-CDs are recognized as natural products and their commercialization in the food sector is restricted only by considerations of purity. In Australia and New Zealand α- and γ-CDs were classified as Novel Foods from 2004 and 2003, respectively (Astray and others 2009). The New Zealand Food Safety Authority {, 2004 #127} defines a novel food as a non-traditional food for which there is insufficient knowledge in the broad community to enable safe use in the form or context in which it is presented.

β-CD, by contrast, is approved as a processing aid. Processing aids fulfill a technological purpose relating to treatment or processing, but do not perform a technological function in the final food. Moreover, the processing aid is used in the course of manufacture of a food at the lowest level necessary to achieve a function in the processing of that food, irrespective of any maximum permitted level specified. This is deemed good manufacturing practice (GMP). In the case of β-CD, it is approved for the function of extraction of cholesterol from eggs at a GMP level, but this clearly would not extend to using β-CD to modify flavour because it would then perform a technological function in the final food. Thus the New Zealand Australia rules are a variance with those of other countries with strict food laws.

Cyclodextrins as food ingredients

CDs are widely used in the food industry. The molecular encapsulation of lipophilic food ingredients with cyclodextrin improves the physicochemical stability of flavours, vitamins, colourants and unsaturated fats (Szente and Szejtli, 2004a). CDs are also used to protect against light-induced decomposition of food ingredients, to protect against heat-induced changes and improves shelf life of food products (Furuta and others 1996) and also undesirable components such as off-flavours or bitter components present in the food in its

natural form can be masked or covered by CD complexation. (Del Valle, 2004). Molecular encapsulation with CDs can also provide an effective protection for multiple flavour constituents present in a multi-component food system even though all are not bound. The molecular scale encapsulation can inhibits molecular interactions between the different components of natural or synthetic systems like flavour concentrates essential oils, etc.

The complexation of CDs with sweetening agents such as aspartame stabilizes and improves the taste. The bitterness of citrus fruit juices is a major problem in the industry caused by the presence of limonoids, mainly limonin and flavanoids, and naringin. Cross-linked cyclodextrin polymers are useful to remove these bitter components by inclusion complexation (Szente and Szejtli, 2004b).

CDs can also protect food ingredients against oxidation. Table 11 shows oxygen uptake by the flavours benzaldehyde and cinnamaldehyde flavour with and without CD encapsulation. These flavourants are prone to oxidation. Oxygen uptake in encapsulated samples was much lower. Also CD complexes are stable over years (Table 12).

Table 11   Effect of β-CD on uptake of oxygen by flavourants

	Cumulative consumption of oxygen ($\mu L \ mg^{-1}$)						
	Exposure time (hours)						
Benzaldehyde	0	26	37	44	48	52	56
Benzaldehyde + β-CD	0	2	4	4	4	4	5
Cinnamaldehyde	0	7	23	30	34	47	53
Cinnamaldehyde + β-CD	0	4	6	9	8	9	11

Data adapted from (Szejtli and Szente, 1987)

Table 12   Preservation of β-CD complexed with anis oil, cinnamaldehyde and citral during long term storage

	Flavour load of the β-CD complexes (%)					Percent remaining encapsulated after 14 years
In year	1976	1978	1980	1987	1990	
Anis oil + β-CD	9.9	10.1	8.9	8.0	7.8	78
Cinnamaldehyde + β-CD	9.2	7.8	7.1	6.0	6.3	68

Citral+ β-CD	8.9	Not avail.	6.0	4.4	4.0	40

From (Szente and Szejtli, 2004a)

CDs are also used for removing of cholesterol from butter, milk and other food products. β-CD is the CD of choice (Dias et al., 2010). Over 90% of cholesterol was removed from commercial milk at refrigerated temperature with 1% β-CD (Alonso and others 2009). β-CD immobilized on glass beads has also shown to be effective (Kwak and others 2004).

As noted earlier, CDs form complexes with FFAs. When milk is stored, the tri-acyls slowly hydrolyse to produce FFAs. This happens spontaneously but is also catalysed by lipases that occur naturally in milk, or are derived from microbial contamination (Ha and Lindsay, 1992). The higher the concentration of free fatty acids, the greater the potential for these to have a flavour impact through odour.

In a study by Meier and others (2001), β-CD improved the quality of goat's milk flavour by minimizing it presumably by binding FFAs. The concentration of β-CD necessary to minimize the goaty flavour was 0.35 to 0.4% which was determined by sensory analysis. Another study was conducted by Gupta (2004) at AUT University on the effects of CDs on goat milk flavour. The study was done by sensory evaluation and associated chemistry experiments. It established that β-CD and α-CD can reduce the perception of goaty note in goat milk and goat yoghurt.

Summary

The commercial value of goat milk can be enhanced, especially for higher value milk products, if its goaty flavour can be eliminated or reduced to an unobjectionable level. In the case of goat milk and goat milk products, the characterizing odorous compounds are short and branched chain fatty acids, with about four to ten carbon atoms. The odour threshold of these BCFAs is very low, so can often detect a goaty odour in goat milk. It becomes much more obvious in goat cheese, where they become liberated by hydrolysis from the fats they 'belong' to, and create the characteristic goaty note of goat cheese for example. Yoghurts made from goat milk may be similarly affected. If the odour/flavour problem of goat milk can be overcome there is potential to create a range of goat milk products.

CDs were suggested as a means of reducing goaty flavour, presumably by complexation of BCFAs. CDs are likely to have the ability to encapsulate small molecules like BCFAs in inclusion complexes, which theoretically should render the BCFAs incapable of sensory detection. β-CD being the cheapest among the three common CDs could be a cost effective

solution. The prior studies with β-CD and goat milk showed that β-CD altered the flavour quality of goat milk flavour but did not alter the basic composition of milk in respect to fat nutritional values. That study emphasized the inclusion process over the perception of goat milk flavour.

The aim of this research was to test the effects of β-CD on the chemistry of goat milk with a view to improve the flavour of goat milk and its products. The following chapter discusses details of the materials, equipment and development of methods used for this study (Chapter 3). That is followed by results and discussion in chapters 4 and 5. That research involves chemical analysis, GC analysis of fatty acids entrapped in β-CD inclusion complexes. A study of the affinity of β-CD with FFAs is the subject of Chapter 6. Chapter 7 summarizes the research and discusses future opportunities for research.

Chapter 3

Materials and Methods for Copper-Salt Method

Introduction

As discussed in Chapter 2, the quality of milk and milk products can be adversely affected by lipolysis. Enzyme-catalysed and spontaneous hydrolysis of triacylglycerols produces FFAs that include volatile short chain FFAs. The flavour impairment caused by lipolysis is the so-called hydrolytic rancidity. In the case of goat milk, the short chain FFAs and branched chain free fatty acids (BCFAs) that define a distinct goaty flavour, which is often considered unpleasant to the un-habituated consumer. Also discussed in Chapter 2 was the concept that goat milk flavour could be altered by entrapment of short and medium chain BCFAs, in cavities of CDs. This concept is by two publications (Meier and others 2001; Gupta, 2004).

Hydrolytic rancidity in milk develops when lipases attack triacylglycerols in fat globules to produce free fatty acids. In fresh milk, the triacylglycerols are protected from lipases by the milk fat globule membrane. If this membrane is damaged, the triacylglycerols are exposed to lipolysis. Damage can occur from temperature fluctuations in the stored milk or by agitation of raw milk at the farm, or during transportation, and processing and can lead to excessive lipolysis. Lipolysis also increases with time of storage.

In testing the role of CDs to bind FFAs it was decided to artificially enhance the concentration of FFAs in milks by the addition of lipase(s). That was frequently done in the experiments to be described in later chapters.

There are three major parts to this study. The first involves measuring the effect of β-CD on the concentration of FFAs in milk as determined by a colorimetric method. The second involves determination of volatile FFAs by gas chromatography, and involves finding which of the volatile FFAs are best bound by β-CDs. The third involves the study of relative affinity of FFAs for CDs.

All this work was supported by materials, chemicals and equipment, and by methods described below.

Materials, chemicals and equipment

Milks

The main milks used in this study were commercial offerings from goat and cow. For reasons discussed in the Introduction (Chapter 2) goat was the preferred milk. But because goat milk was not always available from supermarkets, it was decided to use Blue Top® Anchor milk containing 3.3% fat (w/w) for many of the developmental experiments, and which did not involve the BCFAs as the subject of the experiment. Where goat milk was used it was Nanny Goat Lane (Dairy Goat Co-Operative) containing 3.4 % w/w fat also purchased from a supermarket. It is a UHT shelf-stable product and is packed in 1 L packs, and was usually but not always available. All milk was used before the stated best before date.

Cyclodextrins

α-, β- and γ-CDs were from Cyclodextrin Technologies Development Inc. (High Springs Florida), and were described as technical grade not approved for food use. However, since no sensory trials were conducted, the food exclusion was not a problem.

Enzymes

Enzymes purchased from Sigma-Aldrich were lipase from *Candida rugosa* (L1754, 724 lipase unit mg^{-1}), lipase from *Pseudomonas fluorescens* (Fluka 28602, 282 unit mg^{-1}), and lipoprotein lipase from a *Pseudomonas* species (L965, 79000 unit mg^{-1}) the *Candida rugosa* bought in 2004 and stored chilled at AUT but the other two have bought for this research. One unit of enzyme activity corresponds to the amount of enzyme which librates 1 μmol of oleic acid per minute at pH 8.00 from fat under conditions described in the Sigma catalogue.

All enzymes were prepared fresh for use in sodium phosphate buffer (0.17 M with respect to phosphate) at pH 8.0, and kept in a refrigerator until used.

Other chemicals

Other chemicals were sourced from AUT's chemical store, and these were analytical grade unless specified otherwise. These chemicals were ethylene-diaminetetraacetic acid disodium salt (EDTA) ($C_{10}H_{14}O_8N_2Na_2.2H_2O$, E5134), chloroform Merck, (K 32975445), n-heptane (Unichrom, 1206), NaCl, cupric nitrate (Ajax Finchem, AF508225) and methanol (BDH UN 1230) sourced from AUT's chemicals store.

Fatty acids were from AUT's chemical collection or bought from Sigma Aldrich. Fatty acids used for displacement of phenolphthalein experiments were octanoic, nonanoic,

decanoic, 4-methyloctanoic (Sigma, M-7400), and 4-methylnonanoic (Sigma, W35, 740-5-K). Heptadecanoic acid (069K1703, > 98%) was used as an internal standard for gas chromatography. Phenolphthalein was laboratory grade (BDH, 0016400).

Spectrophotometery

The spectrophotometer used in this experiment was an Ultraspec 2100 (Amersham Pharmacia Biotech) and was used in the visible range. One centimeter plastic cuvettes were used for the phenolphthalein displacement experiments. Quartz cuvettes were used in the copper salt method because plastic cannot be used with aggressive solvents like chloroform.

Gas chromatography

The chromatograph was a Shimadzu GC-17 A, with a FID detector and fitted with a fused silica column. The column was CP-WAX 58 (FFAP)-CB, with an internal diameter 0.25 mm, and 25 m long, used for analysis of acidic compounds, such as free fatty acids. This column provides excellent peak shapes and separates C2 to C24 acids. The stationary phase was polyethylene glycol 20 μm thick.

Analytical method for determination of FFAs

The copper salt method to determine FFAs

This method depends on the creation of soap with FFAs and Cu^{2+}, and the isolation of those soaps in an organic phase, with excess Cu^{2+} ions remaining in an aqueous phase. Cu^{2+} ions that form the soap are reacted with diethyldithiocarbamate to form a yellow pigment and the colour is measured in a spectrophotometer. A volume of milk (0.5 mL) is added to a glass screw top tube containing 5 mL of chloroform: heptane: methanol (CHM) in the ratio of 49:49:2 (v/v/v). To this is added 0.1 mL of 0.8 M Na_2 H_2EDTA and 0.1 mL of 0.7 M HCl. To this is added 2 mL of the copper reagent that comprises 10 mL of 1 M Cu $(NO_3)_2.H_2O$, 5 mL of triethanolamine and 85 mL of saturated solution of NaCl. Triethanolamine prevents the copper from precipitating in the milk. The total volume of 7.7 mL is mixed by shaking for 2 minutes and the two phases are separated by centrifugation. Excess copper remains in lower aqueous phase of the mixture and the Cu^{2+} ions associated with FFAs remain in upper organic phase (Kuzdzal-Savioe & Anderson, 1980). This upper phase is chloroform rich, which would normally be expected to be the lower phase. The aqueous phase is made denser than chloroform by the near-saturated salt solution. An aliquot of the upper phase is removed by pipette to which is added an equal volume of diethyldithiocarbamate, 0.1% in n-butanol.

The yellow colour (Figure 10) develops immediately and is measured in the spectrophotometer at 440 nm calibrated for known concentrations of palmitic acid.
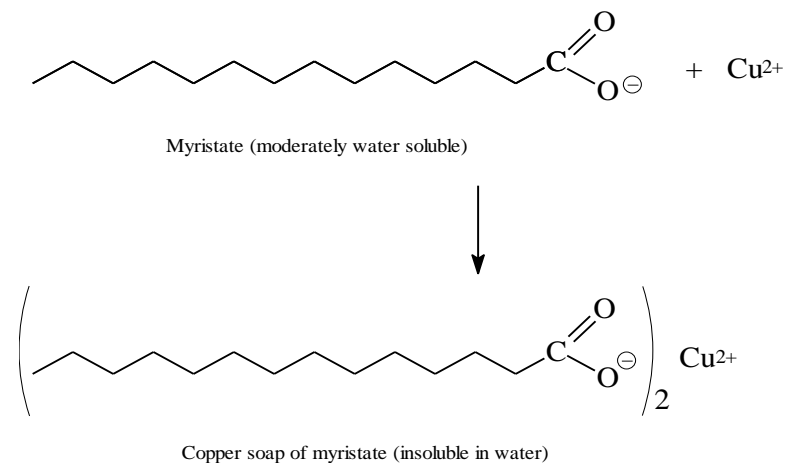

Myristate (moderately water soluble)

Copper soap of myristate (insoluble in water)

Figure 9   Reaction of copper ions with myristate as a representative FFA to form a copper soap

Diethyldithiocarbamate

Yellow complex (absorbs at 440nm)
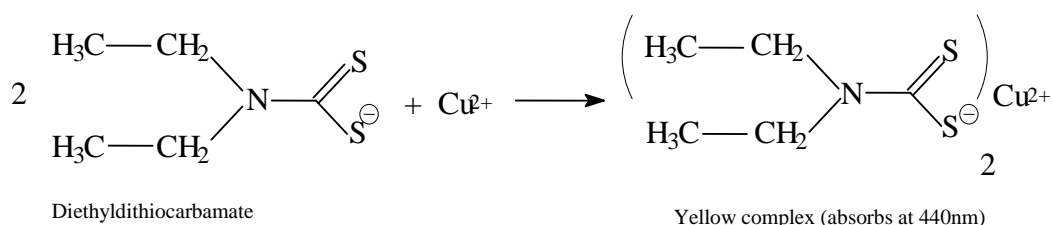
Figure 10   Reaction of copper ions with diethyldithiocarbamate

The gas chromatographic method to determine FFAs in headspace

Gas chromatography (GC), is a common type of chromatography used in analytical chemistry for separating and analysing compounds that can be vapourised without decomposition. The relative amounts of such components can also be determined by comparing peak area (Pavia and others 2006). The objective of this experiment was quantification of volatile FFAs in milk headspace as affected by addition of CD to milk. This was achieved by a purge-and-trap method that was using an internal standard. The purge-

and-trap method is described in Chapter 5 and yielded 1.0 mL of chloroform extract to which is added 500 ppm of heptadecanoic acid as internal standard before GC analysis.

Data analysis

Microsoft Excel was used to marshal and analyse data using simple statistical function in the software. Graphs were also generated by Excel.

Chapter 4

Interactions of β-CD and Fatty Acids after Adding Lipase in Goat Milk

Introduction

UHT goat did not exhibit a strong goat milk odour, so it was suspected that the FFA concentration were too low. Pasteurisation of goat milk at high temperature denatures the enzyme lipoprotein lipase and the UHT process subsequently sterilises the milk. The loss of lipase would have had an obvious effect on FFA development, but additionally sterilisation eliminates microbes that can produce extracellular lipases. For both these reasons, generation of a goaty flavour in UHT milk would be expected to be low or negligible. Two methods were tried to increase the concentration of FFAs. Stirring and bubbling of goat milk with air was tried as a way of generating a goaty flavour, but this did not work within a day of treatment. More positive results were obtained when lipase was added to goat milk. These results are described here, beginning with the calibration of the copper salt method.

Calibration of the copper salt method

Figure 11 shows calibration curve where absorbances due to FFAs are expressed as palmitic acid equivalents. This was used for express absorbance as palmitic acid equivalents where absorbances at 440 nm were less than 1.0.
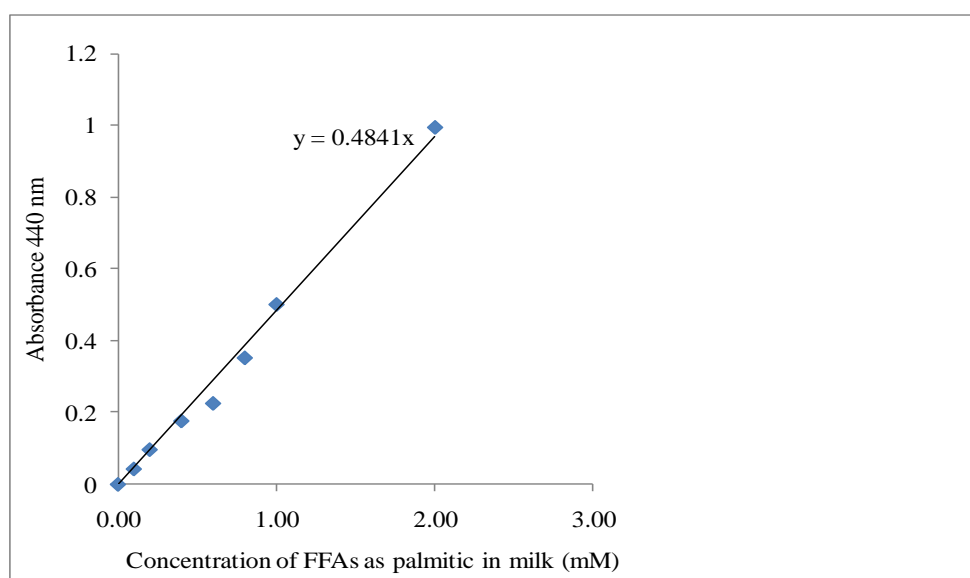

Figure 11   Standard curve obtained from known concentrations of palmitic acid ranging from zero to 2 mM

Effect of lipase from different microbial sources on odour generation and flavour of goat milk

Method

The three microbial lipases were prepared in carbonate buffer as described in Chapter 3 and each was added to UHT milk in the ratio of 30 activity units to 50 mL of milk at ambient temperature. The milks were briefly mixed and allowed to stand undisturbed. Samples (0.5 mL) were taken in duplicate at various times to 24 hours and the FFA concentration measured by the copper salt method.

Results and discussion

Figure 12 shows that the lipoprotein lipase from *Pseudomonas sp*. and lipase from *Pseudomonas fluorescens* were both effective in FFA generation. However, the lipase from *Candida rugosa* was ineffective in liberating FFAs. The reason for this is unknown. The lipase from *Pseudomonas fluorescens* appeared to be very active to the point that there was an immediate increase in FFA formation immediately after the lipase was added.
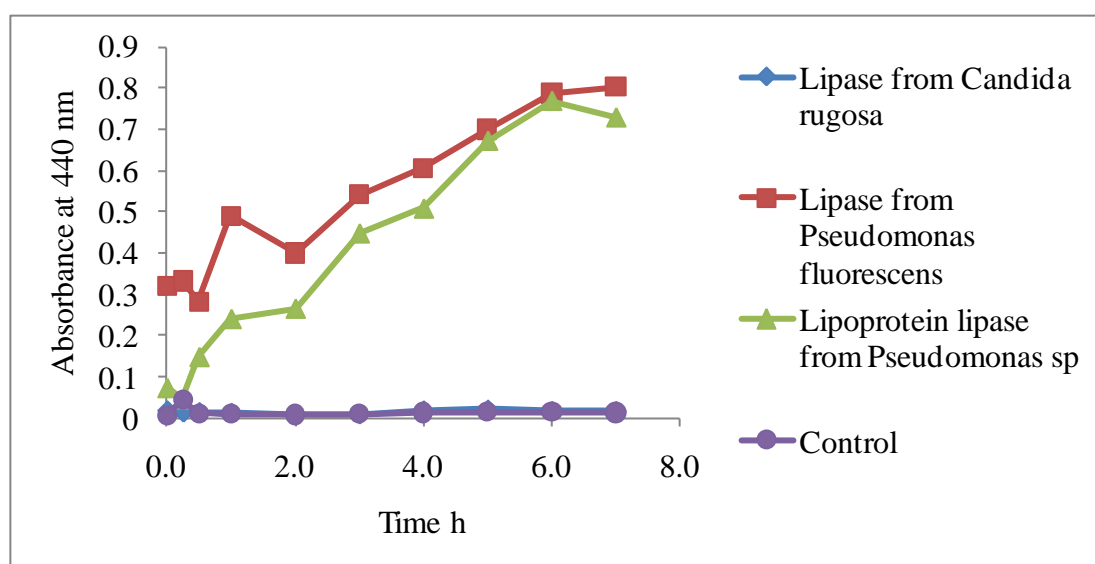

Figure 12   Effect of three lipases, on generation of fatty acids in milk as determined by the copper salt method. Higher absorbance represents more FFA formation
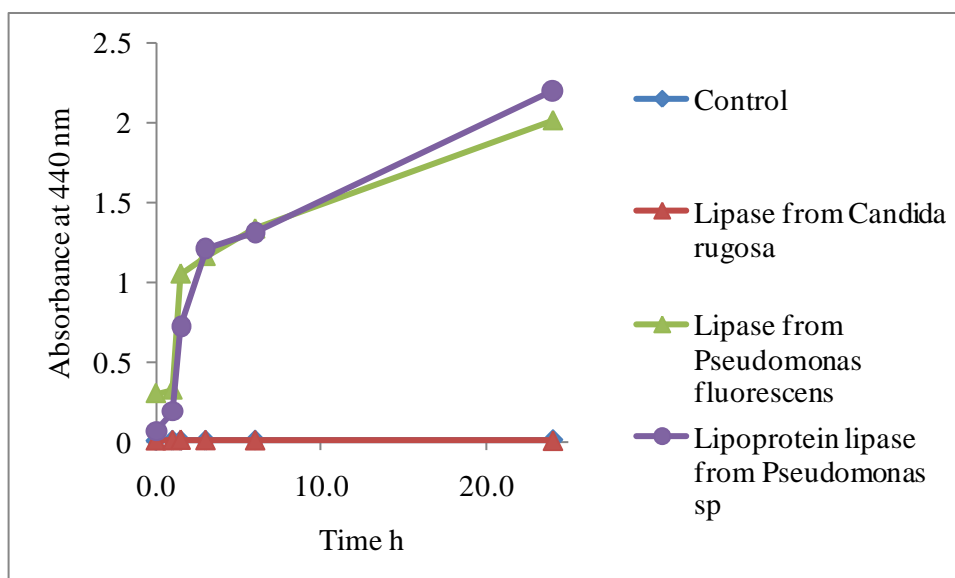
Figure 13   Effect of three different enzyme lipases on lipolysis of fatty acids in milk to 24 hour. Higher absorbance means greater FFA formation. Absorbance due to the lipase from *Candida rugosa* and the control were coincident

Both active lipases (Fig 13) showed signs of saturating activity after about 6 h, corresponding to about 2 mM FFA as palmitic acid. In a repeat experiment that extended to 24 h, the activities of the two active enzymes appeared to saturate after 2 h. The highest concentration recorded was between 3.5 and 4 mM at 24 h, which was calculated from extrapolating the straight line in Figure 11. Although extrapolation is a less-than-ideal way of determining values, it is clear that between 3.5 and 4 mM FFAs is far below the approximate theoretical FFA concentration (as palmitic acid) in this 3.4%-fat milk, had all the fat been hydrolysed. This was calculated as follows. A fat content of 3.4 % fat is equivalent to 34 g of fat L^{-1}, or 42 mM fat, based on a tripalmitate molecular weight of 807 g L^{-1}. Because there are three fatty acids per molecule of fat, the theoretical yield of FFAs would be 3x42 =126 mM, much higher than a maximum obtained at 24 h, say 4 mM. Thus, although the two lipases were active, it seems unlikely that most of the fat is inaccessible to lipase activity, presumably due to the membrane composition of fat globules in this UHT goat milk. Nonetheless, the enzymes were active enough to generate FFAs far above the no-enzyme control (Figures 10 and 11) and this was certainly obvious by odour.

The lipase from *Pseudomonas fluorescens* was chosen for subsequent work because the price per unit of activity was lower.

Inactivation of *Pseudomonas fluorescens* lipase by heating milk

Methods

For experimental purposes it was thought useful to stop enzyme activity so that the milks would have at high and static concentration of FFAs for subsequent experiments. Heating was thought to be the only possible method of stopping enzyme activity while retaining the gross properties of milk. After 2 h incubation with *Pseudomonas fluorescens* at ambient temperature, boiling was performed for up to 150 seconds. FFAs were determined immediately after boiling and rapid cooling, and 2 h later.

Results and discussion

Inspection of the data for Figure 14 confirmed that a 2 h incubation (0 sec boiling) was sufficient to generate useful concentrations of FFAs and confirmed the results in Figure 12 and 13, both again suggested that the results were variable between different lots of UHT milk. The absorbance at 2 h was almost 1.5, and was far above the no-enzyme control (bars at far left).
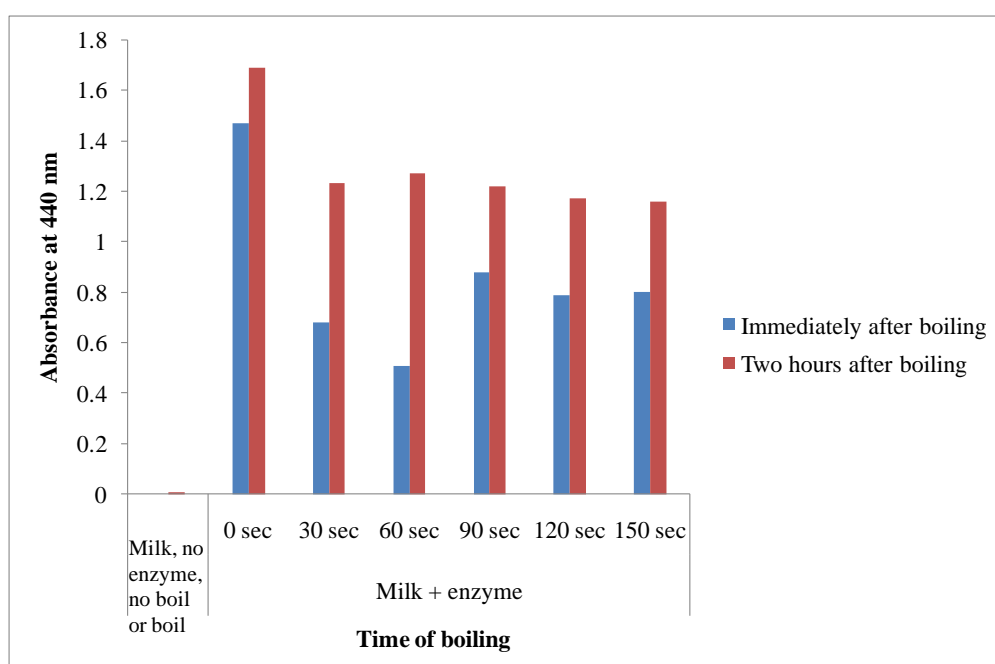

Figure 14   Effect of different boiling times for milk on FFA formation in milk treated with lipase from *Pseudomonas fluorescens* for 2 h at room temperature. Boiling times were as indicated. Control milk with no enzyme was unaffected by boiling, so boil/no boil data are summarised by the two bars at left, where one is so low as to be not visible

Figure 14 showed that boiling was not effective in stopping enzyme activity. Therefore, generation of FFAs continued after boiling. All these treatments were done in scotch bottle with loose lids on. But decrease in the absorbances and FFAs suggests that there is a loss of volatile FFAs. The added lipase produces short and medium chain FFAs with low molecular weight and low boiling points. These short and medium chains FFAs evaporate when heated in microwave.

What happens after further 2 h incubation at ambient temperature? It is clear from these data that boiling did not stop lipase activity. This finding confirms a previous study in which lipase from *Pseudomonas fluorescens* was studied for thermostability at temperatures ranging from 100 to 160 ℃ (Andersson and others 1979). Nonetheless, the results in Figures 12 and 14 strongly suggest that lipase activity plateaus to some extent, probably for reasons of enzyme access to the substrate as was discussed in the previous section. Given that lipase activity was impossible to stop, all copper salt assays on samples generated in kinetic studies were performed within seconds of a set time point, or in some cases the samples were held at -80℃ to prevent further lipase activity.

To summaries boiling of milk samples for two minutes was not enough to stop enzyme activity but FFAs reached the concentration which was high enough to explore the effects of β-CD on FFAs in goat milk.

Effect of β-CD on determination of FFAs by the copper salt method

The copper salt method has been used in many sections of this chapter to monitor the effects of β-CD on FFAs in milk. This method depends on total extraction of FFAs into a liquid organic phase, and it was important to establish that β-CD would not compete with the solvent. In other words by binding FFAs does β-CD lower measured values of FFAs as determined by the copper salt method?
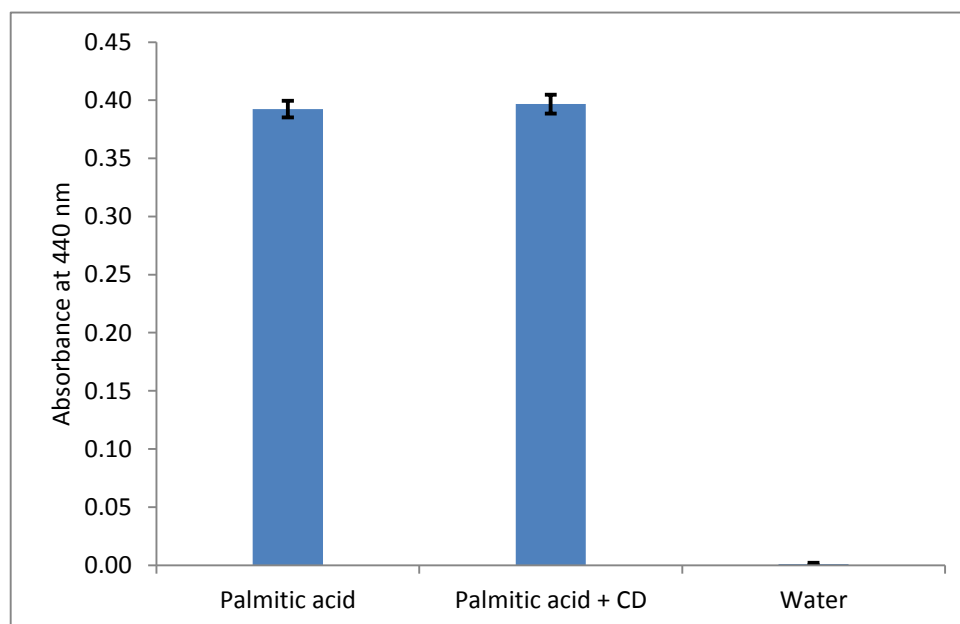
Results and discussion

Figure 15   Absorbance due to 1 mM palmitic acid in the absence and presence
of 0.3% β-CD. Based on means of duplicate, with standard
deviation bars

It is clear from Figure 15 that β-CD did not affect the extraction of palmitic acid into the
organic phase of the copper salt method. This means that experimental effects apparently due
to β-CD – as revealed by the copper salt method – are not due to effects of β-CD on the assay.

Generation of FFAs by lipases in goat milk in the presence of β-CD

Methods

As detailed in Materials and Methods, 30 units of *Pseudomonas fluorescens* lipase in
300 µL of buffer or buffer alone were added to 50 mL of milk (600 units L^{-1}) at ambient
temperature. All milk treatments, which were duplicated, were heated to boiling for 120 to
150 seconds at 2 hours. Solid β-CD was added at 0.30% at various times depending on the
treatment: 0, 1.98, 2.17 and 4 hours. Duplicate sampling to determine FFAs was started
immediately after addition of enzyme and then continued for 8 hours and in some cases to 24
hours. Sampling times were thus 0, 1, 1.98, 2.17, 3, 4, 5, 6, 7, 8 and 24 hours.

Results and discussion

β-CD was added or not to milk samples at different times after addition of lipase at zero time. Figures 16, 17, and 18/19 which are representative of six replicates of this experiment, show that the FFA concentration as palmitic acid equivalents did not change significantly when no lipase was added. In all other treatments, addition of lipase caused an increase in FFA concentration, as expected, which tended to gradually plateau after the 2 min boil at 2 h. Indeed, in one experiment where the incubation was continued to 24 h, all treatments attained a steady state (Figure 17). Immediately after the 2 min boil there was a decrease in FFA concentration. The most likely cause of this is loss of volatile FFAs, realising however, that only a fraction of the FFAs would be protonated and therefore volatile. After heating, the Milk + Enzyme treatment resumed an upward trend, but the rate appeared to plateau at around 4 h, and clearly reached a steady state by 10 h (Figure 17). This result confirms that results in Figure 14, that boiling was not effective in denaturing the enzyme, because fat hydrolysis still occurred after boiling. However, it did plateau after many hours, suggesting that although still active, the enzyme can no longer access substrate. In UHT milk, the fat globules are very small (Paquin, 1999) and are surrounded by caseins and whey proteins (McPherson and others 1984), which may protect the fat from lipase activity. Indeed, based on the palmitate-equivalent values (up to 2.0 mM), it was calculated that only about 1% of the total fat in the UHT milk, with a declared 3.4% fat content, was hydrolysed to FFAs.

Addition of β-CD at 0 hours along with lipase usually resulted in an increase in FFA concentration, as shown in Figures 16, 17 and 18/19. Initial addition of β-CD thus promoted the generation of FFAs. This suggests a mass action effect, where the β-CD trap 'pulls' the reaction in the direction of FFA formation. When additions were made at later times, 1.98 h (before boil), 2.17 h (after boil and cool) and 4 h, the FFA concentrations were always higher than in the Enzyme-alone treatment. These results are consistent with a mass action effect. However, there are some unexplained variations between the effect of boiling and the effect of adding β-CD at different times. Thus for example, at around 7 h, the concentration of FFAs as palmitic acid due to time of addition differed between Figures 16, 17 and 18.

After addition of enzyme, steady generation of FFAs occurs but after 4 h it plateaus. This may be result of saturating effect of FFAs as seen previously (Figures 12 and 13). As a result of saturating the effect of FFAs in milk, the rate of production of FFAs stays almost in steady concentration by equilibrium.
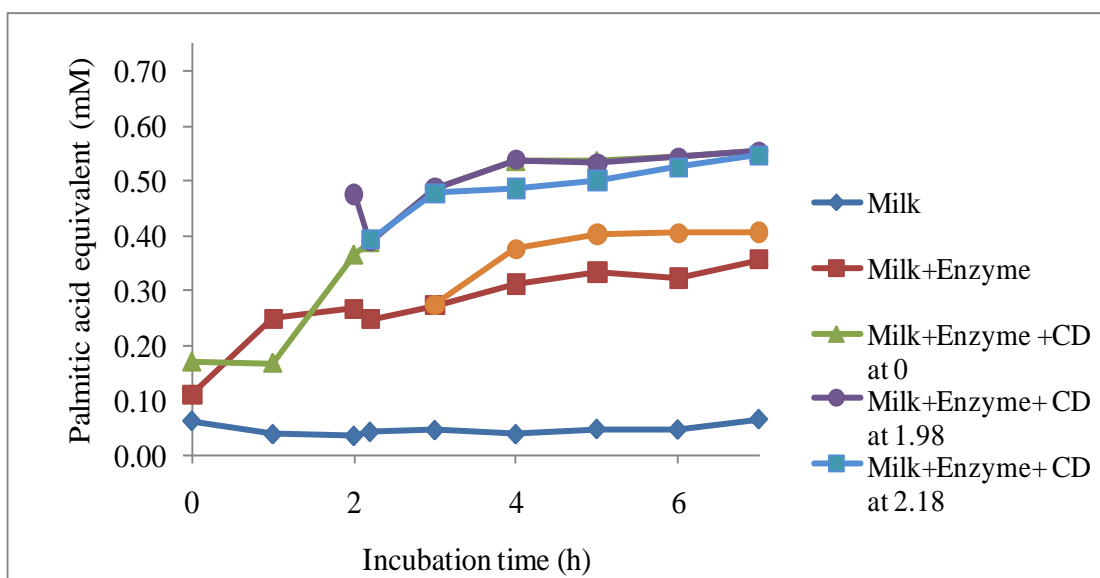
Figure 16   Effect of combinations of lipase, β-CD and boiling in goat milk on FFA
generation as palmitic acid; replicate 1 of 6
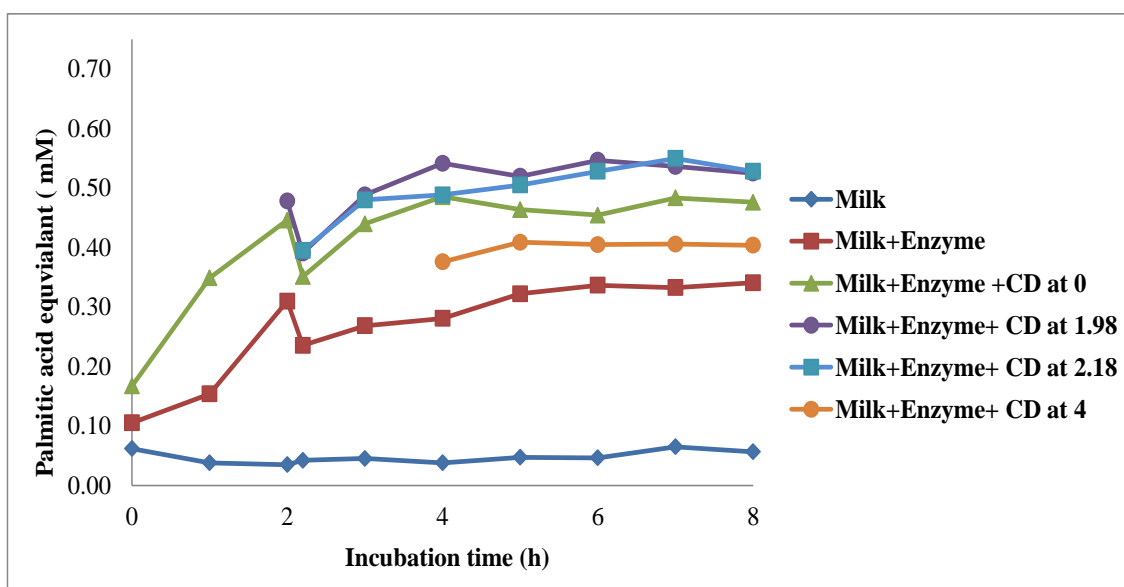
Figure 17   Effect of combinations of lipase, β-CD and boiling in goat milk on FFA
generation as palmitic acid; replicate 2 of 6
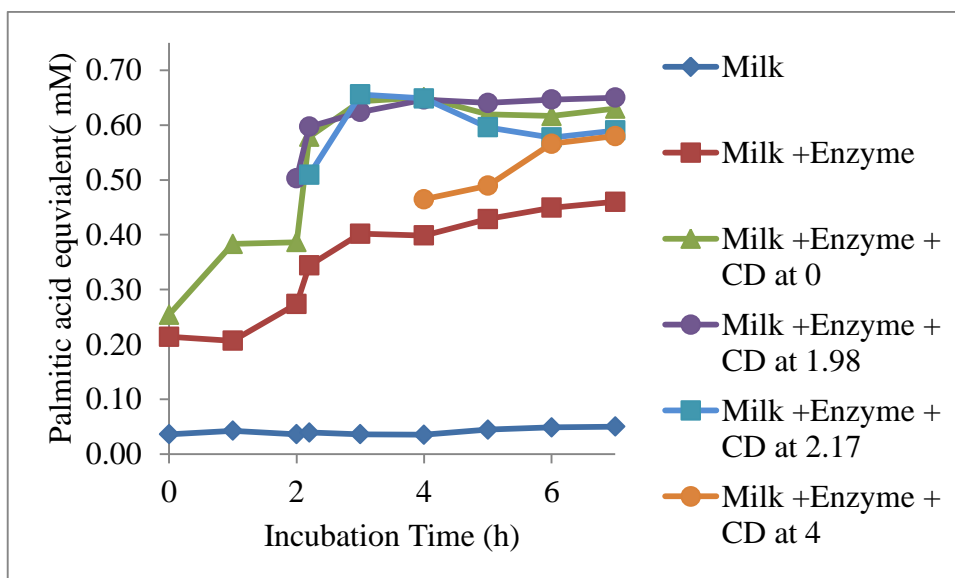
Figure 18   Effect of combinations of lipase, β-CD and boiling in goat milk on FFA generation as palmitic acid; replicate 3 of 6 over 6 h
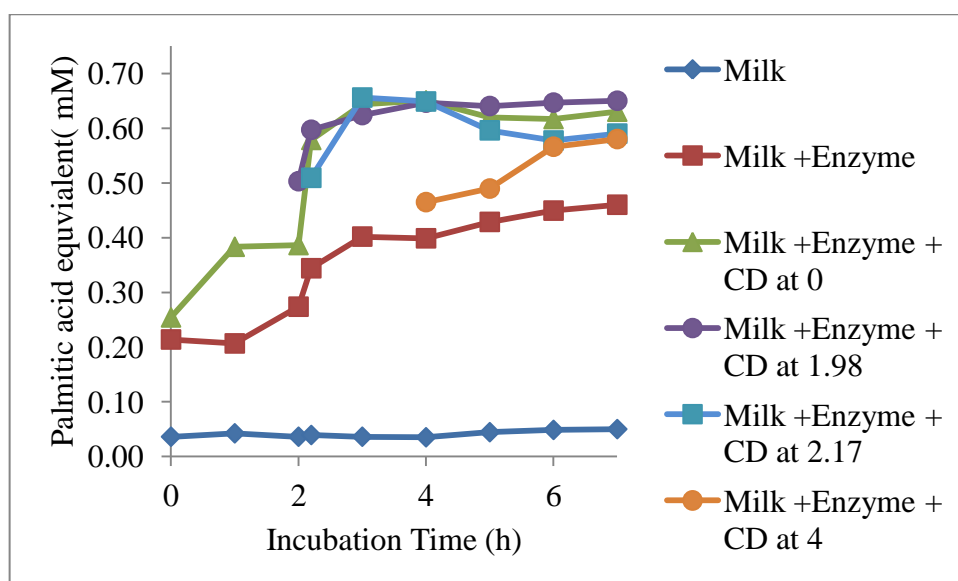
Figure 19   Effect of combinations of lipase, β-CD and boiling in goat milk on FFA generation as palmitic acid; replicate 3 of 6 over 24 hours

To summarise, in all experiments the addition of β-CD increased the generation of FAAs but after some time this effect plateaued. There were however, some unexplained effects due to time of addition of β-CD. Although the results show a higher FFA concentration when β-CD is added, available evidence from Young and others (2011) (Table

14) suggests that goaty odour would not be enhanced, but rather reduced: in that experiment with lipase *Candida rugosa* panelists were asked to rank the odour intensity of (non-UHT) goat milk treated with lipase. A low rank means the odour intensity is reduced, as is clearly the case for β-CD.

Table 13	Effect of three CDs at 3.08 mM on the odour intensity of goat milk treated with lipase[1]. Values are sum-of-ranks from 61 panellists ranking the four treatments
Treatments	Goat milk treated with lipase
No cyclodextrin	162
α-CD	157
β-CD	126
γ-CD	165
Overall statistical effect of Cyclodextrins on sum-of-ranks	$P < 0.001$

[1]Data are abstracted from Young and others (2012)

The results in Table 13 are interesting for another reason. In Figures 12 and 13 it was shown that the lipase from *Candida rugosa* was inactive with UHT goat milk. It was however active in whole non-UHT goat milk (Gupta 2004). The reason for this difference has not been pursued but may be due to the age of the enzyme stored at AUT.

This chapter established the ability of lipase to generate FFAs and showed that β-CD accelerated lipase activity. Gupta (2004) showed that β-CD could reduce goat milk odour (Table 13), suggesting that fatty acids liberated by lipase would be unavailable for sensing, trapped in β-CD and thus not potentially in the head space above milk.

The copper salt method does not distinguish between trapped and free fatty acids. However gas chromatography can be used to measure the FFA concentration in the headspace above a food. In the next chapter gas chromatography has been used to determine the concentration of identified FFAs in the headspace above UHT in the presence and absence of a lipase and β-CD.

Chapter 5

Gas Chromatography

Effect of Lipase and β-CD on Volatile Fatty Acids in Goat Milk

Introduction

FFAs formed by lipase activity on triacylglycerol result in a rancid flavour termed hydrolytic rancidity. The traditional method used in dairy industry to determine the extent of hydrolytic rancidity is the acid value (Vazquez-Landaverde and others 2005), which depends on titration of the acyl groups of FFAs to a phenolphthalein end point. This method does not detect the alkyl chain profile of the FFAs causing off-flavour from hydrolytic rancidity. In the previous chapter it was suggested that β-CD accelerated lipase activity, and thus hydrolytic rancidity, yet the evidence from Gupta (2004) is that by trapping FFAs, β-CD reduced FFAs in the headspace above goat milk. It was therefore of interest to show what was happening in the headspace above lipase-treated goat milk. Gas chromatography is a rapid reliable and precise method to quantify fatty acids, and can be applied to analysis of FFAs in the headspace above foods. Thus the objective of this work was to use headspace sampling to establish the profile of FFAs from goat milk under four experimental conditions, lipase, no lipase, both with and without β-CD.

Methods

Preparation of treatments for GC analysis

UHT goat milk containing 3.4 % w/w fat was used as in previous experiments. There were four treatments. These were milk, milk plus 1% β-CD, milk plus enzyme, and milk plus 1% β-CD plus enzyme each replicated three times, making 12 preparations in all. The volume of each was 100 mL containing as required, 60 units of lipase from *Pseudomonas fluorescens* in phosphate buffer pH 8, and 1% β-CD. The analysis sequence was designed to avoid temporal problems. All 12 preparations were prepared at once and left for two hours at ambient temperature. Then all were transferred to a -80°C freezer and held for up to a week. Preparations were then sequentially and randomly thawed at room temperature prior to immediate trapping of volatile FFAs. Trapping was accomplished by bubbling piped laboratory air for two hours where the milk flask was immersed in a water bath at 60°C (Figure 20). The volatile matter was trapped in a U tube containing 1 g of glass beads about 2

mm in diameter placed in container of dry ice (Figures 20 and 21). Immediately after

bubbling, exactly 1 mL of chloroform containing heptadecanoic acid (500 ppm) and 4-

methyloctanoic acid (100 ppm) as an internal standard was added to the U tube that was

immediately sealed by the valves. After 15 min the lower organic phase was substantially

recovered with a glass Pasteur pipette and held in a glass vial sealed under a Teflon cap

awaiting triplicate injections for each of the three replicates within four treatments.
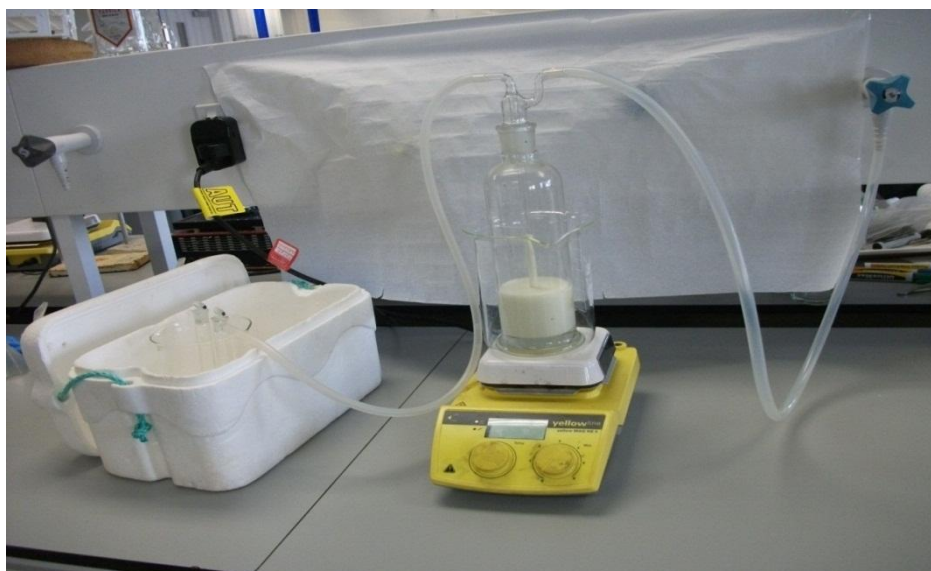

Figure 20   Purge and trap equipment used to trap FFAs in four milk
treatments. The glass bottle contains milk was immersed
in a beaker of water at the controlled temperature 60 ℃

Figure 21   The U tube used in the purge and trap equipment.
This tube could be sealed by valves as required. The
U tube contained in a beaker of dry ice which
hold in a foam container with dry ice

Chromatography conditions

The chromatograph was a Shimadzu GC-17 A with a flame ionized detector and fitted with a Varian-WCOT fused-silica column, with an internal diameter 0.25 mm, and 25 m long. The stationary phase (0.2 µm thick) was CP-WAX 58 (FFAP)-CB. This was column, resolves FFAs. The temperature program used was: injector port temperature 250°C, followed by a column hold at 100°C for 3 minutes, increasing to 180°C at the rate of 30°C degree min^{-1}, followed by a hold at 180°C for 10 min. All the FFAs of interest were detected with 20 min.

Determination of retention time for FFAs

Retention time for each FFA was determined as follows. Each FFA individually prepared at concentration about 100 to 200 ppm in chloroform and 1 µl of solution was injected to GC to establish retention time. Then 1 mL of each of solution was mixed and 1 µl of mixture was injected to GC to determine retention time for a mixture of FFAs.

Data analysis

Microsoft Excel was used for data analysis and calculation of standard deviations The relative areas of detector responses for each fatty acid were normalised to the internal standard heptadecanoic acid and the mean of each triplicate injection was calculated. Thus for each of the three replicates within treatment, there were three values. The treatment means and standard deviations were calculated from these three values. Means and standard deviation expressed as histograms.

Result and discussion
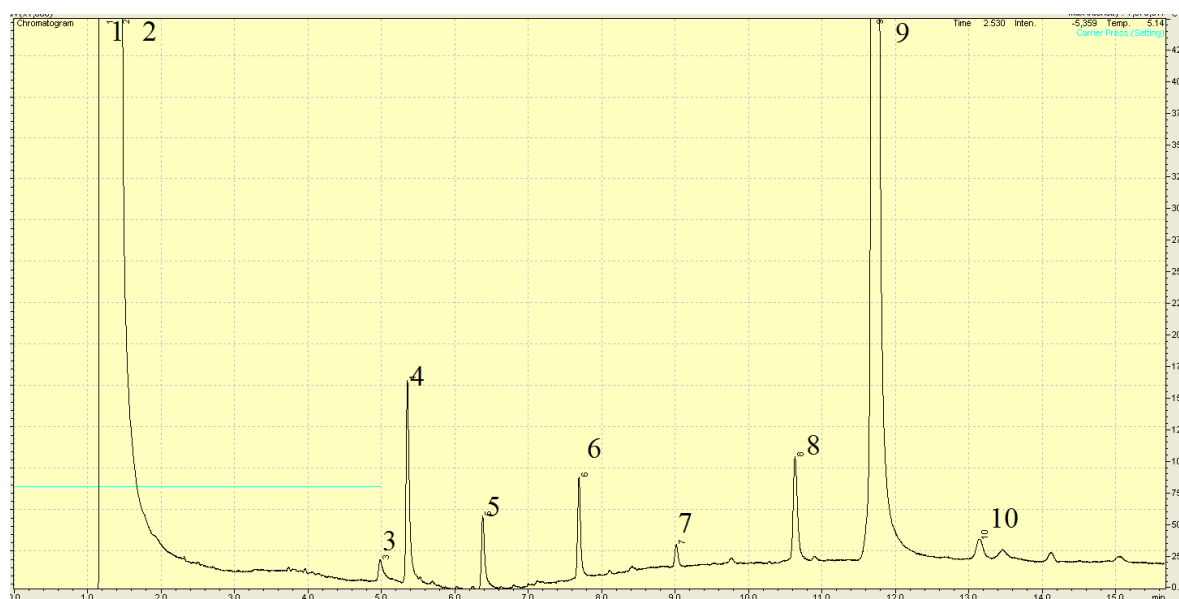
Figure 22   This histogram of detector response of FFAs in the headspace above milk at 60ºC treated with lipase. It shows free fatty acids range. (1) chloroform, (2) butyric acid, (3) hexanoic acid, (4) octanoic acid, (5) 4-methyloctanoic acid.(internal standard), (6)octanoic acid, (7) decanoic acid, (8) lauric acid, (9) palmitic acid, (10) heptadecanoic acid as another internal standard, and (11) stearic acid

The trapping experiment was done many times. The results were somewhat variable, but the general outcomes were the same. Figure 23 shows a summary of one of experiments where the treatments were triplicates.

Figure 23   Histogram comparing the effect of β-CD and lipase on UHT goat milk showing standard divisions between three replicates and nine injections

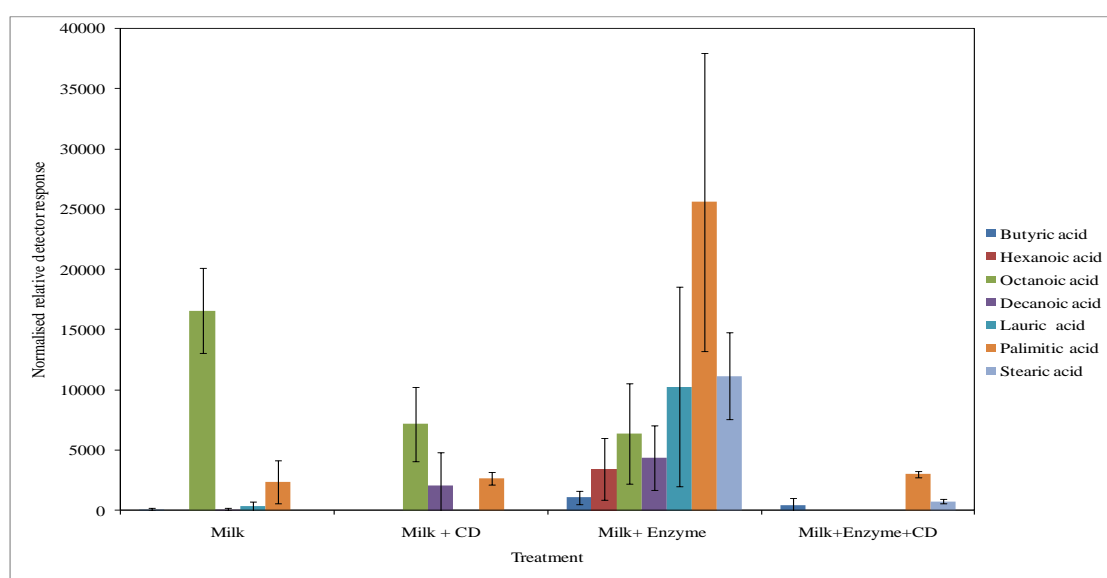The effects of four treatments are shown in Figure 23. Looking first at the situation where the volatile FFAs came from milk alone, there was a clear peak due to octanoic and palmitic acids, with only traces of the other fatty acids. Because the longer chain fatty acids are less volatile it is perhaps not surprising they are absent from the headspace. Thus they may be present in the milk, but would probably be partitioned into the fat globules or the aqueous phase. When β-CD was added the profile of FFAs changed with a clear trend. Because FFAs are trapped by CDs, it is possible that β-CD is promoted the hydrolysis of triacylglycerols, even in the absence of any enzyme. But more likely explanation is that addition of β-CD changes the pattern of partitioning of FFAs. In the absence of β-CD, the FFAs can partition into the aqueous phase of milk, the fat globules, and the headspace. β-CD may simply disturb the pattern of partitioning.

Addition of lipase to the goat milk resulted in a significant increase in concentration of all the saturated FFAs ranging from C4 to C18, except myristic (C14:0) (Figure 23). The approximate fatty acid composition of goat milk fat is shown in Table 14.

Table 14	Fatty acid profile of goat milk. Goat milk fat is composed of many fatty acids, but these eight compromise over 83% of total fatty acids in milk

Fatty acid	Proportion by weight (%)
C4:0	0.88
C6:0	5.35
C8:0	5.39
C10:0	14.43
C12:0	5.60
C14:0	11.15
C16:0	22.19
C18:1 cis	18.41

Data source was Strzałkowska and others (2009)

Comparison of the percentages in Table 14 and the profile shows a rough equivalence, with the notable exception of myristic acid. Assuming for the moment that lipase liberates FFAs is proportion to their overall concentration in triacylglycerols, it is odd that myristic is absent and that palmitic acid is so dominant. The latter is particularly surprising because palmitic is much less volatile than shorter chain fatty acids. It is possible therefore that the enzyme is more specific for some triacylglycerols than others, and it is well known, for cow milk at least that, fatty acids are not distributed randomly in triacylglycerols (Swaisgood 1996). But the absence of myristic acid remains unexplained.

In the treatment containing both lipase and β-CD, the concentration of all FFAs in the headspace was reduced, even though the copper salt method has shown that β-CD accelerated hydrolysis (Figure 16, 17, 18 and 19). Remarkably the treatment Milk + lipase + β-CD were more effective than Milk + β-CD at trapping FFAs. There are clearly some complexities in these treatments that have no obvious explanation.

To summarise, these results broadly explain why β-CD is so effective in reducing goaty flavour, as shown by and Meier (2001) and Gupta (2004). However, there are complexities that have not been explained in this experimental approach, notably why the Milk + β-CD treatment was less effective than the Milk + lipase + β-CD treatment.

The answer to some of this complexity may lie in the relative affinities of FFAs for CDs. Wilson and others (1997), Gadre and others (1997) and Meier and others (2001b) showed that affinity of FFAs for β-CD increased with alkyl chain length, but that work was limited to straight chain fatty acids. Goaty flavour is particularly characterised by the

branched chain fatty acids that were discussed in Chapter 2, and if these were preferentially bound by β-CD, it might explain why β-CD is so effective in reducing odour, when it clearly does not trap all FFAs in the treatment Milk + β-CD (Figure 23). The next chapter researches this possibility.

Chapter 6

Relative Affinity of FFAs for CDs

Introduction

Chapter 4 showed that β-CD was very effective in binding FFAs in milk to the point that it will promote hydrolysis of triacyglycerols through a mass action effect (Figures 16, 17, 18 and 19). However, even though hydrolysis is promoted, the β-CD complexation minimises FFAs in the headspace above milk (Figure 23), and this seems the likely reason that β-CD is so useful in minimising goaty flavour in milk and yoghurt (Gupta 2004).

As described in Chapter 2, goat milk fat contains BCFAs that are responsible for goaty flavour. Whereas these BCFAs have low odour thresholds (Brennand and others 1989a) of these BCFAs present in triacylglcerols at much lower concentrations than straight chain FFAs (Ha and Lindsay, 1991a). When fatty acids are in this form they are not available for sensing, but are when released as FFAs by an endogenous or exogenous lipase, they will be available for sensing. Assuming that the relative rate of producing straight chain FFAs and BCFAs from triacylglycerol by lipase is similar, and then the reason that β-CD is so effective at suppressing goat milk odour could be that BCFAs are preferentially bound by β-CD compared with their straight chain geometric isomers. For example, if 4-methylnonanoic were much better at complexation with β-CD than its geometric isomer, decanoic acid, this might explain why β-CD was so good at reducing goat milk flavour. This possibility was not explored by Gupta (2004) and neither does Figure 23 headspace provide any insights.

Thus, the final experiments in this thesis explore this hypothesis of preferential binding of BCFAs compared to straight chain FFAs and reducing the goaty note in goat milk and goat milk products.

The literature on the effect of cavity size of cyclodextrin in entrapment of free fatty acids is limited. One study by Meier and others (2001) suggested that there is a relation between cavity sizes and association constant of different fatty acids with cyclodextrin. In that colourimetric study, phenolphthalein was used as a competitive binder with CDs. Phenolphthalein does not absorb visible light when it is complexed with CDs, but absorbs light at 550 nm when free in solution. In this experimental approach the competing molecules are FFAs.

Development of displacement method

Materials

The fatty acids were analytical grades of octanoic, nonanoic and decanoic that were sourced from AUT's chemical collection or bought from Sigma Aldrich. These were 4-methyloctanoic (Sigma M-7400), and 4-methylnonanoic (Sigma 35, 740-5-K). The buffer used for all solutions was carbonate buffer at pH 10.5 containing 2% ethanol. This was made from 2.1 g of $NaHCO_3$, 170 mL 0.1 M NaOH and 20 mL of 99.9% ethanol made to 1 L with water.

Methods

In duplicate for each treatment, 0.5 mL of 400 µM phenolphthalein solution and 0.5 mL of 10 mM α-, β- and γ-CD solutions were placed in 10 mL glass tubes and incubated at ambient temperature for two hours. The phenolphthalein was variously decolourised due to complexation with the three different CDs. Four millilitres of FFA solutions spanning 0 to 30 mM in carbonate buffer were then added giving a final volume of 5 mL. The tubes were sealed and left overnight. Absorbances were measured at 550 nm in 1 cm plastic cuvettes.

Comparative affinity of straight and branched chain fatty acids with CDs

When the fatty acid concentration was zero, and CDs were omitted from the mixture, the mean absorbance due to phenolphthalein was 1.15 ± 0.002 (Figure 24). Inclusion of α-, β- and γ- cyclodextrins reduced the absorbances to means of 1.09 ± 0.01, 0.038 ± 0.00, and 0.40 ± 0.01 respectively. β-CD clearly binds the phenolphthalein most effectively and that in turn will reflect the dimensions of the van der Waals force field of the phenolphthalein and the CDs. α-CD is appears be too small to bind any phenolphthalein, and γ-CD appears to be too big to tightly bind phenolphthalein. What little change in absorbance was caused by α-CD may have been caused by β-CD for example as a contaminant of α-CD, or may be a real effect due to α-CD.
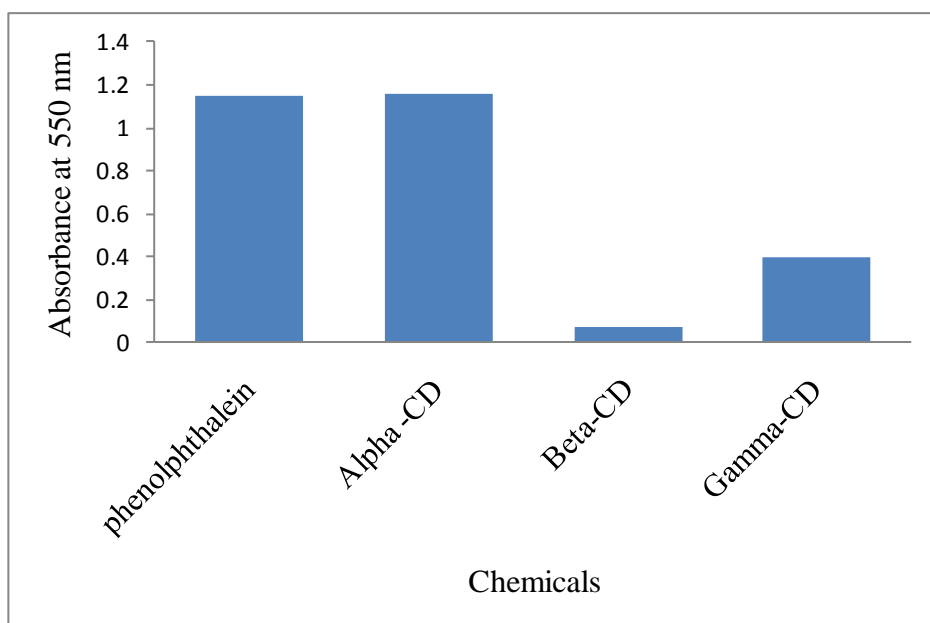
Figure 24   Absorbance for phenolphthalein in buffer and absorbance due to
                   phenolphthalein unbound by α-, β- and γ-CD

Figures 25, 26 and 27, shows the effect of different fatty acids on displacement of phenolphthalein by FFAs in the, α-, β-, and γ-CD cavities. Consider β-CD (Figure 32) where the final concentration of phenolphthalein bound to β-CD or free was 40 μM and the final concentration of fatty acid (soap) ranged between 0 and 24 mM, a 600 (24,000/40) molar excess at highest. As fatty acid concentrations increased, the phenolphthalein was progressively displaced from the cavity, and from these data it appears that the FFAs would never completely displace the phenolphthalein to generate an absorbance of 1.15 (Figure 24).
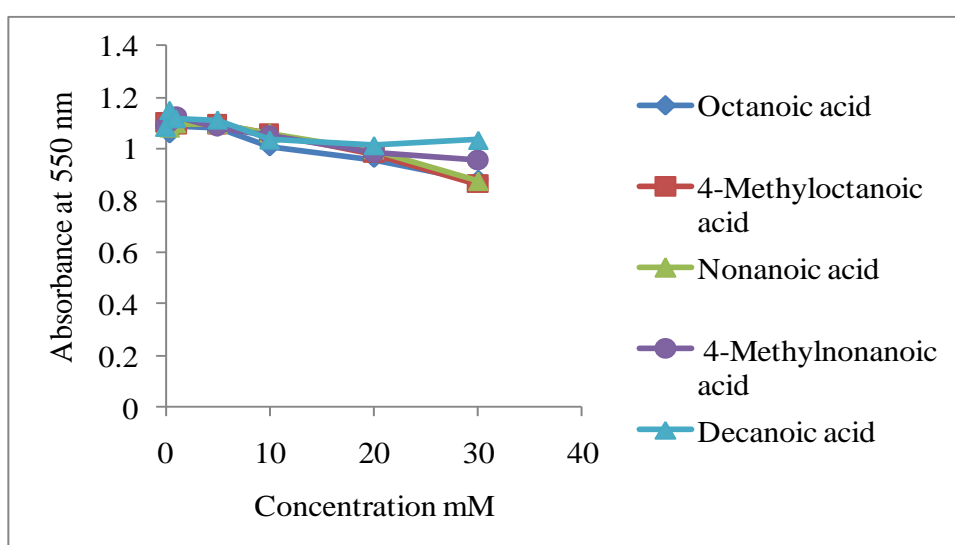

Figure 25   The effect of fatty acid type and concentration on the displacement of
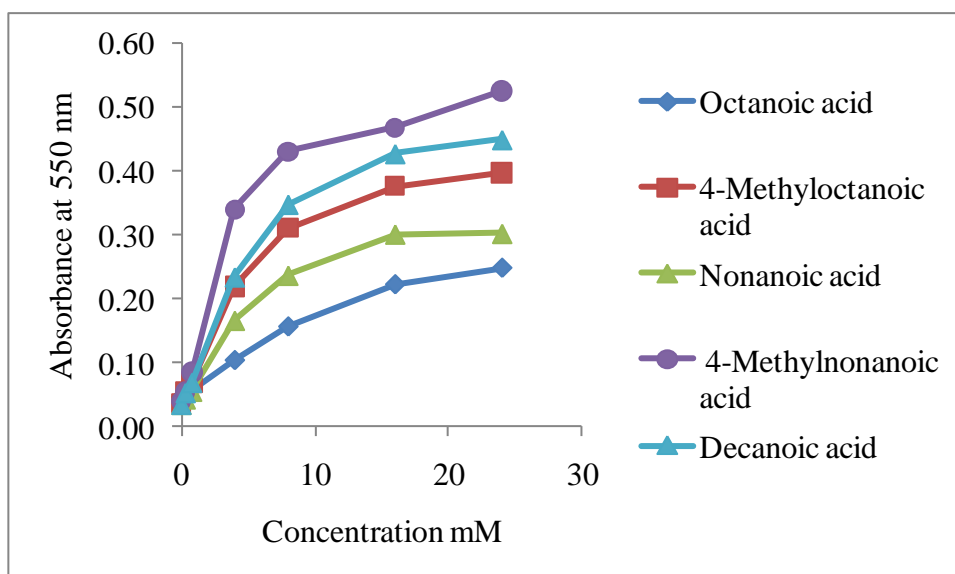   phenolphthalein in α-CD

Figure 26   The effect of fatty acid type and concentration on the displacement of phenolphthalein in β-CD
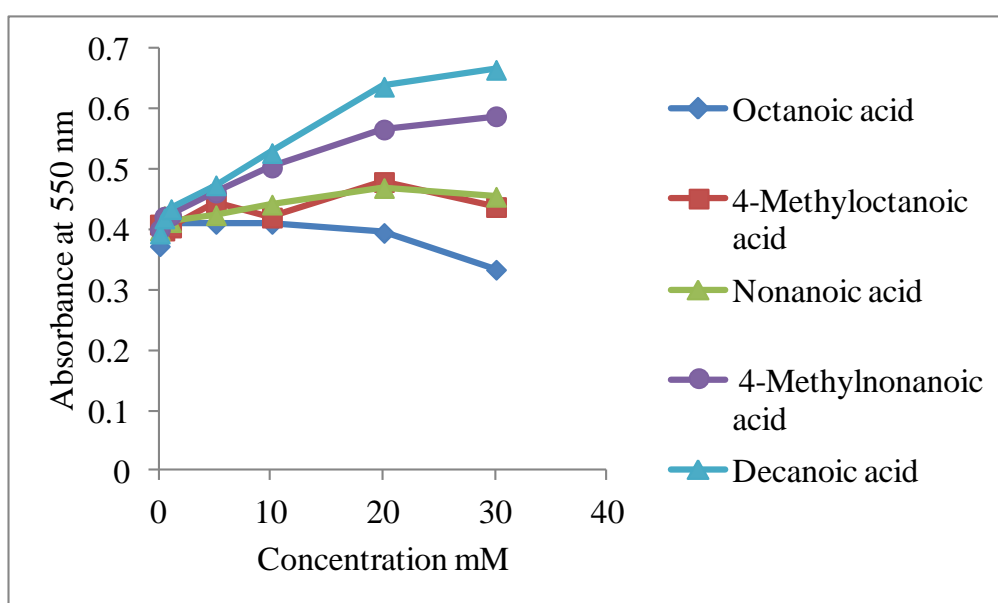
Figure 27   The effect of fatty acid type and concentration on the displacement of phenolphthalein in γ-CD

Among the straight chain fatty acids, affinity for β-CD increased with chain length because more phenolphthalein was displaced from the complex as chain length increased. This result was consistent with results obtained by Wilson and others (1997), Gadre and others (1997) and Meier and others (2001b).

The chain length rule apparently applies to BCFAs too, because more phenolphthalein was displaced from the complex as chain length increased from 4-methyloctanoic to 4-

methylnonanoic acid. Figure 26 also shows that phenolphthalein can be displaced more effectively by BCFAs compared with their straight chain isomers. Thus, 4-methylnonanoic acid was more effective than decanoic acid, and 4-methyloctanoic was more effective than nonanoic acid. However, the effect was minor.

In the absence of fatty acids, the absorbance of phenolphthalein in the presence or absence of α-CD was 1.15 (Figures 24 and 25). However, there was some effect of fatty acids. The pattern of what displacement – if that is what is occurring – was the same for γ-CD as for β-CD, although there was an unexplained fall in absorbance due to higher concentrations of the shorter fatty acids. This fall suggests some negative cooperative effect between fatty acids, and phenolphthalein. It may also occur in the case of β-CD, but not visible in the concentration range chosen. The explanation for this phenomenon, which clearly occurs with γ-CD and α-CD (see next), is beyond the scope of this project.

Data in several tables and figures suggest why the BCFAs are better bound than the straight chain isomers. First consider phenolphthalein (Figure 25).
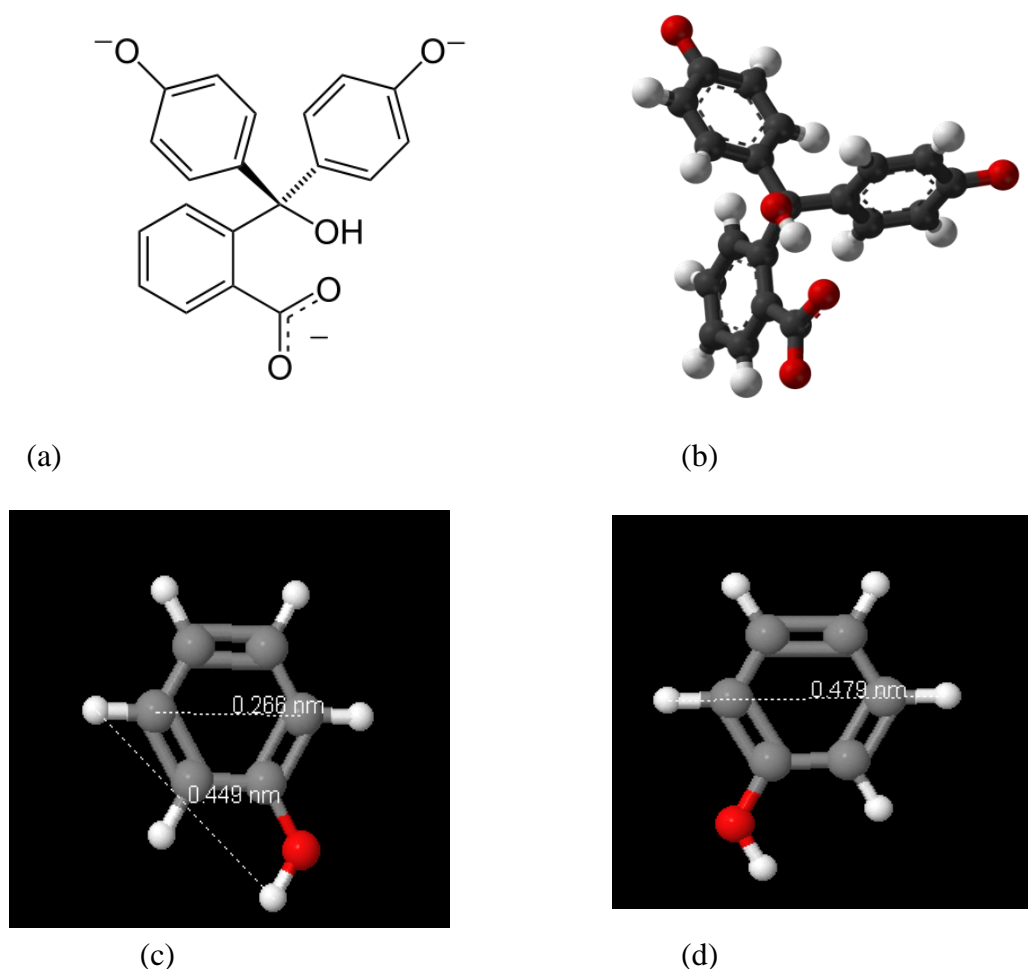

(a)

(b)

(c)

(d)

Figure 28 Dimensions of phenol ring (c and d) measured from
http://www.thegoodscentscompany.com/data/rw1009361.html

The molecule has three aromatic rings that result in at least three ways in which phenolphthalein can interact with CDs. Moreover, the width of the benzene ring from H to H is about 0.48 nm (Figure 28 d), and would fit comfortably in the β-CD cavity but not the α-CD and γ-CD cavities (Table 15) and Figures 26 and 27. Oddly, no comparable image of β-CD could be found in a thorough internet search. It is noted that published dimensions of CDs in Table 15 do not make the start and end points clear, so the dimensions measured here for CDs do not exactly match the dimensions in Table 15.

Table 15   Critical dimensions of CDs

Chemical	Diameter of CD cavity (nm)	Length of CD cylinder (nm)
α-Cyclodextrin	0.47 to 0.50	0.79
β-Cyclodextrin	0.60 to 0.65	0.79
γ-Cyclodextrin	0.75 to 0.80	0.79

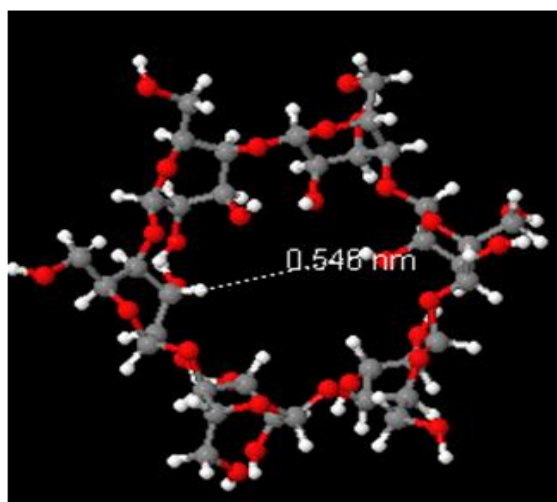Data are from Szejtli (1985) and Connors and Burnette (1997)

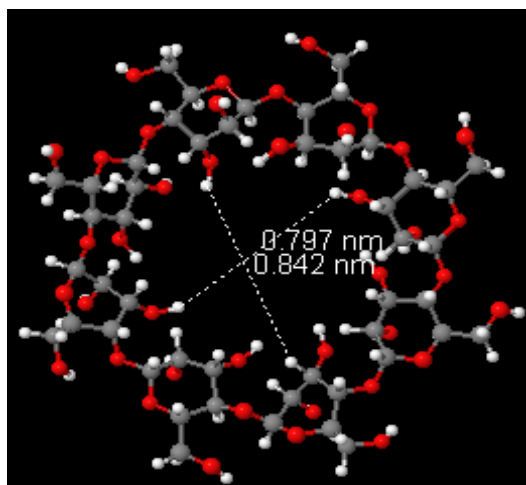Figure 29   Internal diameter the α-CD molecule from http://www.thegoodscentscompany.com/data/rw1607711.html

Figure 30   Internal diameter the γ-CD molecule from
             http://www.thegoodscentscompany.com/d
             ata/rw1041811.html

Table 16 shows that the fatty acids vary in their alkyl chain length as is obvious from the molecular weights, and suggests why longer chains displace phenolphthalein better than shorter chains. There are more ways that the longer chains can be accommodated. Based on the measurement of 0.269 nm shown in Figure 28, the approximate diameter of the alkyl chain calculated by triangulation is 0.24 nm and explains why fatty acids are unlikely to fully displace the better fitting phenolphthalein (0.48 nm, Figure 25) in the clear case of β-CD (Figure 32).

Table 16   Approximate dimension of 5 types of FFAs used in the study of
            affinity of CDs with FFAs

	Diameter of alkyl chain (nm)	Approximate length of alkyl chain (nm)
Octanoic (Fig. 25)	0.24	0.65
Nonanoic	0.24	0.80
Decanoic	0.24	0.87
4-Methyloctanoic	0.48	0.63
4-Menonanoic	0.47	0.91

Data are from measurements made using the facility in
http://www.thegoodscentscompany.com
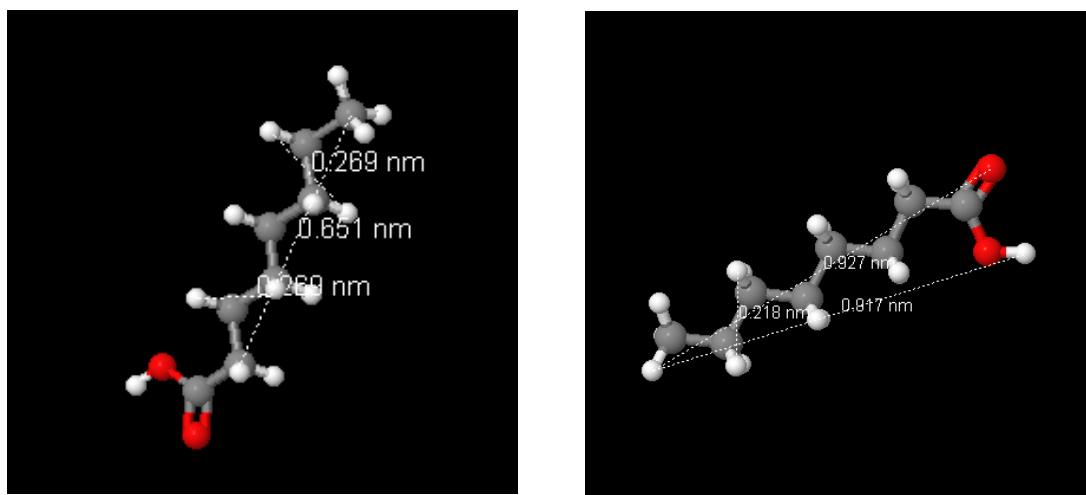
Figure 31   Some dimensions of octanoic acid from
            http://www.thegoodscentscompany.com/opl/124-07-2.html

Turning now to the three dimensional images of BCFAs, the various dimensions shown in Figures 26 and 27 suggest there are more way that BCFAs to fit in the cavity, and moreover match the cavity size of β-CD. Thus a V-shaped molecule might be inserted to the point that the top or bottom of the cavity is fully occupied.

Figure 32   Some dimensions of 4-methyloctanoic acid molecule from
            http://www.thegoodscentscompany.com/data/rw1036901.html
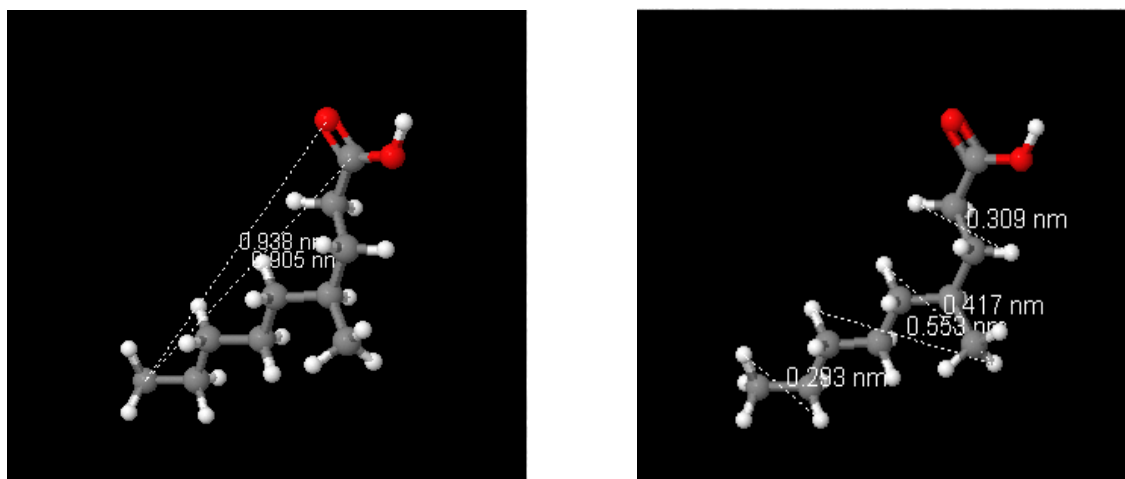
Figure 33   Some dimensions of 4-methylnonanoic acid from
http://www.thegoodscentscompany.com/data/rw1036891.html

Summary

The question posed for this chapter was: are branched fatty acids significantly better at complexation with β-CD than their straight geometric isomers? The answer is that they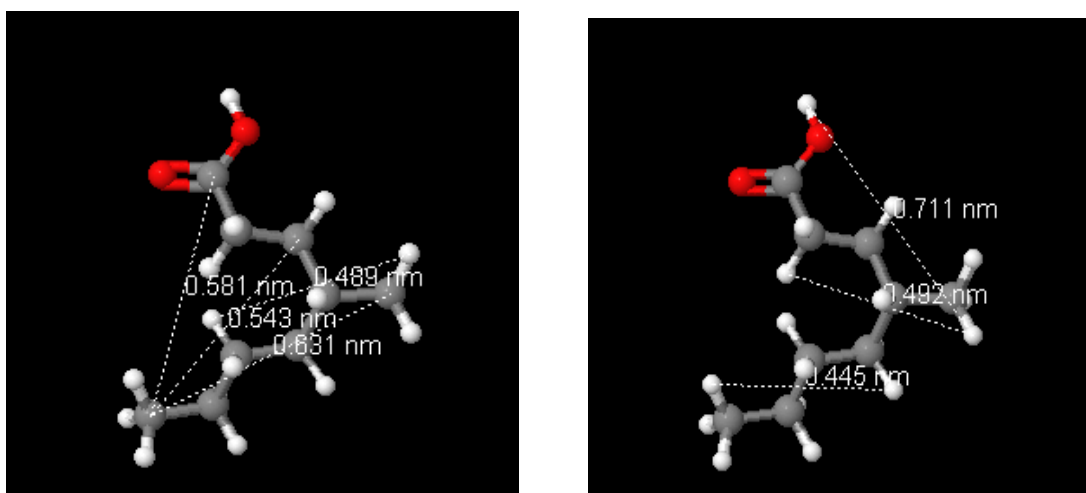 are better but the differences are small. Thus the ability of CDs, β-CD in particular, to reduce goat milk flavour due to fatty acids appears to lie in their ability to bind any free fatty acids, characterising one included, and so reduce the flavour.

The development of product-specific goat dairy will depend on a range of factors such as the availability, cost and consumer acceptance. If any of these factors prohibit on-farm specialisation, this approach of harvesting from bulk milk may prove to be the method of choice used by an industry that deals with very large volumes of milk.

Chapter 7

Conclusion

The commercial value of goat milk may be enhanced, especially for higher value milk products, if its goaty flavour can be reduced to an unobjectionable level. In goat milk and goat milk products, the characterising odorous compounds are short straight chain fatty acids and particularly their branched chain isomers, the BCFAs. Meier and others (2001) and Gupta (2004) showed that CDs as an additive in goat milk and its yoghurt was effective in reducing goaty odour, presumably by trapping these fatty acids and making them unavailable for sensing. The main topic of this research was to understand at the molecular level how CDs can be so effective in reducing goaty flavour. β-CD was chosen as the main CD for study on the basis of the positive experience of other researchers working in same area and on its relatively low price.

The goat milk chosen for research here, shelf-stable UHT goat milk, did not display a marked goat flavour so it was enhanced by addition of lipase. The problem with this approach was that once added there was no way of stopping its activity, and there was no control over what fatty acids were liberated.   However, some results were obtained that made chemical sense. Increasing concentration of FFAs due to lipase activity were enhanced by the addition of β-CD.   This was presumably a mass action effect where the β-CD acted as a chemical sink. This led to the question: if β-CD accelerates lipolysis, does this enhance goaty flavour, or does the β-CD effectively trap the liberated fatty acids that cause this flavour?

This was answered by analysis of the volatile and therefore free fatty acids in the headspace above goat milk that was treated with lipase in the presence and absence of β-CD. The effect of the lipase was marked, but simultaneous addition of β-CD led to a huge reduction in volatile fatty acids, remarkably more so than when compared to milk plus β-CD. Thus, β-CD effectively traps all the liberated fatty acids that cause goaty flavour. Of these fatty acids, the BCFAs are believed to be mostly responsible. It was possible that the effectiveness of β-CD demonstrated by Gupta (2004) might be due to a higher affinity of BCFAs than their straight chain isomers for β-CD. This was testable by displacement experiments with phenolphthalein. These first showed that β-CD was much more effective in binding fatty acids than α- and γ-CD, and this was traced to molecular dimensions of the fatty acid alkyl chain and the cavity size to the three CDS. α-CD was too small and γ-CD was too

big. Second, the displacement experiments showed that the BCFAs had greater affinity for β-CD than their straight chain isomers. This again might be explained by molecular dimensions, but also by the greater degree of freedom that BCFAs offered to the hydrophobic cavity of the β-CD. Being branched there were more ways the BCFAs could bind. Thus if a straight chain fatty acid were to approach the cavity of the CD in an orthogonal orientation – rather than inserting parallel to the CD's central axis – it would fail to bind.   In contrast, a BCFA in that situation could have its branch chain oriented to insert parallel to the CD's central axis and thus bind. However, the difference in affinity between two BCFAs and their straight chain isomers was not great, and it seems likely that the effectiveness of β-CD in minimizing goat flavour is due to its ability to comprehensively bind FFAs, and that is reasonably clear from the headspace experiment.

β-CD can be legally added to goat milk and its yoghurt in many countries, and Gupta (2004) showed how the mandatory pasteurization step for milk can be used to advantage in the preparation of goaty-flavour-reduced milk or yoghurt. The present study has added new information as the mechanism of flavour reduction. The remaining gap in knowledge is the long-term effectiveness of CD treatment.   That should be the focus of new research.

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Abbreviations

Cyclodextrin	CD
Free fatty acids	FFAs
Branched chain fatty acids	BCFAs
Generally Recognised As Safe	GRAS
Food and Agriculture Organisation	FAO