Universität für Bodenkultur Wien

Masterarbeit

Detection of silage feeding by fatty acid profile analysis in consumer milk

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Olschewski Isabel-Luisa

Angestrebter akademischer Grad:

Diplom Ingenieur

Masterstudium: Safety in the Food Chain/ Food Science and Plant Biotechnology

Betreuer: Priv. Doz. Dr. Matthias Schreiner

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Acknowledgement

First of all, we want to thank Matthias Schreiner, Priv.-Doz. Dr., who gave us the opportunity for this research and he was always supportive for advice and explanations. Also we want to thank Claudia Laguna Paredes (Ph.D. student), who provided samples and supported us with data analysis and advice. After all, thanks to the whole “Institut für Lebensmittelwissenschaften und –technologie”, especially Meltem, Nicole and Iris.

We want to thank our families, friends and people who supported us.

Statutory declaration

Wir erklären an Eides statt, dass wir die vorliegende Masterarbeit selbstständig und ohne fremde Hilfe verfasst, andere als die angegebenen Quellen und Hilfsmittel nicht benutzt bzw. die wörtlich oder sinngemäß entnommenen Stellen als solche kenntlich gemacht haben.

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Abstract

There is a growing demand for high-quality dairy products. Dairy production systems vary in the way of feeding and livestock-keeping regimens. They are on the one hand quality parameters and on the other hand important topics for consumer perceptions. Animal feed represents one of the major impacts on the fatty acid (FA) profile in consumer milk. In respect to this, the aim of the present thesis was the research on analytical differentiation of consumers' cow milk according to their feeding system of origin by the FA spectra. Simultaneously, this study compares the FA pattern of retail consumer milk (n= 84) from different feeding systems. Fourty-five samples of non-silage milk, declared as hay milk and 39 samples of conventional milk CON (silage) were analysed across seasons in 2015. The emphasis of this comparison was on the minor, long chain polyunsaturated FAs. Additionally, a differentiation of seasons (4 quarters= Q1-Q4) was done to see if and how they affect the cows feed and the resulting milk FAs. A distinctive differentiation and identification of FAMEs was possible with previous concentration steps, gas chromatography (GC) together with mass spectrometry (MS) and a flame ionisation detector (FID). A seasonal difference between winter and summer feeding is evident in both groups. In HAY- milk, the values of each FA are always higher compared to the conventional samples, during the summer months. The polyunsaturated FAs as well as the omega-3 FA concentrations are higher in HAY than in conventionally produced milk, especially during summer.

Die Nachfrage nach qualitativ hochwertigen Milchprodukten steigt kontinuierlich. Das Herstellungsverfahren von Milch und Milchprodukten variiert nach Art der Fütterungsstrategie sowie Viehhaltungsregime. Es sind einerseits Qualitätsparameter und andrerseits ein wichtiges Thema der Verbraucherwahrnehmung. Den größten Einfluss auf das Fettsäureprofil von Konsummilch hat das Tierfutter. Das Ziel der vorliegenden Arbeit war die Erforschung analytischer Differenzierungsmöglichkeiten von Konsummilch anhand des Fettsäurespektrums, bezogen auf die jeweilige Fütterungsstrategie. Gleichzeitig vergleicht diese Studie das Fettsäuremuster von Konsummilch (n=84) verschiedener Qualitäten. Es wurden Proben von Silage- freier (HAY, n = 45), welche als „Heumilch" deklariert waren, und Silage-Milch (CON, n = 39) im Laufe des Jahres (2015) analysiert. Der Schwerpunkt dieser Untersuchung lag dabei auf den minoren, langkettigen und mehrfach ungesättigten Fettsäuren. Darüber hinaus wurden, um einen Einfluss der Jahreszeiten auf das Futter und das Fettsäurespektrum zu sehen, zudem je 10 Proben pro Jahreszeit (4 Quartale= Q1-Q4) gezogen. Mit Hilfe von Aufkonzentrierungsmethoden, Gas- Chromatographie sowie

Massenspektroskopie war es möglich, die Fettsäuremethylester zu differenzieren und identifizieren. Ein saisonaler Unterschied zwischen Winter und Sommer Fütterung ist in beiden Gruppen zu finden. Während den Sommermonaten zeigte Heumilch jeweils höhere Werte im Fettsäureprofil als die konventionellen Proben. Die Konzentrationen an mehrfach ungesättigten Fettsäuren sowie der omega-3- Fettsäuren sind in Heumilch höher als bei konventionell erzeugter Milch, besonders während des Sommers.

Table of contents

Table of figures

List of tables

List of abbreviations

Acc.	according
Approx.	approximately
BCFA	Branched chain fatty acid(s)
BHT	Butylated hydroxytoluene
CLA	Conjugated linoleic acid(s)
CON	Conventional
DM	Dry matter
ESL	Extended Shelf Life
F-FA	Furan fatty acid(s)
FA	Fatty acid(s)
FAME	Fatty acid methyl ester
FTIR	Fourier transform infrared spectroscopy
GC	Gas Chromatography
IR	Infrared spectroscopy
LCPUFA	Long-chain polyunsaturated fatty acid(s)
MTBE	Methyl-*tert*-butylether
MUFA	Monounsaturated fatty acid(s)
n-3	Omega-3
n-6	Omega-6
PA	Pristanic acid(s)
PHY	Phytanic acid
PL	Phospholipid(s)
PUFA	Polyunsaturated fatty acid(s)
rpm	Revolutions per minute
SFA	Saturated fatty acid(s)
TG	Triacylglycerols/ Triglycerides
TLC	Thin-layer chromatography
tVA	Trans-vaccenic acid
v/v	Volume concentration

List of fatty acids

Trivial name	Abbreviation	Lipid name
Stearic acid		C18:0
Oleic acid		C18:1n9
Linoleic acid	LA	C18:2n6
Rumenic acid	CLA	C18:2(9c11t)
Alpha- linolenic acid	ALA	C18:3n3
Arachidic acid		C20:0
Eicosenoic acid		C20:1n9
Eicosatetrienoic acid	ETE	C20:3n3
Eicosapentaenoic acid	EPA	C20:4n3
Arachidonic acid	AA	C20:4n6
Eicosatetraenoic acid	ETA	C20:5n3
Behenic acid		C22:0
Erucic acid		C22:1n9
Docosapentaenoic acid	DPA	C22:5n3
Docohexaenoic acid	DHA	C22:6n3
Lignocerid acid		C24:0

Abbreviation author

KJ Keplinger Julia

OI Olschewski Isabel

1 Introduction | OI

About 12000 years ago humans started to integrate milk originating from animals in their diet. The main purpose of dietary milk is supplying human nutrition with its macro- and micronutrients and the contribution to satiety. For a long time, the rapid spoilage of milk, caused by microorganisms, was the main issue concerning the safety of milk-products. Through new inventions in methods which extend the shelf life and packaging, the spoilage of milk- products is not a real aspect today. Nowadays, milk is one of the most popular nourishments and thus various product variations, such as yoghurt, cheese and cream cheese, are available on the market. Over the years, the concerns of the consumer towards the amount of fat in foods led to a shift in the image of milk products. When buying food, the health value of food has become one of the most important points. The urge of rebranding milk with a healthy image aroused in order to serve recent consumer requirements. Milk fat shows variation in its content and composition, thus it is the component which agriculture or the food industry is able to alter to a certain extent (BMELV, 2010). In addition, consumers have become more aware and sensitive about the milk origin and conditions of production. Factors like environmental footprint, organic, healthiness and also animal welfare represent the main apprehensions consumers consider today (Mott, 2001). Diaries in Austria serve upon these desires and created a new niche: hay milk products. This type of milk is produced under strict conditions and requires the authorization of a special label (by ARGE). Furthermore, it is sold at a higher price with the help of a tailored marketing strategy (ARGE, 2015). Currently the myth remains about hay milk being healthier than conventionally produced milk and that the difference results in benefits for consumers' health. What is the potential of hay milk and are there measurable differences compared to regular, conventional (CON) cow milk?

With regards to this, the following thesis focuses on the differentiation of consumer milk, originating from different production systems, through the analysis of milk fatty acids (FA). Although many milk FAs are already identified, some still remain unknown. Only little data is available for minor, long-chain polyunsaturated fatty acids (LCPUFA). Polyunsaturated fatty acids (PUFA) are considered as beneficial for health, particularly when saturated dietary FAs are replaced. This study emphasises on the detection of LCPUFAs, their alteration by cow feed and their potential influential factors. The results intent to contribute to the knowledge about potential differences in the FA- spectrum between HAY and CON milk depending on the different seasons throughout the year.

2 Theoretical background

2.1 Milk

2.1.1 Definition and composition | OI

Milk is, per definition, the “milking of the cow or many cows” and is commonly known as cow’s milk. Milk from other mammals is termed with the name of the origin, such as “mare milk; goat milk”.

Milk is a white to yellowish oil-in-water emulsion. Milk is composed of water, proteins and carbohydrates, as well as of vitamins, minerals and trace elements, as demonstrated in Figure 1. The milk fat is secreted as globules, which have a diameter of around 3 µm. The core of these globules consists mainly of triglycerides (TG), the membrane of monoglycerides, sphingolipids and stearins. Together with proteins they operate as emulgators (Bösze, 2008).

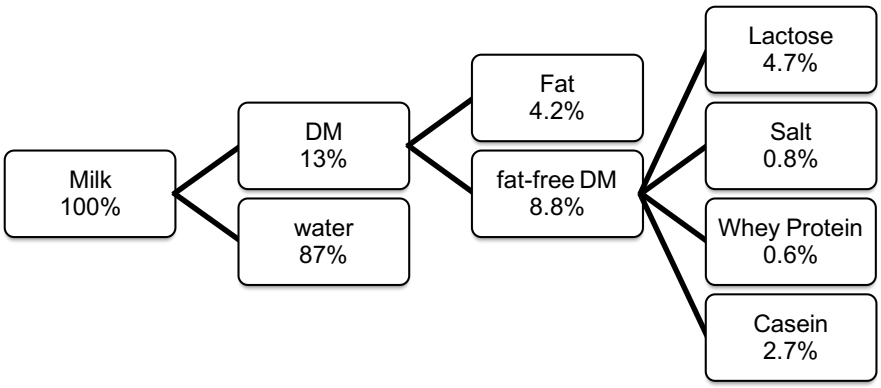

Figure 1: Average composition of cow milk (data from Eckard Schlimme, 1995)

Humans consume milk and milk products of many species, e.g. sheep and goat, but cow milk is the most important one from an economic perspective. Usually, milk is supposed to be the first and only nourishment for a new-born. This „first milk “, also called the colostrum, is essential for the newborn, because it provides all important nutrients and antibodies.

The composition of milk depends on health status and lactation stage, as well as on the composition of feed and genetics. In terms of nutrients, the protein and FA contents are of

special interest and influence the quality of the milk. Studies have already shown that the composition can be changed by altering the diet/ feed and breeding.

2.1.2 Extended shelf life milk | OI

About twenty years ago, extended shelf life- milk came on the market, aiming for a longer shelf-life containing only a minimal loss of off-taste. Per definition, the term “Länger frische” or extended shelf life- milk (ESL) is used for milk, which has an extended durability of about twenty days (unopened and stored at low temperatures at about 5 °C) with a taste like fresh milk. An exact, accepted definition of the term “ESL” and the production methods are not yet defined by legislation by the European Union (Mayer *et al.,* 2009). “Fresh milk” is pasteurised raw milk. Various methods are used for the manufacture of ESL-milk. The extended shelf life is either reached through indirect or direct heat treatment or by the combination of upstream membrane/ depth filtration followed by a subsequent heat treatment (De Vrese, 2010). A newer method represents the bactofugation (Buckenhüskes, 2014). The difference between pasteurisation (15-30 sec at 72-75 °C) and the thermal treatment for ESL-milk is, that for ESL-milk higher temperatures (125-127 °C) for a shorter time (2-4 sec) are used. Like pasteurized milk, ESL milk must be kept before and after opening at low temperatures (around 5 °C). Table 1 shows the currently used production methods of ESL-milk.

Table 1: Technological properties of ESL- milk production (data from Strahm, 2010)

	Indirect heating	**Direct heating**	**Micro-and depth filtration/ past.**	**Bacteria removal/ pasteurisation**
Temperature [°C]	125	127	74	74
Time [sec]	2	3	20	20
			Separation into cream and skimmed milk; cream is heated to 127 °C; skimmed milk is passed through filter	Two bacteria removal separators bevor skimming separator
Peroxidase	-	-	+	+

In former times, the consumer was able to differentiate between “fresh”- respectively pasteurised milk and high-temperature treated milk declaration on the milk carton. These treatments can be distinguished by enzymatic testing, the peroxidase test. Pasteurized milk results in a positive- test, meaning the enzyme is still active. High- temperature (ESL) (and ultra-high temperature) treatments show a negative reaction to the enzyme. Contrary, as seen in Table 1, some ESL production methods result in a positive test result. At this time, both treatments are labelled as pasteurised and fresh milk. Since 2009, a distinction between “fresh”-pasteurised and ESL- milk can be made. Classical fresh milk is labelled with “traditionell hergestellt” and ESL- milk is labelled with “länger haltbar/ frisch”. However, within this regulation, the applied production procedure of ESL-milk is not labelled. This lack of the regulation leads to a confusion of the consumer and inadequate information, because only the high- temperature treatment leads to minimal a loss of taste. This could cause a loss of appreciation of the gentle production methods.

Other milk qualities, like the ultra-high temperature- milk (UHT) uses even higher temperatures for three to four seconds. Depending on the applied treatment, the milk has a divergent shelf life. Pasteurised milk has a keeping quality of about one week and UHT milk can be kept at room temperature for several months. The longest period of storage, with up to one year, is reached with sterilised milk. Here, milk gets heated for ten to 30 min at 110 to 120 °C. Regardless which procedure of heat treatment is used, after the milk arrives at the diary, the overall aim is to reduce the microbial population and to minimize chemical, enzymatic and physical changes. The microorganisms are responsible of the spoilage of milk and dairy products, because they are able to produce heat-stable proteinases and lipases (Molina *et al.,* 2009). However, thermal treatments furthermore can affect the nutritional quality of milk. These effects include, vitamin destruction, changes in taste (cooked, off- flavour), hydrolysis of proteins and lipids. Only little research exists on the effect on the FA profile of cow milk. Costa *et al.*, found no significant alterations in the FAs through heat treatment, only successive treatments reduced the FA contents (Costa *et al*.,2011) Also other authors did not find any changes in the FA profile through heat treatments and state that other factors are more relevant for the changes in the FA spectra (Pestana *et al.,* 2015).

2.1.3 Milk production in Austria | OI

Milk production in Austria contributes to a large extent to the income of Austrian farmers. In 2015, more than 3 million tons of milk has been produced, (see Figure 2; Figure 3), and the trend is still on the rise. Comparing the milk- production of May 2015 and May 2016, the results shows almost 20.000 tons more milk.

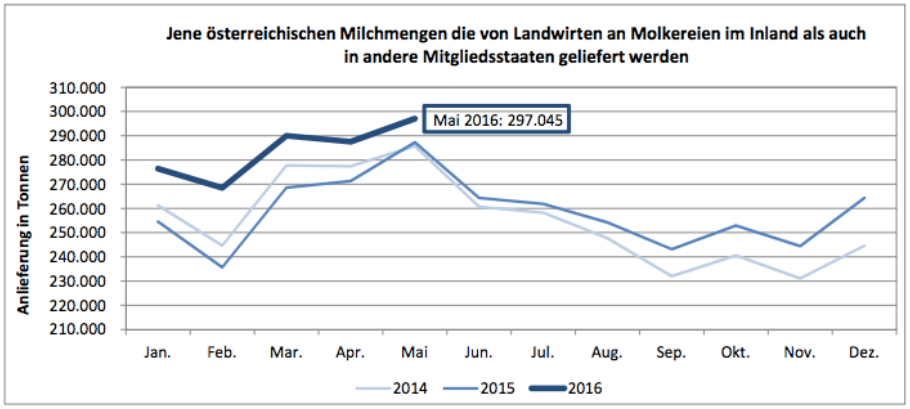

Figure 2: Milk supply in Austria (https://www.ama.at/getattachment/7265c403-a0f8-4cc2-acfb-138e20ec95e0/05_Marktbericht_Milch_Milchprodukte_05_2016.pdf)

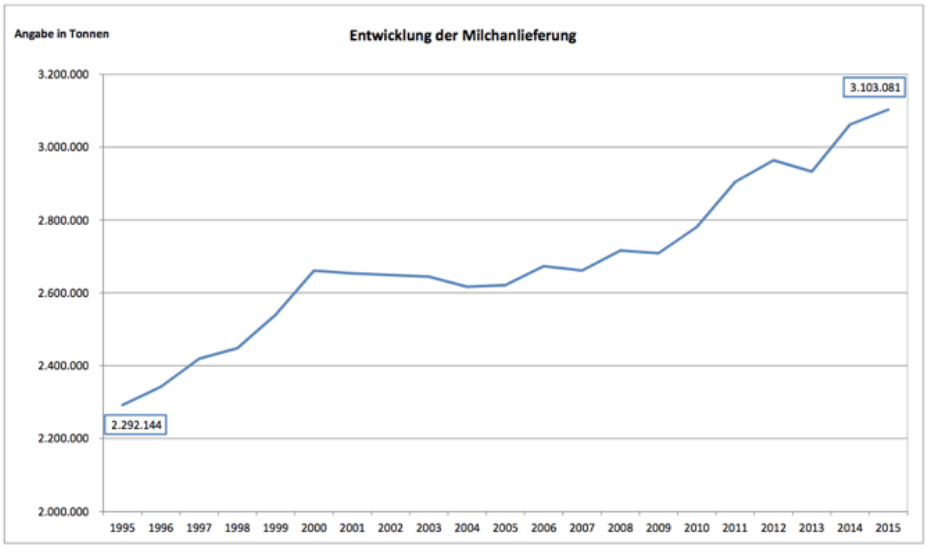

Figure 3: Development of milk supply in Austria 1995-2015 (https://www.ama.at/getattachment/3265d00f-c705-4a3f-9add-a7e84be5854a/170_Entwicklung-der-Milchanlieferung_1995-2015.pdf)

Comparing the single provinces of Austria, most of the milk is produced in Upper- (33 %) and Lower Austria (including Vienna, 18 %). Furthermore, the biggest amount of milk- cows is kept in those areas.

Over the course of the past years, the sector of milk and milk products has expanded. The consumer is not only able to select between whole milk and low- fat milk; they are further able to choose, which type of farming system they intent to support with their products. Nowadays, the diary sector in Austria undergoes a real boom in terms of demand in hay milk and the production is still rising. By this time, hay milk represents 11 % of the total milk production in Austria. Nearly every supermarket offers hay milk products (ARGE, 2016).

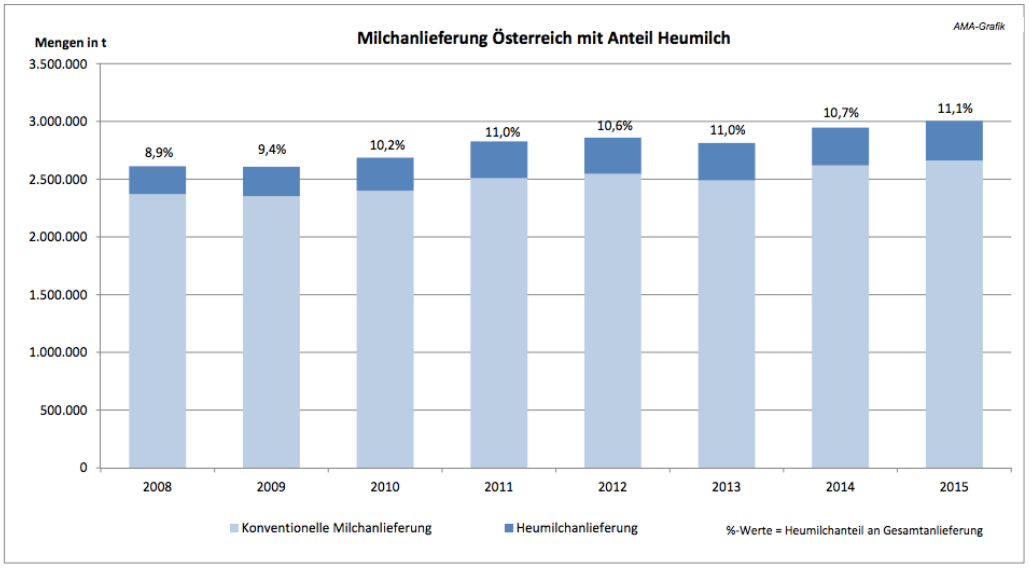

Figure 4: Milk supply in Austria, April 2016 (https://www.ama.at/getattachment/0ba4bb78-ae99-4728-9238-db7a7b38a6d1/181_Bio_Heumilchanlieferung-mit-zuschlag_1998-2015.pdf)

2.1.4 Brief overview of the origin of the fatty acids in milk | OI

Milk fat is a major component of milk and has an impact on energy and manufacturing properties to milk and milk products. Raw cow milk contains about 3-5 % fat. Until today, more than 400 different FAs have been detected in milk, but many still need to be identified (Jensen, 2002).

Milk fat consists mainly of FAs which are bound to glycerides as triglycerides (98 %). These FAs can be classified due to their chain length and their degree of (un-) saturation. The main FAs have a straight chain and an even number of carbons. Odd chain and branched-chain FAs (BCFA) occur only in trace amounts (Bösze, 2008). The remaining content consists of diglycerides, phospholipids, monoglycerides, free FAs, cholesterol and fat soluble substances.

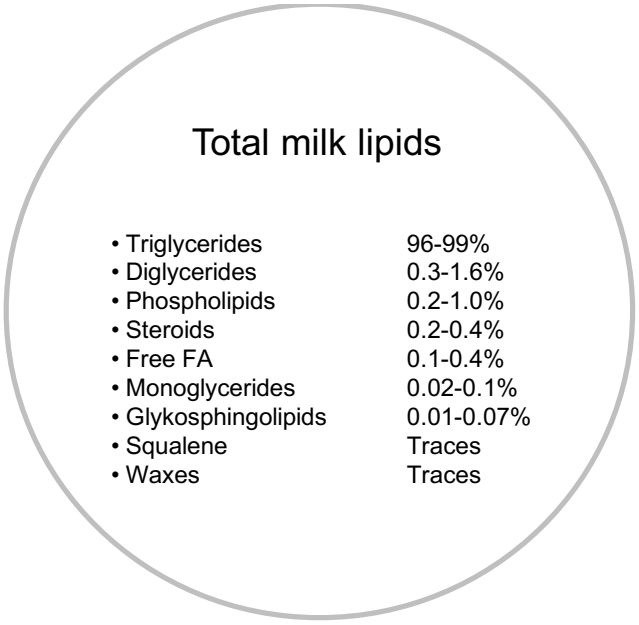

Figure 5: Composition of the total milk lipids (data from Eckhard Schlimme, 1995)

From the around 400 detected FAs the major part are minor components (“minor FAs”) and about 15 FAs occur in a higher share than 0.5 % considering the total FA content in milk. These FAs can be described as the “main FAs” cause over all they represent 95 % of milk FAs, see Table 2.

Table 2: Composition of FAs in bovine milk fat, quantitative categorization in main and minor FAs

main FAs (~15 FAs that contribute ~95% of total milk FAs)	**minor FAs** (~415 FAs that contribute ~5% of total milk FAs)
n-alkanoic acids, even-numbered C (C4:0-C18:0)	n-alkanoic acids, even and odd-numbered FAs (C2:0-C28:0)
n-alkanoic acids, odd-numbered C (C15:0, C17:0)	Monomethyl-branched FAs (C11-C28)
n-mono-alkanoic acids (C14:1, C16:1, C18:1)	Poly-branched FAs (C16-C26, altogether 115 isomers)
n-polyene-alkenoic acids (C18:2, C18:3)	Mono-, di-, polyene-, keto- and hydroxy- FAs

The composition of the FAs in milk is influenced by many specific intrinsic and extrinsic factors. These factors include genetic variations (breeding, race), feed composition, physiological conditions (lactation stage) and influences of the season and feeding system (Tschager and Dillinger, 2001).

Most of the FAs incorporated in milk are influenced by the cow's diet. Forages are one main source of FAs in ruminant diets and contribute in two ways. Firstly, microorganisms ferment roughage to acetate and butyrate, which are the precursors for the *de novo* synthesis (Mansbridge and Blake, 1997). Secondly, LCPUFAs cannot be synthesised *de novo* and their precursors (LA and ALA) have to be included in the diet (Palladino *et al.,* 2009; Elgersma *et al.,* 2006). In fresh grass, 75 % of the fat consists of C18:3 (Elgersma *et al.,* 2003). In maize, soybeans and sunflower C18:2 dominate (Elgersma *et al.,* 2006). The levels of FAs in plants vary with environmental factors, such as season, light and age (Dewhurst, 1998).

The milk FAs originate from two main milk fat synthesis sources. They can be newly synthesised in the mammary epithelial cells (40%). This includes the short chain FAs (C4:0 – C14:0). They can further be derived from blood/ plasma (60%), either from dietary lipid absorption or body reserve mobilisation (<10%) (C18:0- long chain FAs). The palmitic FAs come from both sources (50% respectively), but the ratio is strongly dependent on the metabolic situation of the cow. Lipids are transported in the organism via blood-lipids and free FAs (Chilliard *et al.,* 2000; Collomb *et al.,* 2002). Blood lipids can be derived from fat tissues or from feed.

Butryic acid is quantitative and qualitatively an important and specific FA in milk fat. Approximately (approx.) one third of all milk TG contain butyric acid. Butyric acid is formed in

the rumen by microbial fermentation (Parodi, 1996). Together with propionic acid, and acetic acid their content influences the ruminal pH-value: an increase reduces the pH- value.

Essential FAs cannot be newly synthesized by the cow itself and originate directly from dietary sources. For an optimal lipid concentration in milk, the pH- value of the rumen is important and has to be at 6.5- 6.8. This can be reached by chewing, which influences the salivary flow and this in turn influences the pH of the rumen. A diet rich in cellulose keeps the pH at the optimal stage and acidic acid is built. On the contrary, a diet consisting of a high concentrate (rich in starch) content results in a pH shift towards a slightly more acidic value (around 5.2). More propionic acid is built and the content of microbes is decreasing, resulting in milk with a low fat content (Weerasinghe *et al.,* 2012; Lock and Bauman, 2004).

Table 3: Origin and quantitative distribution of fatty acids in bovine milk fat

fatty acid	C-atoms	content g/100g fat[a]	origin [b]
Butyric	C4:0	4.5	rumen; microbial fermentation
Caproic	C6:0	2.3	mammary gland
Caprylic	C8:0	1.3	mammary gland
Capric	C10:0	2.7	mammary gland
Undecanoic	C11:0	0.3	mammary gland
Lauric	C12:0	3.0	mammary gland
Tridecanoic	C13:0	0.2	mammary gland
Myristic	C14:0	10.6	mammary gland
Tetradecenoic	C14:1n5	0.9	mammary gland
Pentadecanoic	C15:0	1.0	mammary gland
	C15i	0.7	mammary gland
Pentadecenoic	C15:1	0.3	mammary gland
Palmitic	C16:0	28.2	mammary gland and adipose tissue
Palmitoleic	C16:1n7	1.8	mammary gland and adipose tissue
Margaric	C17:0	0.6	adipose tissue
	C17i	0.7	adipose tissue
Heptadecenoic	C17:1	0.4	adipose tissue
Stearic	C18:0	12.6	adipose tissue and diet
Elaidic	C18:1t	1.7	intermediate product from rumen
Oleic	C18:1n9	21.4	from C18 desaturation
LA	C18:2n6	2.9	diet
Linoeaidic	C18:2t	0.4	diet
CLA[c]	C18:2, c9 t11	0.8	diet and rumen
ALA	C18:3n3	0.3	diet
Gadoleic	C20:1n9	0.6	diet
AA	C20:4n6	0.2	diet
EPA	C20:5n3	-	diet
DPA	C22:5n3	-	diet
DHA	C22:6n3	-	diet

[a] *= Chow, 2007*

[b] *= Meyer, 2005*

[c] *= Schlimme, 1995*

For the mammary *de novo* synthesis, acetic acid and ß-hydroxyl butyrate represent the substrates (= initial four carbon unit), originating from microbial fermentation of carbohydrates (cellulose and hemicellulose) in the rumen. Only acetic acid is used later in the elongation procedure. They are carried via the blood stream to the mammary epithelial cells, which synthesise short- and medium- chain FAs. There are two main steps in the

synthesis pathway. Acetyl-CoA carboxylase and FA- synthetase are the two key enzymes that are necessary for the synthesis.

Firstly, acetate gets converted into acetyl-CoA in the cytosol via acetyl-CoA synthetase.

Secondly, an elongation takes place by the malonyl-CoA pathway. Acetyl-CoA gets converted into malonyl-CoA by acetyl-CoA carboxylase. For further elongation malonlyl-CoA and also acetyl-CoA are used. Each cycle in this pathway results in two carbons being added to the FA chain and the FA- synthetase complex is responsible for the chain elongation. The required reducing agent is $NADPH_2$ (Harstad and Steinshamn, 2010). Finally, C16:0 is generated by the separation under enzymatic action (thioesterase I) (Smith, 1980). Thioesterase I can form FAs of various chain length, dependent of synthesis stage during release of FAs.

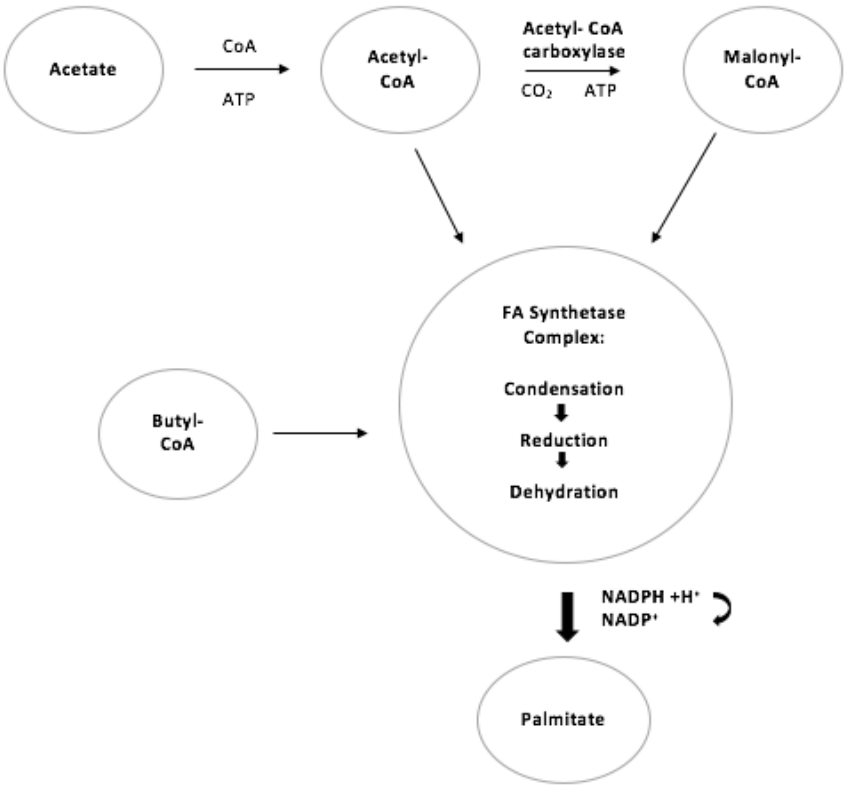

Figure 6: Overview de novo synthesis of Palmitate (data after Harstad and Steinshamn, 2010)

The main substrates for biohydrogenation are PUFAs, like C18:2n6 and C18:3n3. These long-chain FAs, originating from the diet, get hydrolysed by lipases and undergo a second transformation called biohydrogenation (Elgersma *et al.,* 2006). At this stage two different bacteria act. One group hydrogenate the C18:2n6 and C18:3n3 and the other group,

hydrogenate the *cis*- and *trans* isomers of the unsaturated FAs to stearic acid (Collomb *et al.,* 2002).

The biohydrogenation of PUFAs to monounsaturated fatty acids (MUFA) and saturated fatty acids (SFA) happens stepwise, where couple of intermediate products like vaccenic acid (VA) or conjugated linoleic acid (CLA) (by Δ^9 -desaturase from VA) are generated. These may escape during further hydrogenation and can then be found in milk fat. The detailed biohydrogenation of PUFAs are descripted in chapter 2.2.1.2. Generally, the double bonds of PUFAs occur in *cis*- configuration. Through biohydrogenation also *trans*-PUFAs occur. The reduction of biohydrogenation is therefore also of interest (Elgersma *et al.,* 2006; Baars *et al.,* 2015). Conversion of SFAs in unsaturated FAs is possible with desaturase enzymes (Δ^9) which are also built up by the mammary gland. Controversy, FAs which originate from the mammary gland are saturated (Chillard, 2003; Grummer, 1991).

Finally, all FAs get esterified with glycerol, resulting in triglycerides. These are secreted as milk fat globules (Harstad and Steinshamn, 2010).

Desaturation of saturated preformed FAs is possible via Δ^9 -desaturase in the mammary gland, which applies to most CLAs and endogenous FAs, especially the mono-unsaturated FAs (Soyeurt, 2008). Stearoyl-CoA is synonymously used for the Δ^9 -desaturase and its activity is responsible for the relation of mono-unsaturated and SFAs (Chillard 2003). Δ^9 -desaturase regulates occurrence of CLA isomers and the conversion from 18:1 *trans*-11 to C18:2*cis*-9, *cis*-12. Besides C18:0, the enzyme desaturates C10:0 to C10:1, *cis*-9, C12:0 to C12:1, *cis*-9, C14:0 to C14:1, *cis*-9 and C16:0 to C16:1, *cis*-9. According (acc.) to findings of Miyazaki and Ntambi Δ^9 -desaturase preferably desaturates C16:0 and C18:0 (Miyazaki and Ntambi, 2003). There are a couple of studies investigating the influence factors of Δ^9 -desaturase activity, which is highly variable in cows. Short mentioned, breed, lactation stage, feed is influential for desaturase activity (Soyeurt, 2008). Considering feed Chouinard *et al.* observed and increased Δ^9 -desaturase activity using C14, C16:0 and C18:0 as substrate, when cows received dietary supplementation with CLA (Chouinard *et al.,* 2001). Another study found an increasing enzyme activity when cows were fed fat supplements high in C16:0.

2.1.5 Human health: milk as “functional” food | OI

The term “functional food” became more and more popular during the last decades. Consumers have developed an increasing awareness towards food containing components which may have a beneficial effect on their health. As nutrition can play an important factor in the prevention of chronic diseases, such as obesity, cancer, insulin resistance and cardiovascular disease, there is a need to develop food which helps to keep the human population healthy.

Generally, milk contains many components e.g. antioxidants, probiotic bacteria, vitamins, peptides, which are thought to have a health benefit.

The German society for nutrition (DGE) recommends the daily lipid uptake till 30 % of the total energy intake. 7 % should be supplied by PUFAs, 2.5 % from omega-6 (n-6) and 0.5 % from omega-3 FAs (n-3) to reach the optimal ratio of 5:1 (DGE, 2015).

As it is generally known, unsaturated fats are beneficial for human health and studies stated a connection between SFAs and adverse effects on the human health. They can alter the cholesterol level and increase the risk for cardiovascular diseases. Hence, the nutritional quality of milk (- fat) has been criticised for years, because of its association with cholesterol, SFAs and *trans*- FAs and coronary heart diseases. Therefore, the attention of the consumers towards characteristics of dairy products is growing. But milk fat has a unique composition, because most of the SFAs are short chain FAs, which have not been negatively associated. The negative association with *trans*-FAs does apply to animal origin as well, but it is excluded from the *trans*-regulation in Austria. There is a considerable interest to modify and optimise the FA composition to keep improving human health (Harstad and Steinshamn, 2010). In regards to that, milk is an important source of PUFAs, particularly of n-3 FAs and n-6 FAs, like the C18.3n3 and C18:2n6. These FAs are essential and cannot be synthesised by the organism itself, because of the lack of Δ^{12} - and Δ^{15} -desaturases. They must be ingested by nourishment. Therefore, increasing these FAs might improve the nutritional value of milk. Other FAs which are associated with a positive effect on health are CLAs. CLA is the most important substance concerning human health benefits in the milk fat and dairy products are the main source of it in the human diet (Gierus, 2009).

C18:3n3, C20:5n3 and C22:6n3 are considered as the most relevant n-3 FAs. The physiological benefits of n-3 FAs have been proven in several studies. They are positively associated with a better blood flow because of reduced thrombocyte aggregation, reduction of blood triacylglycerides (Backes *et al.,* 2016), reduction of inflammatory processes,

improvement of skin (Wolters, 2005) and a reduction of all-cause mortality (Burr *et al.,* 1989). Eicosanoids, which are synthesised by the n-3 pathway tend to have a vasodilatory as well as an anti-inflammatory effect. Whereas eicosanoids coming from the n-6 pathway tend to have inflammatory and vasoconstrictive effects. Again, the ratio between both is important.

Also, BCFA are described to have a positive effect. They are associated with a decreased risk of cancer. Phospholipids (PL) do also have health promoting effects, but these are not completely assured, yet. They can act in the prevention of chronic diseases like cardiovascular diseases, atherosclerosis, obesity or diabetes type II (Pereira *et al.,* 2002; Küllenberg *et al.,* 2012).

Table 4: Health effects of FAs in milk (data after Shingfield, 2008; Harstad and Steinshamn, 2010)

FA	effect	content in milk	recommended daily dose % of energy intake
CLA C4:0	↓ cancer		0.5
CLA	↑immune response ↑ bone health		0.5
SFA	↑ LDL and ratio LDL/HDL	70 %	10
MUFA	↓ LDL and ratio LDL/HDL	26 %	15- 20 depends on total fat intake
n-6 PUFA	↓cardiovascular health	1.6 %	2.5- 9
n-3 PUFA	↑cardiovascular health	2.4 %	0.5- 2

2.1.6 Safety of milk: clostridia in milk | KJ

Milk and its potential spoilage or even harmful microorganisms are an essential issue to food safety. There are several critical points, at which milk can be contaminated from the primary production until it reaches the end-consumer. Particularly raw milk, independent of production system or feeding regime, provides risks for foodborne diseases. Although raw milk (-products) only represent a smaller part of dairy production in Austria and Europe, it has a dedicated position on the dairy market. For some traditional types of cheese exclusively raw milk is used, e.g.: for Roquefort or Camembert, in this cases milk gets heated or warmed up to 40 °C at maximum. There are also other types of cheese in which raw milk is used as starting product, but during cheese production a further heat treatment (with temperatures exceeding 40 °C) is conducted. Moreover, an increase of lactic acid bacteria would cause a lower pH-value in the cheese that leads to a limited growth of several undesired and potential pathogen bacteria. So ripening (influence on water activity) and heating are mainly influencing the presence and constitution of bacteria in raw milk cheese. In Austria the potential risk of getting a food-borne illness (like tuberculosis) from consumed raw milk products is rather low but not completely be excluded (Fuchs, 2008) For minimizing risks of food borne illnesses and an increasing shelf life, consumers milk that is available in supermarkets usually is pasteurised. By application of this heat treatment most bacteria that could have adverse influence on dairy products get inactivated. These bacteria mainly are: *Escherichia coli, Campylobacter, Listeria monocytogenes, Salmonella, Staphylococcus aureus or Yersinia enterocolitica, etc.* (Oliver *et al.,* 2009). Nonetheless, not all bacterial risk is eliminated by pasteurisation or even a higher heat application like UTH, especially spores are likely to remain. Clostridia are spore-forming bacteria and in context to this thesis a closer look is taken on them, because they are relevant for pasteurised consumers milk, which applies to the experiment (analysed samples) of this study. Further, these bacteria are of special interest, because their appearance in milk and dairy products is sometimes related to silage-fed cows (Klijn *et al.*, 1995).

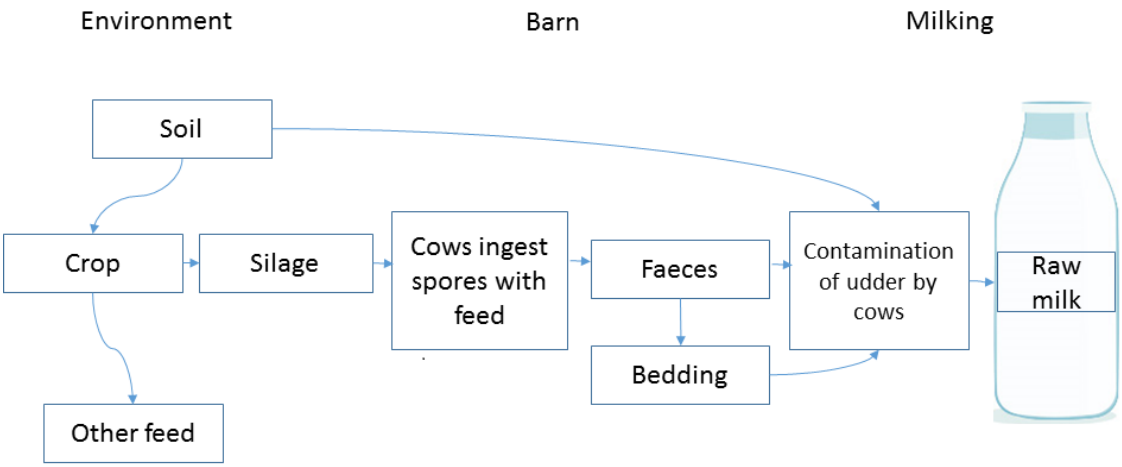

Figure 7: Potential way of contamination with bacteria including spores of raw milk trough feed (data from Driehuis 2013)

Clostridia are gram-positive and mostly anaerobe bacteria that are able to form endospores. These bacteria occur in natural environment like in soil and can also be found in animal feed and dairies, so the occurrence of Clostridia in milk is realistic (AGES, 2016). One critical point is the analysis and identification, as well as the differentiation of clostridia strains and their spores. Especially the detection of spores represents a challenge in dairy products and their safety monitoring. Moreover, the discrimination of clostridia (and its spores) is relevant, because not all strains imply a potential threat to human health. For this purpose, the field of next generation sequencing has been developing and keeps going on with improving methods (Xu and Wu, 2015).

The presence of most Clostridia strains is rather a question of spoilage than a risk for human health. Mainly Clost*ridium tyrobutyricum* is responsible for spoilage and following economic losses in dairy products, but in some cases Clostridia strains have already caused foodborne outbreaks. Even groups of the pathogen *Clostridium botulinum* have been detected in the dairy processing chain, which represents a complete exception. *Clostridium botulinum* can cause botulism in animal (cow) and human by bacterial toxins. *Clostridium botulinum* have also been detected in non-acidified silage that was wrapped in plastic (Lindström et al., 2010). Another severity with the Clostridium botulinum toxin is, that their toxins are often not detectable by human (not visible, no specific smell/taste). After all, a contamination with botulism causing bacteria from dairy products is very rare (AGES, 2016). There are 3 species that are categorized as the main clostridia contaminants in milk, namely *Clostridium butyricum, Clostridium sporogenes* and the main influential is Clost*ridium tyrobutyricum*, (Cocolin et al. 2004 in (Drouin & Lafrenière 2012). The presence of Clost*ridium tyrobutyricum* in silage enables growth of less acidic tolerant clostridia and other potential unwanted microorganisms. When silage gets contaminated with *Clostridium*

tyrobutyricum respectively its spores are ingested by the cow they survive digestion. Then the spores are in the faeces and can contaminate the milk, often trough contact with the udder. The presence of these three mentioned Clostridia species and their spores that are termed as “butyric acid spores”, cause loss of value in dairy products, mainly in cheese. In raw milk or drinking milk these bacteria are less influential regarding spoilage, but during cheese fermentation and the presence of lactic acid spores experience optimal circumstances for germination. This adverse effects in semi-hard and hard cheese like in Gouda, Emmentaler or Parmesan and others are referred to as “late-blowing defect” (Driehuis & Oude Elferink 2000). The blowing effect can happen within 3-10 weeks of cheese ripening. In that case the present lactic acid gets degraded to butyric acid and hydrogen gas, resulting in this visible blowing and the cheese has to be discharged (Seelhofer 2013).

Butyric acid can also develop during the process of silage production as an unwanted product. Basically silage production with its various influence factors like season, weather and storage offers chances for a diverse bacterial colonialization, so there is requirement for controlled production in order to provide safe feed. Hence, milk from cows that are fed with silage are more likely to contain Clostridia (Pahlow *et al.,* 2003 in Drouin and Lafrenière 2012). The primary concept of hay milk production was to get a milk that is optimal for cheese production and to eliminate or reduce the chance of the blowing effect (Geisler and Ginzinger, 2010). So an exclusion of silage in cows feed leads to milk with less butyric spores per litre. Studies confirmed values of spores counting under 200 per litre in hay milk, whereas in silage milk values are higher (1000 to 100.000 spores/litre). Spores that do not exceed 200 per litre would not negatively influence cheese processing. There are other ways to use milk with higher amounts of butyric spores for cheese making, e.g. by adding preserving agents like nitrate or lysozymes or filtration methods. Since hay milk production usually needs less processing steps or less addition of preserving agents it provides advantages in cheese making. Moreover, the occurrence of off-favours is less frequent. Off-flavours are by tendency more recognized in silage milk, due to defects during silage production. If defect silage is ingested by the cows, it is transferred to the mil k, also a transfer via respiratory air is possible (Ginzinger and Tschager, 1993). It also has to be mentioned, that the processing of silage has improved over the last years, consequently cows receive silage of better quality which influences milk quality (Driehuis and Oude Elferink, 2000). In conclusion, conventionally produced milk, meaning no hay milk declaration does not imply constant or permanent silage feeding. Silage production and composition can be quite different (composition, procedure) so that no clear or discriminative statement can be done on food safety conditions of HAY vs. CON consumers milk.

2.1.7 Influence of the different feeding strategies on the fatty acid pattern | OI

There are two systems of farming. One is called “extensive farming” which represents the traditional, organic and ecological way. In this practise the cows are fed with pasture and fresh grass/hay. This system also significantly contributes to a country’s culture. The other system is called “intensive farming”. In this style of farming, the cows are kept indoors and are predominantly fed with silage and concentrate to reach a high milk performance. In this feeding regime, the performance can be kept on a constant level during the whole year (Hofstetter *et al.,* 2014). Due to geographical circumstances, the milk production in Austria is mostly cultivated by extensive farming. Therefore, one can find special dairy products, like hay-milk-products, in usual supermarkets.

Hay milk is referred to “milk from cows, fed without the use of silage (fermented grass or corn)”. These cows are allowed to graze and eat fresh grass and herbs outside during the summertime and are fed with hay and concentrate (cereals- and protein crops) during the winter period. The exact definition is listed in the “Heumilchregulativ” from ARGE. ARGE was established in 2004 and includes 8000 hay milk farmers and over 60 diaries in Austria.

The following has been set in relation to the diet (ARGE, 2009):

- No production and feeding of silage; the sale directly from the field is not permitted
- No preparation and feeding with moist hay or fermented hay
- No feeding of by-products from breweries or other food industries
- No use of feed materials of animal origin (milk, whey, flour)
- As supplements, green rape, maize, rye, fodder beet, hay-, lucerne and maize pellets are allowed
- Field beans, peas, oil plants and extraction-cake can be used

Hay milk farmers obligate themselves to stick to the *ÖPUL* (*Österreichisches Programm für umweltgerechte Landwirtschaft)* –sanction. Hay milk products with the hay milk label fulfil these requirements.

There are three advantages by hay milk products:

- less odour and taste faults
- less harmful clostridium traces for cheese
- higher n-3 FA concentrations (Geisler, 2010)

The diet fodder can be seen as the main source of FAs in the cow's diet and many studies have shown, that FAs can be a quality criterion of the different production systems. Also the fat content itself can be significantly influenced by the cow's diet and is the major factor regulating the milk fat composition, besides the lactation stage (Banks *et al.,* 1976; Palmquist *et al.,* 1993). For example, the low milk fat syndrome is caused by diets that contain a lot of fermentable carbohydrates. By now, it is well-established and proven in many studies that the grazing allowance and the intake of fresh grass has a positive influence on the FA pattern of milk, especially in the CLA and n-3 FA concentrations (Kelly *et al.,* 1998; Kraft *et al.,* 2003, Chouinard *et al.,* 2001; Elgersma *et al.,* 2003; Elgersma *et al.,* 2004). Cows, which are allowed to walk and graze outdoors have a diet which is richer in PUFAs (Chilliard *et al.,* 2007). Therefore, the milk is also richer in PUFAs, in particular CLA, C18:2n6 and C18:3n3, compared to milk from the intensive farming system and also contains less SFAs. C18:3n3 and C18:2n6 are taken up directly from the feed into the milk. CLA is synthesized from C18:2n6 in the rumen and also in the mammary gland from VA and thus not a component of the fodder (Elgersma *et al.,* 2003). Further it was observed, that the Δ^9- desaturase activity is proportional to the substrate uptake in the mammary gland (Glasser, 2008). Since the content of C18:2n6 is higher, the CLA content rises consequently. A higher amount of PUFAs can lower the ratio between PUFAs and SFAs and enhances the quality of the milk. Also the ratio of n-6 and n-3 FA has an influence on the quality (Baars *et al.,* 2015). The German society for nutrition recommends a ratio of less than 5:1 (n-6 to -n3). Only feeding pasture does not provide an optimal mix of nutrients for high- producing dairy cows. Studies have also shown, that within all types of fodder, the composition of FAs also varies. That is due to the differences in botanical composition, which additionally depends on the season (Wyss, 2007). The lipid fraction of plants is mostly located in the photosynthetic tissue of the plants, the chloroplasts (Gierus, 2009). Hence, leaves contain the most FAs (Wyss, 2012). During spring and summer time the concentration is higher than in autumn (Bauchhart *et al.,* 1984). Till the first cut, the content of PUFAs is decreasing, because the stem grows bigger and stronger (Boufaïed *et al.,* 2003). Morel *et al.,* found out, that swards with Lucerne and swards with red/ white clover have a positive effect on the PUFA content (Morel *et al.,* 2005). Also a higher concentration in C18:3n3 had been reported. As Baldinger

et al., found out, that the botanical composition also plays a role within silages. Italian Ryegrass silage contributed to less PUFAs (Baldinger *et al.,* 2012).

Another factor, which influences the FA composition is the forage conservation method. When the grass gets cut or wilted, the FA concentration decreases due to oxidative reactions and leaf shatter (Dewhurst *et al.,* 2006; Shingfield *et al.,* 2005; Wyss, 2007). The reactions which take place are called lipolysis and result in free FAs. Losses are especially encountered in the C18:1n9 and C18:3n3 FAs. It can be up to 75 %. Pre- wilting has also an impact on the FA profile of plants (Elgersma *et al.,* 1998; Elgersma *et al.,* 2003; Dewhurst, 1998). Another issue is that the grass is harvested relatively late for an optimal yield of dry matter. The longer the grass is able to grow, the less effort is put into the leafs by the plant. The stem becomes thicker and thus, the FA content decreases. In conclusion, it's advantageous to have a high herbage content in the diet. Shingfield *et al.,* found out, that the transfer from C18:2n6 and C18:3n3 from diet into milk was higher in diets based on hay than silages. There are only a few studies on the different feeding strategies, especially hay vs. silage fodder (Shingfield *et al.,* 2005). Bernadini *et al.,* found out, that animals fed with a grass- hay diet showed an increase of CLA and n-3 FAs compared to maize silage fed cows (Bernardini *et al.,* 2010).

A way which allows to increase the n-3 FA content in milk is adding linseed oil or other supplements to the feed (Goodridge *et al.,* 2001; Kennelly 1996). An overall reduced energy uptake results in higher n-3 content in milk, which was observed in cows grazing in alpine regions where the grass had a reduced fat content. One reason why an energy deficit causes higher n-3 concentration, is the increased release of C18:3n3 from the cow's fat tissue. The influence of certain alpine plant components could also affect biohydrogenation, respectively certain ruminal bacteria leading to higher C18:3n3 concentrations (Leiber *et al.,* 2005; Wit *et al.,* 2006). Overall, the reduction in energy uptake is more effective for higher n-3 concentration in milk than the addition of feed supplements like linseed oil (Chilliard *et al.,* 2000).

2.2 Fatty acids in milk

The following chapter gives a short introduction to lipids, with an emphasis on the subgroup of FAs. Those FAs that mainly occurring in cow's milk are considered closer. Furthermore, some single FAs are described with their possible influence on human health.

2.2.1 Overview | KJ

The class of lipids is heterogeneous and their definition varies dependent on literature source. A common characteristic of lipids is their solubility in organic, unpolar solvents and solubility among themselves. Contrary is their behaviour in unpolar solvents like water, where they are not soluble (Krömker, 2006). There are different classification systems and scientists are not agreeing on one general system. But one categorisation by Fahy *et al.,* is mentioned, which distinguishes in eight classes of lipids:

- Fatty acyls
- Glycerolipids
- Glycerophospholipids
- Sphingolipids
- Sterol Lipids
- Prenol Lipids
- Saccharolipids
- Polyketides (Fahy *et al.,* 2011).

A quite simple categorisation of lipids can also be done in: simple and complex components and derivatives e.g.: FAs, alcohols, fat soluble vitamins. Complex lipids include phospholipids (PL) and glycolipids. Simple lipids are waxes and fats, whereas fats comprise TGs. The quantitatively highest share of the milk lipids with about 98% are TGs, which consist of 3 FAs and one glycerol molecule. In milk FAs are esterified, but by enzymatic hydrolysis e.g. lipase activity, free FAs are generated. The amount of free FAs in milk is a quality parameter because lipolysis leads to off-flavours like rancidity. Additionally free FAs

can be an indicator for extended or/and inadequate storage conditions (Antonelli *et al.,* 2002).

Longer chain FAs occurring as TGs, are used as energy storage in tissues (e.g. animal or human fat tissue). TGs can be described by their FA composition and their structure is influential for their physical and metabolic behaviour. In a dietary context some FAs are essential for human metabolic pathways and other functionalities.

FAs can differ in:

- The length of the carbon chain (short, medium, long chain)
- Degree of saturation (e.g. saturated/ unsaturated FA)
- Relative location of double bonds (conjugated, isolated)
- Geometry of double bonds (*cis*- or *trans* position) (Matissek, 2010; AOCS, 2013)

FAs are categorized by their number of carbon atoms (3 - 7 carbon-atoms), medium (8 to 13 carbon-atoms) or long-chain (≥ 14 carbon – atoms). The most important short FAs are Butyric and Capron acid; the most important medium chain are Caprylic- and Caprinc- and Lauric- and for the long chain it is: Myristic-; Palmitic-, and Stearic acid. Generally, double bonds in FAs are distributed in a methylene- interrupted isolated way, meaning this scheme: C-C=C-C-C=C-, in which two double bonds are separated by two singe bonds. A conjugated double bond is structured like this: —C=C—C=C—. It always has one single bond between two double bonds. Considering the double bond, about 90 % of unsaturated FAs occur in *cis*- configuration, whereas *trans*- occur mainly through mechanical treatment (heating; hydrogenation or bio-hydrogenation in the rumen).

Figure 8: Cis and trans configuration in FAs

Unsaturated FAs can be named by the delta-notation, in which all double bonds are numbered from the carboxy- side of the FA, whereas in the omega notation only the terminal double bond is counted from the methyl-side.

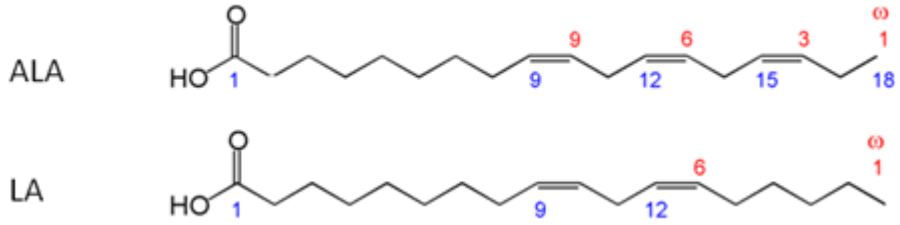

Figure 9: Structure of unsaturated FAs: ALA (n - 3 FA) and LA (n - 6 FA), online http://www.gbhealthwatch.com/Science-Omega3-Omega6.php (accessed on 02.11.2016)

Functional groups are also used for categorization, e.g. hydroxy-, epoxy-, keto- or furan-groups. FAs found in food are mostly even-numbered, unbranched, aliphatic and monocarboxylic acids. Additionally, they usually do not have more than three double bonds and they mostly are *cis* configured (Frede, 2010). In many cases FAs consist of two to 26 carbon-atoms, whereas these with 16 and 18 carbon-atoms occur more frequent. Palmitic acid (C16:0) and oleic acid (C18:1) are the two main FAs in cow's milk and make up about 50% of FAs (Belitz, 2008; Töpel, 2005).

2.2.1.1 **Saturated fatty acids** | KJ

Approximately 70% of milk fat consists of SFA (Månsson *et al.,* 2008). These are FAs which do not contain any double bond. Conventional milk derived from Austrian supermarkets contains about 65 g SFA per 100 g milk fat (Velik *et al.,* 2014). The content of UFAs in milk is also influenced by the livestock breeding, hence from the feed. In contrast, monounsaturated FAs (MUFA) contain one DB and PUFAs contain more than one. Due to their unsaturation, these FAs are more reactive. This influences the characteristic of milk. The "Deutsche Gesellschaft für Ernährung" (DGE) and also many other nutrition guidelines and institutions (like the American Heart Association or the World Health Organization) advice a limitation of SFA intake and they should be replaced by food that Contains more unsaturated FAs (DGE, 2015). This advice for reduction in SFAs is based on studies showing a connection of SFA intake and negative effects on human health. Thus the total fat- and SFA dietary intake is associated with adverse effects on cholesterol levels hence cardiovascular diseases (Capita and Alonso-Calleja, 2003). A recently conducted study also found lower mortality rates when saturated fat was replaced by unsaturated fat in human nutrition and therefore justifies the present nutritional guidelines (Wang *et al.,* 2016). But some studies do not conclude on a definite benefit caused by a reduction of dietary SFAs,

like the results of Ramsden *et al.;* indicate. Ramsden found, that a substitution of SFAs with C18:2n6 showed a reduction of cholesterol levels in human, but this does not necessarily mean a lower prevalence for heart diseases (Ramsden *et al.,* 2016). The differences in these study results and their interpretation about the influence of SFAs in food on human health is not clear, also because there is variability in SFAs (e.g. chain length, origin). Some studies show no health effect in connection with saturated dietary fat intake (De Souza *et al.*, 2015; Siri-tarino *et al.,* 2010).

2.2.1.2 Polyunsaturated fatty acids | KJ

PUFAs are important for the human organism due to their necessity in metabolic pathways. Both, n-3 and n-6 PUFAs, are necessary for building up cell membranes and for synthesis of eicosanoids. Eicosanoids can be regarded as fat-derived hormones and they are so called signalling molecules and are involved in immune- and inflammatory processes. Humans can synthesize EPA (n-3) and DHA (n-3) from ALA (n-3), but not from LA (n-6), which is the precursor of Arachidonic acid (n-6). This synthesis happens with desaturation and elongation enzymes to a different extend that depends on e.g. genetics, nutrition. For an optimal supply of essential FAs, the relation of n-6 to n-3 FAs is crucial. In order to build up Arachidonic acid and EPA. Omega-3 and n-6 FAs compete for the same set of enzymes, which are Δ^6 -desaturase, Δ^5 -desaturase and elongase. As it is visible in Figure 10, eicosanoids are built up from n-3 and n-6 but their effect on the human organism is different, antagonistic. Eicosanoids originating from C18:3n3 tend to reduce inflammatory actions and have a vasodilatory effect, whereas eicosanoids originating from C18:2n6 promote inflammations and are vasoconstrictive (Simopoulos, 2004; EUFIC, 2008).

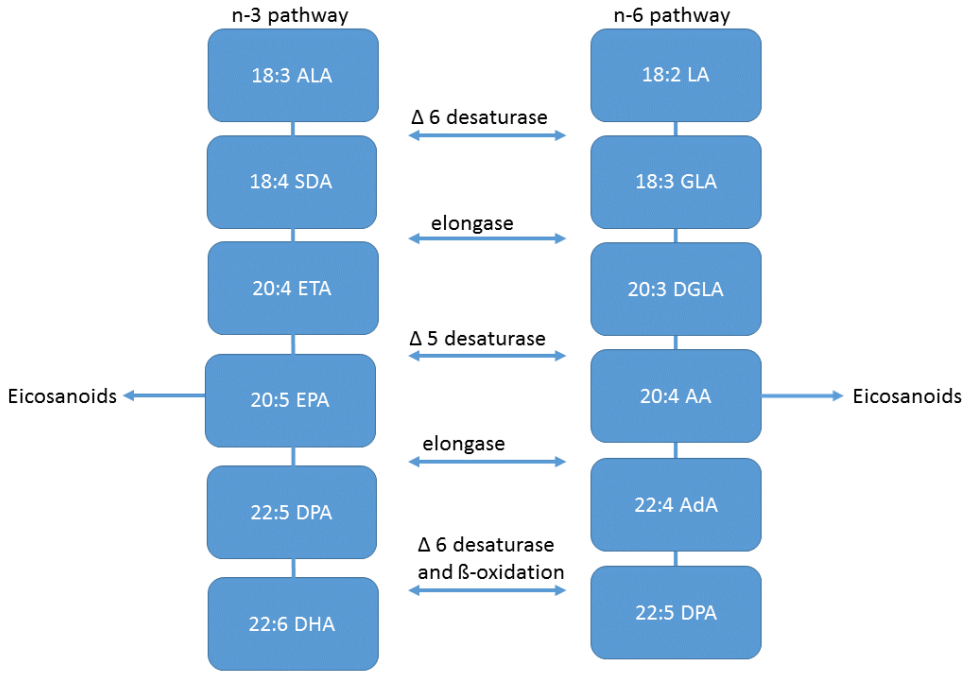

Figure 10: Scheme of LCPUFA synthesis, data after (Lau *et al.,* 2013)

Considering all n-3 FA s the German society of nutrition (DGE) advices a supply of 0.5 % of the daily nutritional intake for healthy adults. The best observed and most advised food source of n-3 is fish and the DGE advices to consume about 200 g fish per week for an efficient n-3 supply. Since the conversion rate from C18:3n3 to the physiological important C22:6n3 and C22:5n3 is limited to approx. 5 % conversion rate in humans (Burdge and Calder, 2005). So milk is obviously no comparable alternative to fish as efficient n-3 source.

Hauswirth *et al.,* showed that alpine cheese contains relatively higher amounts of n-3 FAs, because of the cow's feeding- and keeping regime. In addition, this study mentioned the "alpine paradox", which could explain low rates of cardiovascular diseases in the alpine population because of their sufficient n-3 supply. In connection to our study, Hauswirths findings are an example of how the FA composition in cow's milk can be influenced and probably optimized for human nutrition (Hauswirth *et al.,* 2004). CLA is another important group of FAs studied in milk fat, also because it can be modified by feed and shows notably differences in its variations and quantity. CLAs include several conjugated octadecadienoic acid isomers. It can either be synthesized through incomplete biohydrogenation of PUFAs. This is done by ruminal bacteria like *Butyrivibrio fibrisolvens*, which isomerizes *cis*-12 to *trans*-11 bonds and *Megasphaera elsdenii* that isomerizes *cis*-9 to *trans*-10. Secondly, an enzymatic synthesis of CLA from VA is possible via Δ^9- desaturase. This enzyme is in present in all tissues of the cow as well as in humans, especially in the epithelial cells that are responsible for lactation (figure 10) (Jahreis *et al.,*1999; Baars *et al.,* 2012).

In the ruminants' gastro intestine C18:2n6 gets converted in its isoforms. The result of this partial biohydrogenation is a single bond between one or both of the double bonds, that means *cis*-9, *trans*-11 or *trans*-10, *cis*-12 (Viviani, 1970; Kennedy *et al.,* 2010). Parodi described this conjugated FAs as CLAs including its isomers. The configuration of CLAs can be *cis* or *trans*. The most frequent in ruminants is *cis*-9, *trans*-11 CLA, also called "rumenic acid" (Parodi, 1977).

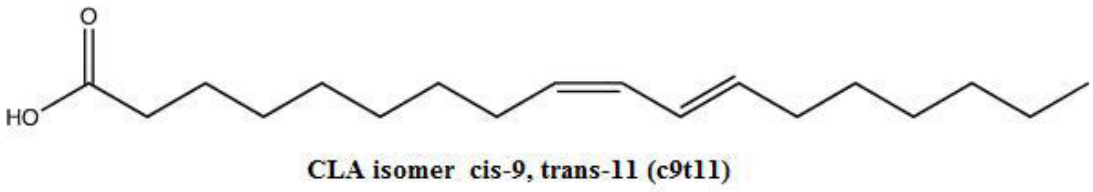

Figure 11: Chemical structure of the most common CLA isomer: cis-9, trans-11 (rumenic acid)

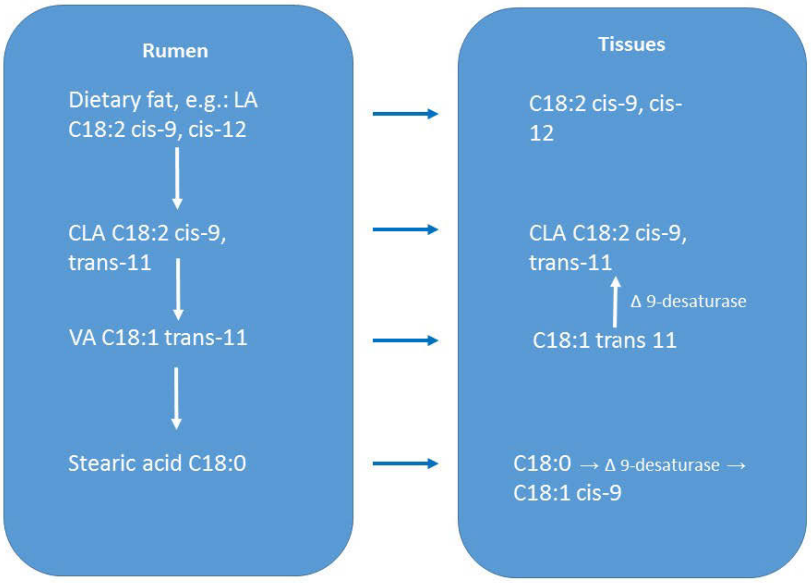

Figure 12: Role of rumen biohydrogenation and tissue ∆9--desaturase in the production of cis-9, trans-11 conjugated linoleic acid in ruminant fat, modified after: Bauman1999,: https://examine.com/supplements/Conjugated-linoleic-acid/ (accessed on 09.08.2016)

As milk and its products are a main supplier for CLAs in food (Chin *et al.,* 1992), the content of CLAs in milk shows a big variation, which depends on the CLA concentration of the used raw milk (Whigham *et al.,* 2000). Several studies confirmed the influence on CLA content in milk caused by production system and feed. There is a clear correlation between the CLA content of pastures and its concentration in milk (Dhiman *et al.,*, 1999; Jahreis *et al.,* 1999; Jahreis *et al.,* 1997a). Especially the main isomer in cow's milk, cis-9, *trans*-11 CLA, can be

increased when cows consume higher amounts of PUFAs, so when they are mainly grazing and do not/hardly get concentrated feed (Figure 13).

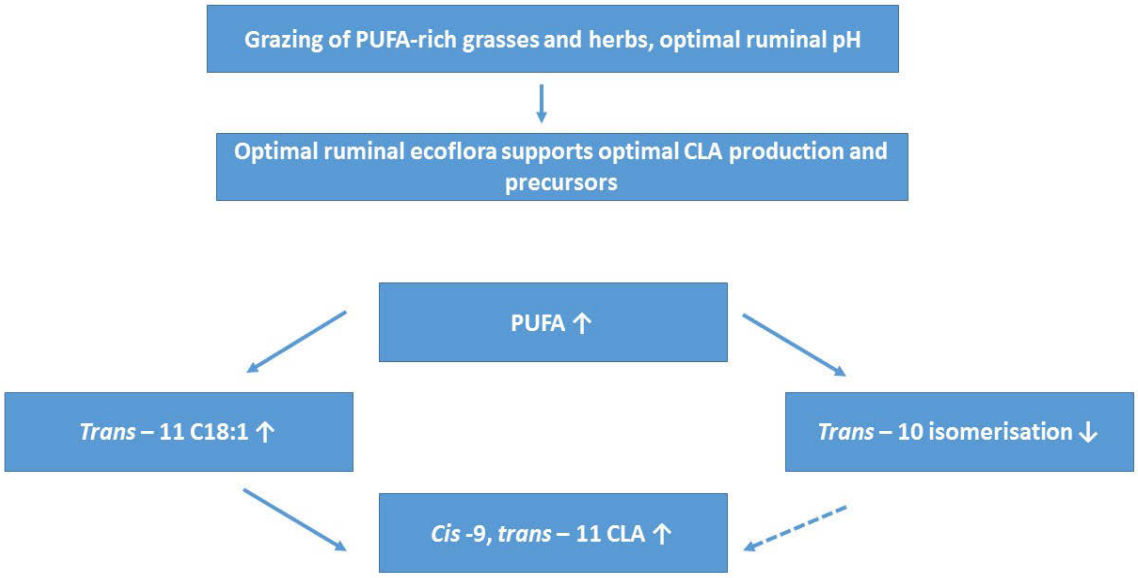

Figure 13: Influence of optimal farming management on desaturation of trans-11 18:1 to cis-11 CLA (inhibition of Δ9--desaturase by trans-10 bonds), (data form Bauman et al., 1998; Kraft et al., 2003)

Table 5: Distribution of CLA isomers in milk fat (%)(Kraft et al., 2003)

Fatty acid distribution of milk fat	**Ratio/percentage**
Ratio of *trans* – C18:1/*cis* – 9, *trans* - 11	~ 0.5
Trans – 11, *cis* - 13	~ 5 % of total CLA
Trans – 10, *cis* - 12	< 0.1 % of total CLA

The occurrence of other CLA isomers in milk fat is very probable the result of ruminal activity. The *trans*-7 *cis*-9 isomer, which appears to be the most frequent isomer, is the product of endogenous synthesis (Sehat *et al.,* 1998; Corl *et al.,* 2002). Another noticeable observation is, that the *trans*-11, *cis*-13 isomer seems to occur in much higher amounts in alpine cow milk than in cows that are kept indoor (Kraft *et al.,* 2003).

Table 6: Overview on dietary factors that (potentially) alter the CLA content in milk (Kraft et al., 2003)

Dietary factor	Effect on CLA Content of milk fat
Lipid substrate	
Saturated vs. unsaturated fat	Increase by addition of unsaturated fat
Type of plant oil	Greatest with oil in 18:2
Level of plant oil	Dose-dependent increase
Ca salts of plant oils	Increased as with free oils
Fat in animal by-products	Minimal effect
High oil plant feeds	
- high oil corn	- minimal effect
- soybeans	- heat processing will increase
- rapeseed vs. soybean	- similar effect
Modifiers of biohydrogenation	
Forage Concentrate ratio	Increased with high ratio
Nonstructural carbohydrate	Minor effect
Restricted feeding	Increased with restricted feeding
Fish oils	Greater increase than with plant oils
Monensin-ionophore	Variable effect
Dietary buffers	Little effect
Combination	
Pasture vs. conserved forages	Higher in pasture
Growth stage of forage	Increased with less mature forage

Since CLAs occurs in *trans*-configuration, they could be associated with adverse health effects, because *trans*-FA consumption is linked to diseases like coronary heart disease (Willnett, 1993; Mozaffarian *et al.,* 2006). As reviewed by Kennedy *et al.,* there are controversial findings in health effects of CLAs. Firstly, there is a lack of human studies, secondly the mechanisms/interactions of the single CLA isomers in humans are not completely clear. Additionally, CLAs in food or dairy products operate differently than supplements which are sometimes used in studies (Kennedy *et al.,* 2010). In the USA and in Canada packed food products containing *trans*-FAs have to be declared on nutritional labels to raise awareness of the potential adverse health effects. This mandatory declaration according to the U. S. Food and Drug Administration (FDA) does not include CLAs or "*trans* fatty acids of ruminant origin with either a single double bond or non-conjugated double bonds" (FDA, 2013).

2.2.1.3 Furan fatty acids | KJ

Furan fatty acids (F-FAs) are FAs that include a furan moiety and they are considered as bioactive components. F-FAs differ in length of their side chains and in substitution of the furan ring (Pompizzi, 1999). Naturally occurring F-FAs consist of a carboxyl chain with 9, 11 or 13 carbon-atoms and a propyl or a pentyl moiety. The most common, naturally occurring F-FA has 11 carbon-atoms, one acidic group and two methyl groups. F-FAs that are unsubstituted can be a result of CLA oxidation, then the F-FAs have 18 carbon-atoms (Yurawecz *et al.,* 1995). Furan fatty acids function as hydroxyl- or peroxyl scavenger, so they can protect PUFAs from lipid peroxidation (Okada *et al.,* 1996). This means, that F-FA have an antioxidant effect, which is one explanation for the positive influence on human health. For instance an anti-inflammatory impact was proven in an *in-vivo* study (Wakimoto *et al.,* 2011). Several food groups originating from pants but also from animals contain F-FAs. Furan fatty acids are synthesized by plants and certain bacteria, the relatively high content in marine animals or in other animals happens through the food chain. The consumption of algae leads to the relatively high content of F-FAs in fish (Guth and Grosch, 1992; Spiteller, 2005). In context of this work only dairy products are considered. The proportion of F-FAs in milk fat is therefore also influenced by the plants/grass consumed by the cow. A study of Wedlinger and Vetter found, that CON versus organic regime influences the F-FA content in cow's milk. They observed a significant difference in F-FA content between the two regimes: Furan fatty acids were higher in organic butter samples than in CON ones. The highest amount of F-FA was observed in organic butter during summer, whereas the lowest level of F-FA was during winter and in CON butter. Overall the content of F-FA was within high variation and very probably caused by different feed. Furan fatty acids reached from 4.5 to 47.6 mg per 100 g butter (Wendlinger and Vetter, 2014).

2.2.1.4 Branched chain fatty acids | KJ

The dietary intake of milk, dairy products or products from ruminants are almost an exclusive supplier of BCFAs for humans. So did BCFA as bioactive components and their content in milk find increased attention over the last years (Bainbridge et al., 2016). There is only a small proportion of odd- and BCFAs in milk. These FAs (mainly C13:0 to C17:0) are the results of microorganisms' ruminal activity, a smaller part derives from *de-novo* lipogenesis (Vlaeminck*,* 2006). BCFAs occur in marine animals and in ruminants. The categorization of BCFA can be done in mono-, di-, and multi-methyl BCFA. Considering the monomethyl-BCFA, which have one methyl-group as side chain, most of them can be categorized as *iso, a smaller proportion as ante-iso*. *Iso* means, that the side chain is at penultimate position counted from the carboxy- end of the FA chain. Whereas *ante-iso* describes the side chain in antepenultimate position (Vlaeminck *et al.,* 2006). The analysis of BCFAs in milk helps to measure feed strategies with their influences on the ruminal microbiome, but also for human health the content of BCFAs is considerable. In a physiological context BCFAs are considered as bioactive components and are associated with a decreased risk for several diseases like cancer and Alzheimer's disease. Additionally, the content of odd- and BCFAs could influence the physical, technological properties of milk (-products), e.g. decrease the melting point of butter (Bainbridge *et al.,* 2016).

Phytanic acid 3,7,11,15 -tetramethylhexadecanoic acid (PHY) is a saturated branched chain isoprenoid FA. It is common in animal tissue and occurs in nearly all dairy products, certainly depending on their total fat content. On average commercial milk contains about 1.5 mg PHY per 1 g fat (Brown *et al.,* 1993; Capuano *et al.,* 2014; Vetter and Schröder, 2010). PHY is built up through enzymatic degradation of chlorophyll by ruminal bacteria. Mammals are not capable of a *de novo* synthesis of PHY, they cannot metabolise/ degrade chlorophyll. In the cow's rumen the phytol is generated from chlorophyll and through oxidation of phytol PHY is built up. Therefore, the content of PHY is a potential indicator for the feeding regime, particularly for the intake of grass respectively hay. More concretely the evaluation of PHY and the relation of its diastereomers: 3R,7R,11R-PHY (RRR) and 3S,7R,11R-PHY (SRR) should allow backtracking of the cows feed. A difference in animal keeping was observed showing that PHY tends to be higher in organic production than in conventional (Vetter and Schröder, 2010). However, it seems that the determination of the total PHY content is not a valid indication for feeding and keeping regime in cows. A different distribution of the PHY isomers was detected, a higher consumption of clover increased the RRR isomer (Che *et al.,* 2013). The experiment of Capuano *et al.,* confirmed that total PHY and its metabolite pristanic acid (PA) are no suitable parameter for drawing conclusions on the feeding. A clear

correlation of the grass consumption and the relation of the SRR to RRR was found, which was smaller in cows that consumed more grass respectively in organic keeping. This ratio of SRR to RRR is, in combination with other analysis, a suggested parameter for distinguishing HAY and/ or organic from CON produced milk (Capuano *et al.,* 2014). In connection to human physiology the evaluation of PA is also interesting. Pristanic acid has signalling functions and is involved in the glucose and the retonic acid metabolism (Zomer *et al.*, 2000; Heim *et al.,* 2002).

2.2.2 Phospholipids | KJ

Phospholipids are often classified as complex lipids and have a share of about 1 % considering the total lipids of milk fat (Grosch and Belitz, 1987). The properties and structure of milk (-fat) are influenced by PL, because they built up the characteristic globule membrane together with proteins. PL are amphiphilic and polar and their ability as emulsifier is used in food production and technology (Kanno, 1989; Singh, 2006). There are subgroups of PL varying e.g. in chain length or degree of saturation (Dewettinck *et al.,* 2008), which will not be discussed in further detail in this work. The two main quantitative subgroups of PL in dairy products are glycerophospholipids and shingolipids (Gallier *et al.,* 2010). The influence of cows feed on PL in milk is not clear due to a lack of studies. PL respectively some subgroups seem to be effected by feed. Graves and colleagues for instance found out, that Sphingomyelin, a PL, is at its highest concentration during summer, when cows have access to fresh feed (grazing) contrary to the winter period (Graves *et al.,* 2007). Additionally, PL are important for human health, some are essential and others also have a preventive or palliative effect on chronic diseases like cardiovascular diseases, atherosclerosis, obesity or diabetes type II. Some of these health promoting findings are not completely assured, because many results are based on animal studies or show controversies (Pereira *et al.,* 2002; Küllenberg *et al.,* 2012).

2.3 Fatty acid analysis | OI

In general, the most common method for the analysis of FAs is gas chromatography (GC) paired with a flame ionisation detector (FID). Further, it is a standardised method for the analysis of FAMEs, because it is cheap, easy and fast. With GC-FID it is possible to determine the FAs qualitatively as well as quantitatively. Similarly, a GC paired with a mass-spectrometer is frequently used. But this method is mainly used for the qualitative characterisation of fatty acid methyl esters (FAME). For the separation of the FAMEs, long, high cyanopropyl- polar capillary columns should be used, e.g. SP2560. Before the FAs can be measured by GC-FID, the samples must be derivatized. The treatment includes the isolation of the lipids followed by methylation (acidic or alkaline) to FAMEs. For correcting relative peak areas to relative mass amounts, theoretical response factors (TRF) can be applied (Schreiner and Hulan, 2004). For the quantification of milk FAs they must be used.

Generally, FA analysis are well established methods regarded as chemical-analytical routine. The analysis of milk shows some challenges. Milk contains a broad range of FAs e.g.: different chain length, degree of saturation or FAs of *cis* – and *trans* configuration. These FAs subgroups require different analysis, meaning one method would not detect or analyse these various FAs properly. The analysis of short chain FAs needs to consider their increased volatility, especially their quantification is error-prone (Simionato *et al.,* 2010).

3 Aim | OI

The aim of the present thesis was to differentiate milk from different feeding systems (silage (CON) and non-silage (HAY)) by their FA profile and further the identification and characterisation of minor long-chain FAs in cow's milk. The main focus was on the estimation of the difference in the minor, long-chain FA composition regarding the declaration of hay milk and CON milk depended on season. By consideration and analysis of these minor FAs an improved statistical discrimination between hay milk (no silage feed) and conventional milk (potentially silage-fed cows). Some studies have already been conducted proving a feed-induced difference in the FA-pattern in bovine milk, without considering LCPUFAs. An additional aim was the contribution of knowledge about variables which influence the FA pattern and showing new differentiation opportunities in the basis of LCPUFAs.

The main hypothesis of this research was, that discrimination of CON and HAY is possible by FA analysis and that taken in consideration minor LCPUFAs as well, statistical significance can be improved.

4 Materials and methods

4.1 Samples

4.1.1 Milk samples

Samples of consumer milk were collected from Austrian supermarkets in the season of 2015.

For the isolation of the milk FAs, 84 whole milk samples produced by different diaries in Austria, all declared as “länger frisch” – ESL, pasteurised and peroxidase positive, from the retail marked were used. 39 of them were potentially produced with silage fed cows and 45 samples were hay milk samples, guaranteed by TSA (traditional specialities guaranteed), which at least did not have the hay milk label. The samples were drawn in every quarter (spring, summer, autumn and winter) of the year 2015. During the winter feeding period (January till March; October till December; Q1 and Q4), 20 CON milk samples and 25 hay milk samples were collected. During the summer feeding period (April- September, Q2 and Q3) there were 19 CON and 20 hay milk samples collected. All of them have been analysed from April- July 2016.

4.1.2 Butter

For preliminary tests conventional butter from a local supermarket was used.

4.2 Equipment

4.2.1 Gas chromatograph

The quantitative analyses of FAMEs were carried out by a gas chromatograph from Thermo scientific (Trace GC ultra) with a flame ionisation detector. The used column used for minor FAs was a RTX-225 (30 m; 0,25 mm id; 0,25 µm df) from Restek. Because compared to packed columns they have a better separation capacity. It is also possible to use longer columns with less amount of sample. The longer the column, the better the resolution. On the other hand, the analysis takes longer and results in broader peaks. The choice of the carrier gas, as well of the carrier gas flow is of importance. Hydrogen was used as carrier gas, because of the optimum in correlation with the Van-Deemter equation. Further an auto sampler from Thermo Quest (AS2000) was used. The main FAs were already analysed with the SP2560 column. Components were identified using a 37- component standard from supelco.

4.2.2 GC- mass spectrometer

The structural analysis of FAs in milk were performed using the Trace DSQ GC-MS from Thermo Scientific fitted with a RTX-225 (30 m; 0,25 mm id; 0,25 µm df) column from HP and a FID.

4.3 Reagents and chemicals

Chemicals for the milk fat extraction, trans- methylation methods, concentration techniques and for the chromatographic analysis.

The particular devices and expendable items are listed in Table 8 and Table 9. All chemicals have a P.A. quality (see Table 7).

Table 7: List of reagents

chemical	manufacturer
2,7- dichlorofluoresceine	Sigma Aldrich
Acetonitrile	VWR
Acetyl chloride	Sigma Aldrich
Acetyl chloride	Sigma Aldrich
Ammonia solution 25 %	Carl Roth
Chloroform	Carl Roth
Diethl ether	VWR
Ethanol	Chem-Lab NV
Extran®	Merck
Florisil® adsorbent	Fluka
Glass wool	Supelco
Glass wool	Supelco
Methanol	Merck
n- heptane	Carl Roth
n- hexane	Carl Roth
Petrolether	Carl Roth
Potassium carbonate	Carl Roth
Potassium hydroxide	Sigma Aldrich
Silica gel 60	Fluka
Silver nitrate	Carl Roth
Sodium sulfate	Sigma Aldrich
Soldium chloride	VWR
Tetr, Butylmethylether	Carl Roth
Toluol	Carl Roth
Urea	Merck

Preparation of the used solutions:

1. 5% methanolic HCl

8 mL acetlychlorid was added dropwise into 80 mL methanol and stirred by a magnetic stirrer.

2. 6% potassium carbonate

6 g of potassium carbonate were weighed into a screw-top bottle with a magnetic stirrer and dissolved with 100 mL dest. aqua.

3. KOH in methanol (2M)

56.10 g of KOH were weighted into a screw-top bottle and with a magnetic stirrer dissolved with 500 mL of CH_4O.

4. KOH in methanol (6M)

168 g of KOH were weighted into a screw-top bottle and with a magnetic stirrer dissolved with 500 mL of CH_4O

Table 8: List of used equipment

device	manufacturer/ type	position
Centrifuge	Funke Gerber SuperVario- N	-
Cooling pump	Thermo scientific WKL 26	0°C
Heating bath	Büchi B-480	40°C
Oven	Thermo scientific Heraeus	130°C
Rotavapor	Büchi	-
Scale	AandD; FX-300	-
Small evaporator	Pierce	N_2 Gas
Solid phase extraction vacuum manifolds	Supelco Visiprep	
Vacuum Controller	Büchi V-850	-
Vacuum pump	Büchi V-700	-

Table 9: List of consumables

consumables	manufacturer	details
DC- ready-to-use plate	Macherey- Nagel	20 x 20 cm
Filter paper	Whatman	-
Funnel	-	-
Magnetic stirrer	-	-
Monnojer flask	-	20 mL
Pasteur pipettes	Kimble	-
Pipettes	-	2 µl 10 µl 25 µl
Pointed flask	Schott Duran	250 mL
Pyrex tube	-	10 mL
Volumetric flask	-	50 mL

4.4 Analysis of fatty acids in milk

4.4.1 Sample preparation

First, the frozen samples collected in plastic tubes were defrosted to room temperature and then placed in a water bath and stepwise heated up to 40°C and mixed thoroughly. After letting them cool to room temperature again, they were ready for use (see Figure 14).

Bligh and Dyer and the acidic transmethylation were only used for pre- testings'. For the identification concentration methods like, Ag^{+}-TLC and urea adduct formation were used. An adapted Röse- Gottlieb method and the alkaline transmethylation were used for the final sample analysis. The method from Bligh and Dyer is especially applicable for preparative analysis and used for this experiment (pre- testings), because the relation of utilized sample and the received amount of fat is expedient.

Figures 14 and 15 represent the sample preparation and the scheme of analysis.

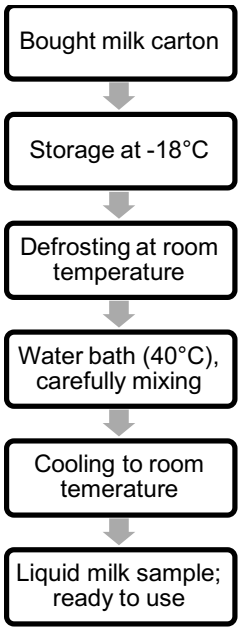

Figure 14: Scheme of milk sample preparation

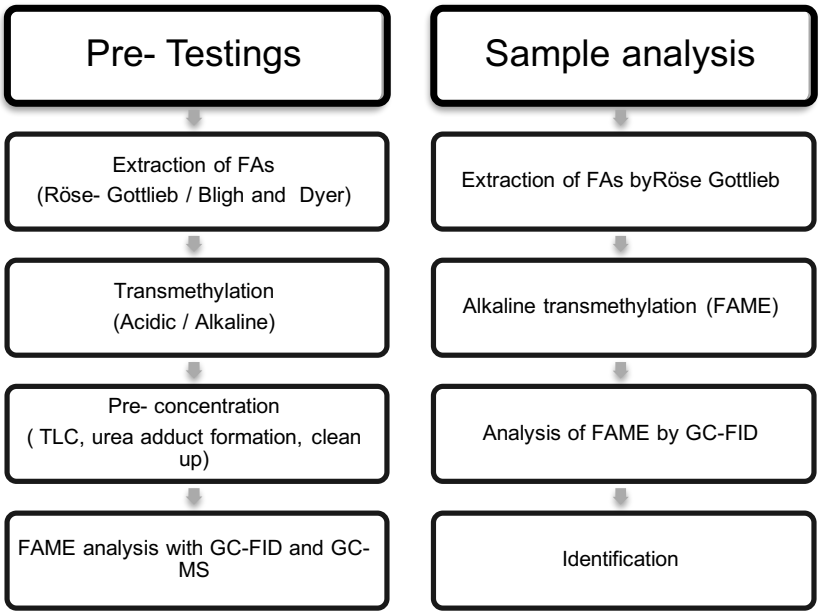

Figure 15: Scheme of pre-testing and final sample analysis methods

4.4.2 Isolation and extraction of milk fat

In order to have the milk fat present, it must get separated from the rest of the milk compounds. The isolation of the total fat was done in two different ways. One way was the isolation by an adapted Röse- Gottlieb method, the other by Bligh and Dyer (Bligh and Dyer, 1959). These extraction methods were chosen, because FAMEs were needed mainly for the preparative analysis and because no quantitative evaluation was needed.

4.4.2.1 **Bligh and Dyer method |** KJ

Four mL of methanol and 2 mL of Chloroform $CHCl_3$ were added to 2 mL of milk sample in a pyrex tube. The pyrex containing the mixture was shaken for 1 minute. Afterwards 2 mL of $CHCl_3$ and 2 mL of dH_20 were added, followed by a centrifugation step for 5 min at 1100 rpm. A pasteur pipette was cut with a knife to fit into the pyrex. Another pasteur pipette was used to reach the lower phase of the two-phase system in the pyrex tube. The lower phase was taken up, put in a clean pyrex tube and vaporized with the use of N_2 to remove the $CHCl_3$. The remaining part which contained the extracted fat was then acidly trans methylated (Bligh and Dyer, 1959).

4.4.2.2 Adapted Röse-Gottlieb method | OI

Some steps were adjusted from the original method, to use the method as a preparative technique. These adjustments were taken from a former student. Following steps had been changed:

- Only one extraction step per sample was performed (instead of 3), since no quantitative method was needed
- For the prevention of “Ghost Peaks”, ethanol was replaced by methanol.
- The solvents were evaporated in a rotary evaporator with a bath temperature of 40°C and 156 mbar
- The fat- residual got solved in n- hexane without prior drying

By this method, the sample is solubilized under alkali conditions, whereas the milk emulsion is broken and proteins are denaturated. 10 mL of each milk sample (84) were transferred into a mojonnier flask. 2 mL ammonia (25 %) were added and mixed. 10 mL methanol were added and mixed thoroughly again. Then 25 mL MTBE were added and shaken for about one minute. At last, 25 mL petroleum ether were added and mixed thoroughly again. It is of importance to mix the solution after every addition very good, to release the embedded fat from the proteins. The mixture was then allowed to stand for 1 h until the layers got separated. The upper layer (= organic) was carefully decanted and transferred into a pointed flask. The solvent in the layer was then evaporated by a rotary evaporator at 40 °C. Afterwards, the fat residue was dissolved with 5 mL n- hexane and transferred into a 8 mL glass tube, filled with one spatula sodium sulphate, to remove water. The solvent, n- hexane, got again evaporated under steam of nitrogen. The remaining fat was transferred into a vial for further analysis.

4.4.3 Transmethylation of milk fat

4.4.3.1 Alkaline transmethylation | KJ

One drop of extracted milk fat was pipetted in a pyrex tube and the addition of 2 mL hexane followed. Then 0.2 mL of methanolic KOH (2 M) was added and the mixture was vortexed at room temperature. Then two phases could be observed, the upper one was taken off with a pipette into a Pyrex containing sodium sulphate to remove any possible traces of water. Finally, the methyl- esters were transferred to a vial (Christopherson and Glass, 1969).

4.4.3.2 Acidic transmethylation | OI

The acidic transmethylation was run as described in the one-step methylation method by (Christie, 1993). Contrary to conventional methods, first extraction with subsequent trans-methylation, this method offers the advantage to unite both steps into one single step. 1 mg of lipids was suspended in 1 mL toluene. As methylation reagent, 3 mL methanolic HCl were added and mixed. For the trans- methylation reaction, the mixture was heated in a water bath at 70°C for two hours. Afterwards, the reaction got stopped by the addition and mixing in of 5 mL 6% K_2CO_3 solution. The upper layer, which contains the FAMEs, was carefully removed by the help of a Pasteur pipette and dried over anhydrous sodium sulphate until the solution became clear. The solvent in the layer was then evaporated by a rotary evaporator. Subsequently the solvent was transferred into a GC- vial and sealed.

4.4.4 Identification by silver ion thin layer chromatography | OI

The principle of this method is, that the separation of the compounds is based on the number and configuration of DBs. They built up complexes with metals. Unsaturated compounds act as electron- donators and the silver ions as electron- acceptors. The quantity of DBs determines the stability and retention of the built complexes and therefore the migration on the plate. The obtained fractions can be further separated by other chromatography methods, like gas chromatography coupled to a mass spectrometer.

First, the silica gel TLC plates were conditioned at 120°C for 30 min. 5 g of silver nitrate were weight in and suspended with 50 mL acetonitrile.

The solution was then transferred into a glass tank, which was made fat-free by extran® before. The thin layer plate was then immersed into the vessel and closed completely. The tank was placed at a dark place for one hour. By this step the plate got impregnated. For activation, the plate was moved back into the compartment dryer for 30 min.

After cooling the plate, a line was drawn 2 cm above the end of the plate. There about 50 µL of the *trans*- methylated butter- sample was applied as a 3- cm band by using a pasteur pipette. After the application, the prepared mobile phase (hexane-diethyl ether 9:1, v/v) was poured into the chromatography tank. For the fractionation, the plate was also placed in the tank until the solvent front nearly reached the end of the plate. After the development, the plates were left to air dry.

For the detection of the bands, the plate was evenly sprayed with a solution of 0.1% (w/v) 2,7- dichlorofluorescein in 95% methanol and viewed under UV light (366 nm). The fluorescent bands (6), saturated and *trans/cis* -monoenes, were marked and for identification they were scraped from the plate.

For the extraction of the FAs, pasteur pipettes (6) were used as columns. First, the pipette was placed into a pyrex tube and filled with some glass wool. Then, sodium sulphate, florisile and sodium chloride were added in this order, by the help of a funnel. Finally, each of the scraped band was filled on top of one column. Afterwards the FAs got extracted by a solution of hexane and diethyl ether (1:1) and collected in the pyrex tube (ca. 5 mL). The six extracts were evaporated under nitrogen steam to about 1 mL and transferred into a GC-vial.

4.4.5 Concentration - enrichment of polyunsaturated fatty acids

4.4.5.1 Concentration of phospholipids | KJ

1.31 g melted butter was dissolved in 50 mL solvent agent. The solvent agent was prepared with: 25 mL hexane plus 25 mL diethyl ether and 1 % acid. The column was fist cleaned with pure hexane, connected to a gentle vacuum, then the sample and solvent got rinsed trough the column. The liquid that passed through the column was discarded. The column was rinsed with 20 mL methanol and after passing through the column it was collected in a glass flask.

4.4.5.2 Urea adduct formation

4.4.5.2.1 Urea adduct formation (triglycerides) - 4 hours | KJ

3.4 g melted butter was dissolved in 100 mL methanol where 20 g urea was added. For a complete dissolving of urea, the mixture was warmed and afterwards cooled down to room temperature with swirling in between. The mixture stood overnight, approx. 20 h. Overnight, the urea-complexes were formed. The complexes were filtered through a Buchner funnel with stepwise addition of overall 6 mL methanol in potassium hydroxide (KOH). To remove the water, sodium sulphate was added and the solution was filtered through glass wool into a glass eprouvette. For removing of the solvent the mixture was put evaporated by using the rotavapor (water bath at 42 °C and vacuum at 156 mbar).

4.4.5.2.2 Urea adduct formation (methyl esters) – overnight | OI

The enrichment of polyunsaturated FAs via urea adduct formation overnight is a good method, when only small amounts of esters are available (Christie, 2011).

100 mg of the methyl esters were dissolved in 4 mL hexane. 1.5 g urea were moistened with methanol (15 drops) and added to the dissolved methyl esters. The mixture was allowed to sit overnight. Next day, the solid was filtered off and thoroughly washed with hexane (two times). The filtrate and the washings were combined and dried over a spatula anhydrous sodium sulphate. The solution was then evaporated using a rotary evaporator at 40°C.

4.4.5.3 Clean up | KJ

A glass pasteur pipette was cut at its thin end, plugged with glass wool and silica and treated with 4 mL hexane. Approx. 1 mL prepared methyl esters was put through the pasteur pipette Then the methyl esters were eluted in 10 mL of a mixture of hexane and diethyl ether (relation 95:5 v/v). After rinsing though the column, the purified liquid was collected in a vial.

4.4.6 Determination of the fatty acid pattern

4.4.6.1 Gas chromatograph injection | OI

The injector, also known as the sample inlet system precipitates non- volatile compounds from the sample and ensures the sample being applied gaseous and in correct concentration. The injection temperature should be over 220 °C.

For the pre- tests with butterfat the injection was done by hand.
The syringe was purged with heptane (three times) and then filled up with 0.8 µL heptane. Afterwards the syringe got filled with air until the 1 µL scale. Then, 1 µL of the sample got filled into the syringe. Subsequently, the syringe was put into the injection slot and waited for 5 seconds before the sample got injected. While injecting the GC- programme was started simultaneously. Before releasing, it was counted to 5 seconds again, after injection.

The FAMEs- milk samples were injected by auto sampling. There, the vials were put into the auto sampler equipment and the auto sampler injected in the same way the injection like it is described (Grob, 2008).

Split (SP) and splitless injection | KJ

Gas chromatographic analysis often results in chromatograms showing overloading effects, especially when higher amounts of sample should be injected. For avoidance of these effects the sample can either be diluted or/and an optimized injection should be conducted.

By using the SP injection, the sample gets into a hot evaporation chamber to reach a quick evaporation. Also it is introduced to the column while the injector is opened. Peaks result in higher resolution due to an increased flow rate. The evaporated sample gets diluted with the carrier gas, so only a small part of the sample volume reaches the column. The bigger part of the volume is vented from the system. Advantages of the SP injection is a narrow injection

zone and the prevention of overloading; due of a reduced amount of sample volume. The main disadvantages are the mass discrimination of components with different volatility, limitations in trace analysis and potential systematic failures in quantitative analysis. Furthermore, SP injection is unsuitable for trace amounts, because the losses might be too high for proper detection. For analysis of trace amounts SL is appropriate.

The injection is done in a solvent that has a higher boiling point compared to the column during which the split exit is/valve is closed. The sample evaporates and nearly a compete transfer to the column takes place. Using SL “tailing” of the solvent can be minimized, almost the entire sample gets into the detector and so it is more suitable for trace analysis. SL is often chosen for injection of diluted samples, as we had them in our study. Disadvantages of SL is that the stationary phase can be damaged due to condensation of the solvent and only columns with chemically bonded phases can be used (Horak, 2012; Badertscher, 2015).

4.4.6.2 Testings with column RTX-225 | OI

Two different temperature programs were used for the pretesting. One for Spilt and one for Splitless injection (see tables below).

Table 10: Pretesting conditions using RTX-225 Splitless

Splitless	
Temperature Program	120 °C for 1 min 20 °C/ min to 170 °C 2.5 °C/ min to 220 °C for 1 min
Injection volume	1 µL
Carrier gas	H_2
Constant flow	2 mL/ min
Velocity	100 mL/ min
Detector	FID, 240 °C

Table 11: Pretesting conditions using RTX-225 Split

Split	
Temperature Program	120 °C for 1 min 20 °C/ min to 170 °C 2.5 °C/ min to 220 °C for 1 min
Injection volume	1 µL
Carrier gas	H_2
Constant flow	2 mL/ min
Velocity	100 mL/ min
Detector	FID, 240 °C

4.4.6.2.1 Gas chromatograph method for milk fat analysis

Final FAMEs were analysed using a gas chromatograph from Thermo Scientific, with a FID and a fused silica capillary column, RTX-225.The carrier gas was hydrogen and the spilt ratio was 1:50.

Table 12: Final GC-FID method

Splitless	
Temperature Program	120 °C for 1 min 20 °C/ min to 170 °C 2.5 °C/ min to 220 °C for 1 min
Injection volume	1 µL
Carrier gas	H_2
Constant flow	0.5 mL/ min
Velocity	10 cm/ s
Detector	FID, 250 °C

4.4.6.2.2 Quantitative analysis

The single FAMEs were identified through comparison of the retention times of an authentic 37- component FAME Standard. The quantification was done by the peak areas. The results are represented as area percent. Also theoretical response factors (Schreiner and Hulan, 2004) have been used for the correction of the peak areas. The calculation of the content of total FAs from the GC-FID FA- profile was done by following:

1. Mass- percentage calculation FAME of all FAs with a retention time < C18:3n3 (Split injection)

➔ peak area of FAME / sum of all peak areas * 100%

General equation of the calculation of peak area percent

$$W_i = (100 * F_i) / F_N$$

W_i= mass fraction of FAME i

F_i= peak area of FAME i

F_N= Sum of all peak areas

2. All FAs with a retention time ≥ C18:3n3 (Splitless)

➔ Peak Area FAME / C18:3n3 *100 %

3. Results of FAs with a retention time ≥ C18:3n3 related to the qualitative amount of C18:3n3 from the first chromatogram (Split)

➔ (FAME/ 100) * C18:3n3

4. Finally, all peak areas were normalized to 100%

4.4.6.3 Qualification with gas chromatograph - mass spectrometry

4.4.6.3.1 GC- MS injection

The GC-MS injection was done like the injection GC-FID. The chromatograms of both methods can be used for identification, because the same type of column (RTX225) was used. The pattern of elution stays therefore the same, only the temperature and the carrier gas (He instead of H_2) had to be adjusted.

Table 13: Pretesting conditions for GC-MS using RTX-225 Split

Split: SP Rate 1:100; T= 250°C	
Temperature Program	140 °C for 1 min 3 °C/ min to 220 °C hold for 5 min.
Injection volume	1 µL
Carrier gas	Helium
Constant flow	1.5 mL/ min
Mass transfer line	250 °C

Table 14: Pretesting conditions for GC-MS using RTX-225 Splitless

Splitless: SL time= 20 sec	
Temperature Program	120 °C for 1 min 3 °C/ min to 220 °C hold for 5 min.
Injection volume	1 µL
Carrier gas	Helium
Constant flow	1.5 mL/ min ≙ 75 cm/ sec
Mass transfer line	250 °C

4.5 Evaluation and statistical methods

4.5.1 Evaluation of the chromatograms

The qualitative determination of the FAMEs in the samples was run with a standard. A 37-component standard got injected in the GC-FID and through comparison of the retention times the FAMEs in the samples could be identified. Because both analyses used the same type of column, the elution pattern is the same.

The GC-MS chromatograms were analysed using the installed software "XCalibur™" and by using data from the online data bank (AOCS), it was possible to identify the peaks of the GC-MS according to their mass spectra. Various fragments, from the subject of interest, are produced by electron impact and are separated according to mass (strictly speaking mass/charge (*m/z*) ratio in which $z = 1$) in a magnetic field. "A spectrum is obtained, that in effect is a bar diagram showing the masses of the fragment ions and their abundances relative to the most abundant ion (base ion) (Christie, 2014)". Therefore, every FAME has a characteristic mass-spectra. The molecular ion is the ion with the heaviest ion (greatest *m/z* value) and represents the relative mass formula of the compound (sum of the masses that make up the molecule). An ion at $m/z = 74$, also called the McLafferty rearrangement ion indicates and confirms a methyl ester. An ion at $m/z = 108$ represents an n-3 FA and the n-6 ion is at $m/z = 150$ from the carboxyl end.

4.5.2 Statistical methods

All calculations were conducted with Microsoft Excel (Version 15.24) and the Statistical Software XLSTAT.

Outlier-Test:

After the calculation of the Peak area percentage, a Grubbs- outlier test (application in XLSTAT) was run, only for the long chain FAs (starting at C18:3n3), followed by the deletion of the outliers.

Afterwards, the missing values (pink) and the outliers (yellow) were replaced with the mean value of each FA (HAY and CON separated) and calculated back to 100 % (see Table 24-28).

t-Test:

A two- sided, two sample t-test was run with XLSTAT. Single FAs of HAY and CON milk samples were compared. (see Table 35 and 36)

5 Results and discussion

5.1 Extraction and isolation of milk fatty acids | OI

Both extraction methods worked quite well and the same amounts of fat could be extracted. Röse- Gottlieb method was chosen, because this method is less time intensive, effortless and a more common, reference method. Further, the process is the recommended extraction method from the EU.

Based on reaction time the alkaline trans- methylation method was chosen. It is a rapid and well-established procedure and for analysis of esters from TG and PL. Moreover, it is recommended for PUFAs and conjugated FAs (like CLAs), which are of main interest. This study focuses on the minor FAs (Ichihara *et al.,* 1996). Also alkali *trans*- methylation does not generate isomerisation of *cis/trans* UFA or artifacts and methanolic KOH would not interact with free FA (Simionato *et al.,* 2010; Christie, 1993).

5.2. Concentration and identification methods

To analyse these minor FAs in milk fat concentration methods were applied. For purposes of this study these two methods were used: thin layer chromatography (TLC) and urea adduct formation followed by an evaluation with GC-MS. Results of the concentrations methods were used for FA identification on HAY and CON samples (main experiment).

5.2.1 Silver- ion thin layer chromatography | OI

After the GC-FID analysis, there were many not yet identified FAs. These should be identified TLC coupled with a GC-MS.

For the segregation of FAs according to the number of double bounds and their geometrical configuration, silver ion chromatography is a favourable method for it. Highly unsaturated fatty acids retain at the starting line, SFAs migrate towards the solvent front.

Figure 16 shows the 6 fluorescein bands, which could be separated by TLC.

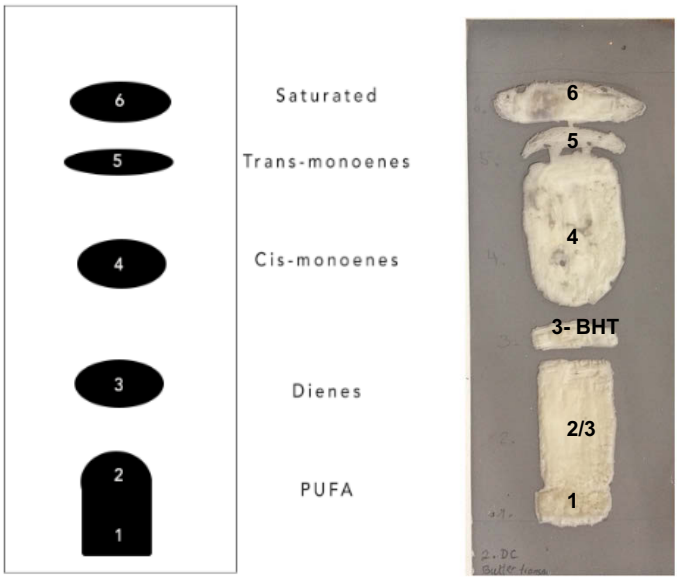

Figure 16: Schematic separation of FAME by Ag+-TLC

For qualitative evaluation of the Ag^+-TLC the retention factor (R_f) was used. The R_f value is defined as follows:

$$R_f = \frac{distance\ starting\ line - middle\ of\ spot}{distance\ starting\ line - solvent\ front} = \frac{a}{b}$$

The obtained R_f values for the fractions 1-6 are: 0.08; 0.29; 0.40; 0.73; 0.88; 0.94

After the extraction from the silica gel, it was possible to detect and determine the fractions by GC-MS. Further, it was possible to identify branched- isomers.

After the extraction from the silica gel, it was possible to detect and determine the fractions by GC-MS. Further, it was possible to identify branched- isomers.

The first (from the bottom) contained the PUFAs with 3 to 5 double bonds. The second band contained the 2 to 3 unsaturated FAs. Mono- and dienunsaturated FAs could be found in the third band. The bands 4 and 5 contained monounsaturated FAs, whereas band 4 contained the *cis*- and band 5 the *trans* isomers. Band 6 contained all SFAs, as well branched isomers.

Figure 17, Figure 18, Figure 20 and 21 start at minute 16, because only the LCFAs are of interest. In Figure 22 the whole chromatogram is shown, because all SFAs in milk are shown here.

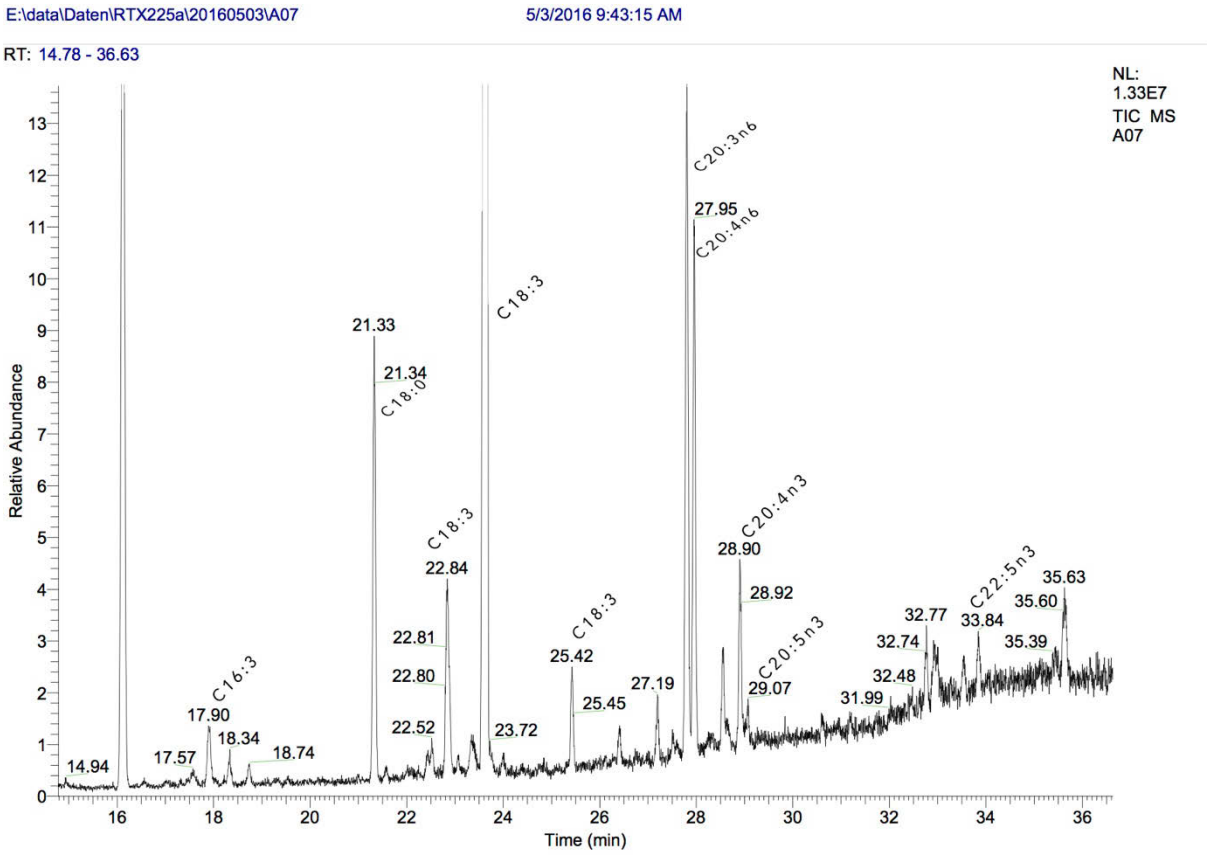

Figure 17: TLC- chromatogram of band no.1, acc. to figure 16: PUFA

Figure 17 shows the peaks of the first band of the TLC. Mostly, PUFAs can be seen in this chromatogram. Some SFAs can also be found. It is possible that they did not migrate on the plate.

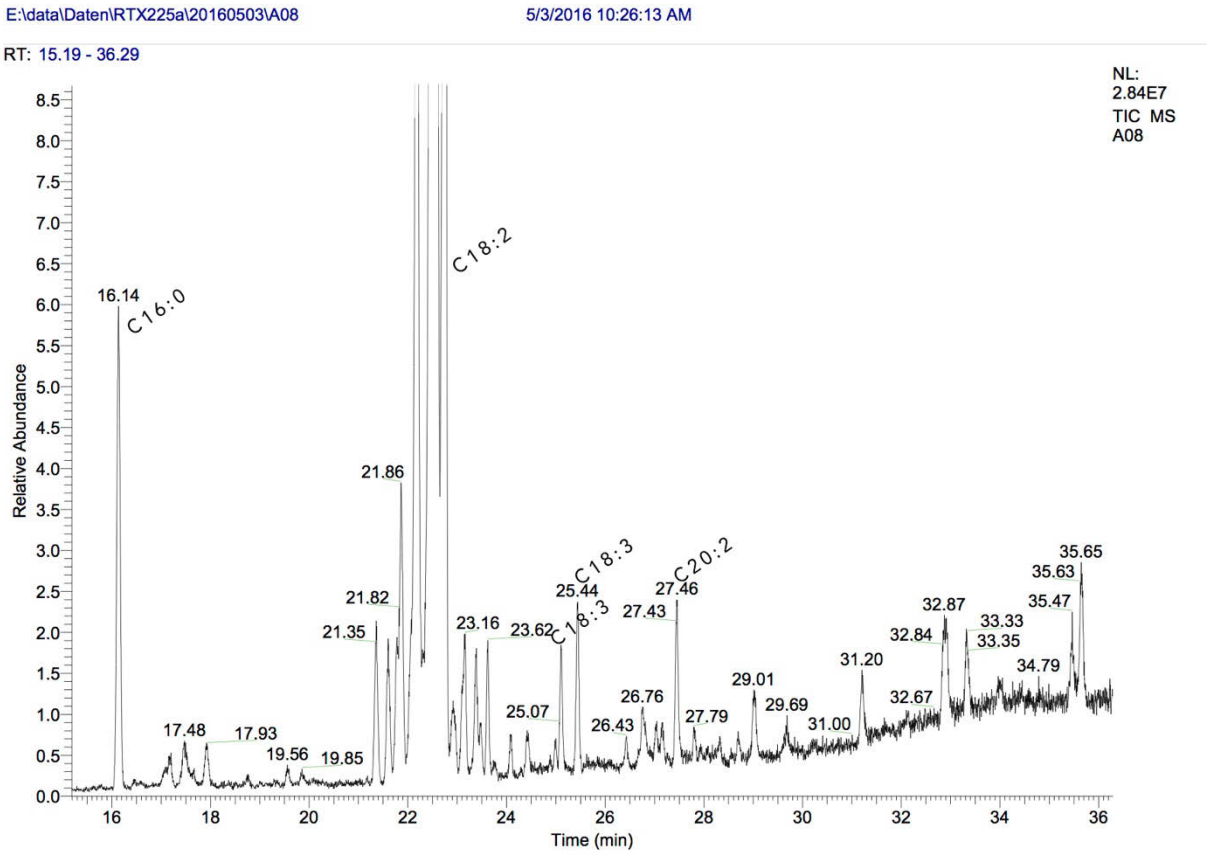

Figure 18: TLC- chromatogram of band no.2/3, acc. to figure 16: PUFA and dienes

Figure 18 shows the con band of the TLC. Dienes and PUFAs can be detected as well as still saturated FAs.

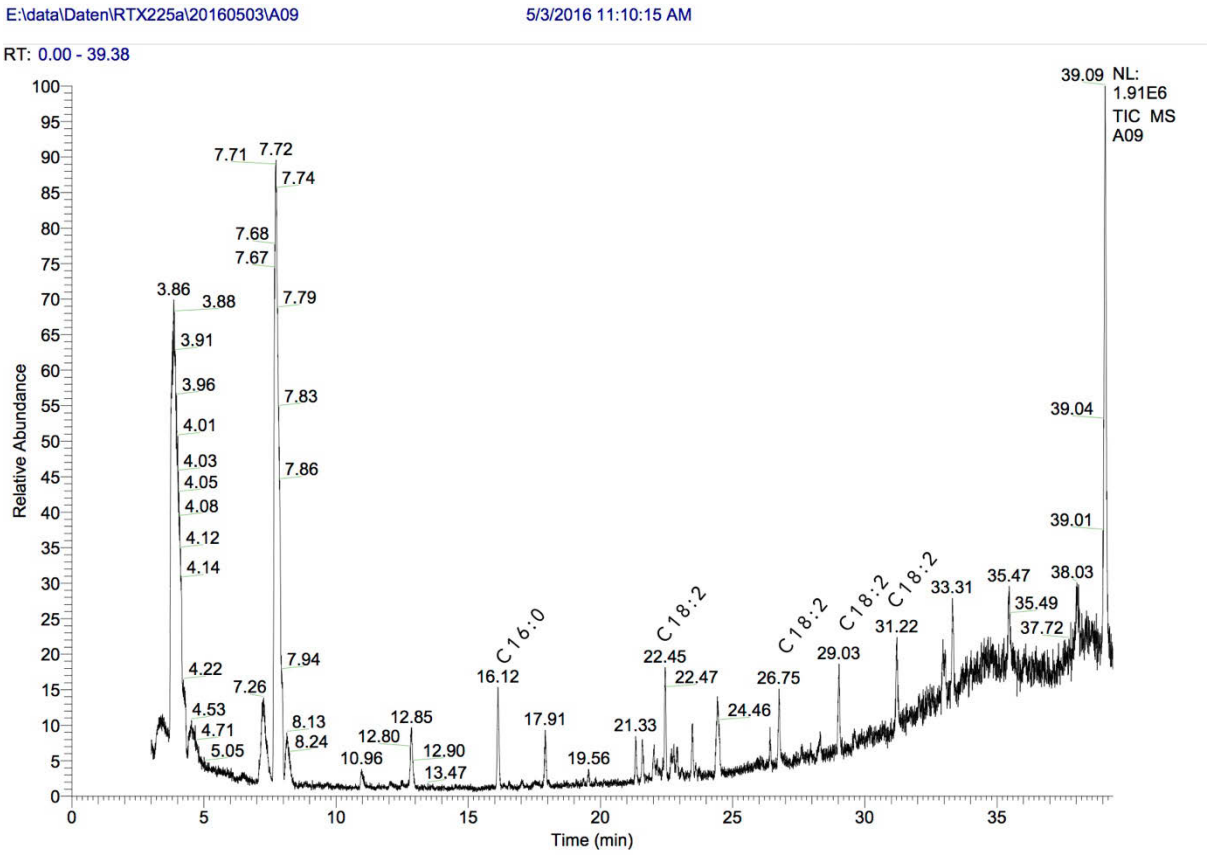

Figure 19: TLC- chromatogram of band no.3, acc. to figure 16: BHT

As visible on Figure 19, representing band 3, many C18:2 isomers can be found. These are not correctly identified, as at minute 23 long chain FAs should be located. It seems that the column got contaminated. Further, the peaks at minute 3.86 and 7.72 might cause the spot on the plate. The peak at minute 3.86 is C10:0 and the peak at minute 7.72 is butylated hydroxyltoluene (BHT). C10:0 stopped migrating on the plate because the concentration might be very high.

It is hard to find/ identify other dienes, because, in milk, until today there are not many other long-chain dienes found yet. C18:2n6 is present in a higher content than e.g. C20:2n6 in milk. That fact makes it further difficult to identify the other dienes.

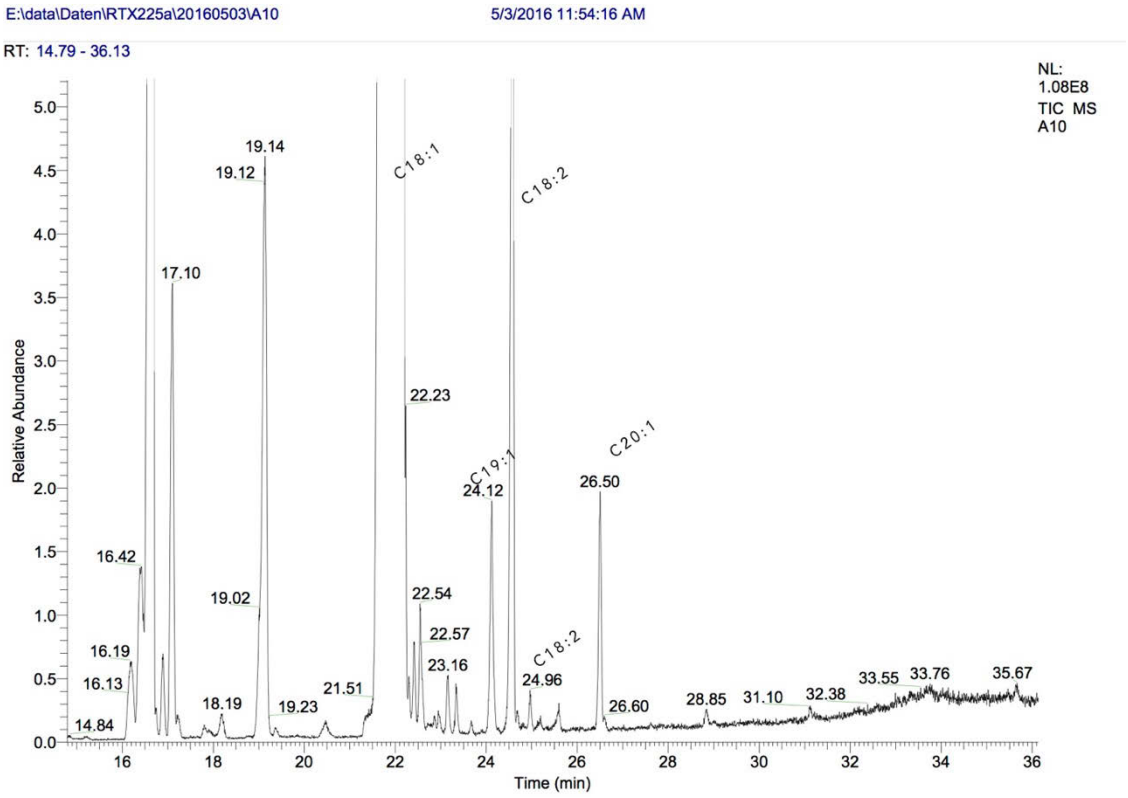

Figure 20: TLC- chromatogram of band no.4, acc. to figure 16: MUFA and cis-isomers

Band 4 Contains the MUFAs- *cis* isomers.

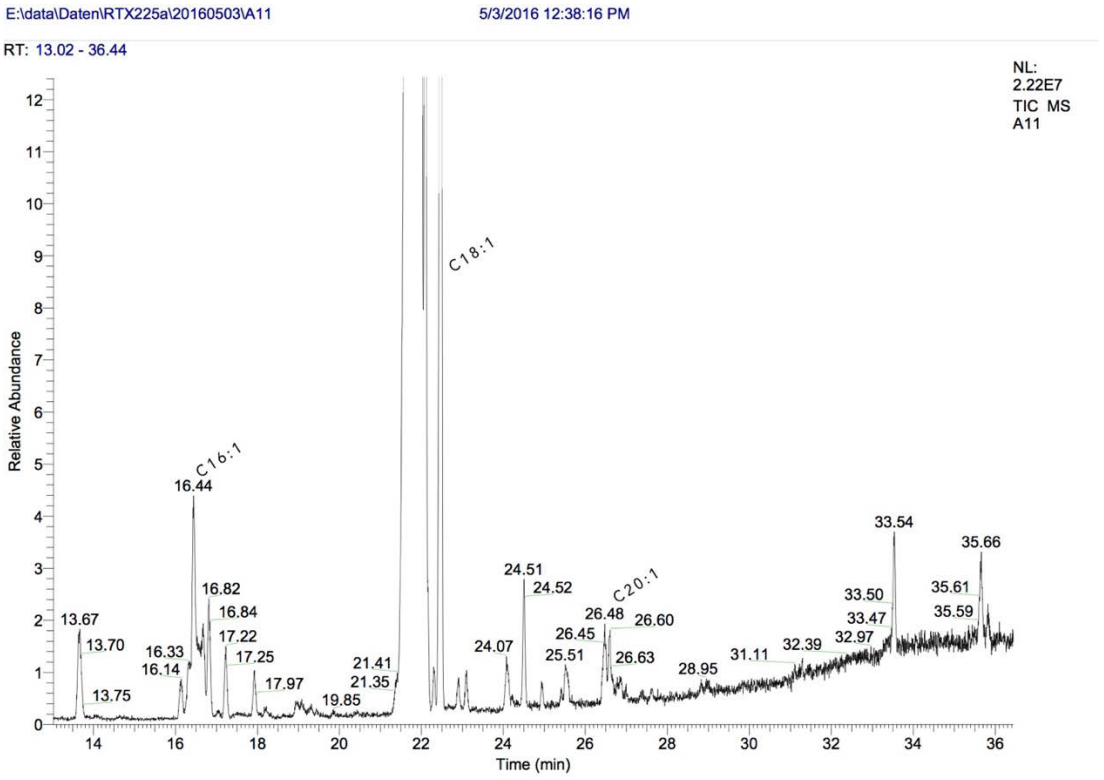

Figure 21: TLC- chromatogram of band no.5, acc. to figure 16: MUFA and trans isomers

Band 5 contains the MUFAs- *trans* isomers.

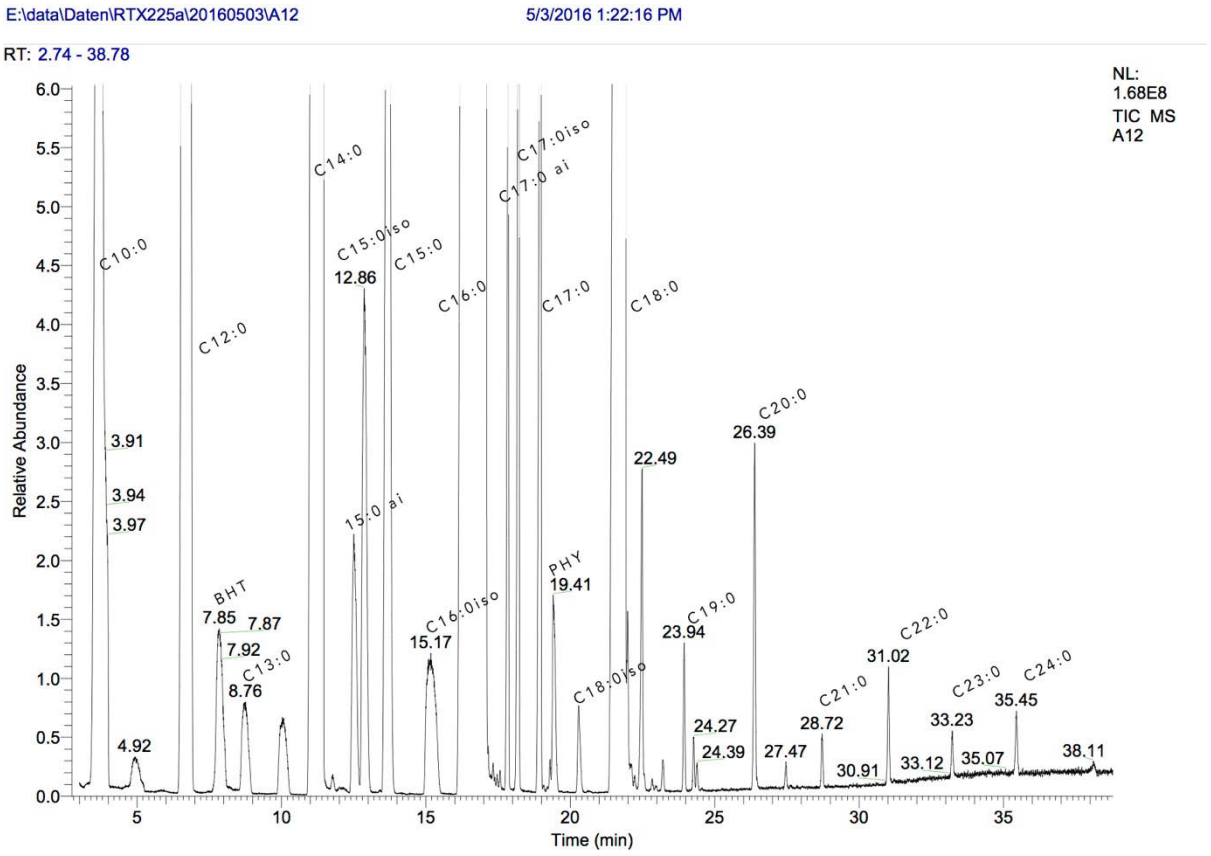

Figure 22: TLC- chromatogram of band no.6, acc. to figure 16: SFA

The figure of band no. 6 shows the branched and odd- chain SFAs and SFAs. Also the antioxidant BHT is detected at minute 7.85 and PHY at 19.41 min.

It is not easy to detect and determine minor components, because they are not always sufficiently resolved by GC-FID (Christie, 2011). By prior concentration steps, the problem can be solved.

Ag^+- TLC is a technique to separate the FAs according to the number and configuration of DBs. The principle is, that unsaturated compounds built a polar complex with metals. The greater the number of DBs is, the stronger the complexation effect is. In other words, the retention is determined by the number and configuration of the DBs. *Cis* bound stronger than *trans* and migrate therefore less far than *trans* on the plate.

Though spraying the plate with an UV-dye, the fractions become visible under a UV-lamp (366 nm). From this visual inspection, it could be seen, that a separation was achieved on the basis of degree of unsaturation. Six bands could be found on the plates. After scratching-off the fractions and extraction of the FAMEs they could be identified by GC-MS.

Like expected, it was possible to see, that band no.6 contained the SFAs and band no.1 the highly unsaturated FAs. PUFAs retained at the starting line, SFAs migrated towards the solvent front. Also, *iso* and ante-*iso* isomers could be detected in band no. 6. All of them are found between two SFAs, for example C15:0iso is between C14:0 and C15:0 and C15:0ai between C15:0iso and C15:0. As it is visible from the figures below, it is not always easy to determine all the peaks with GC-MS. The abundance is very low and therefore very hard to identify the peaks. Also the content of the LCFAs is very low, which reflects another difficulty of the work.

Overall, the aim of the Ag^{+}- TCL was to separate the SFAs from the unsaturated FAs and this could be achieved. The unsaturated FAs are not well separated but as it can be seen, band 6 contains all SFAs.

Table 15 shows the found FAs and the corresponding retention time, starting at minute 21.50, because only the LCFAs are of interest.

Table 15: Retention times and corresponding peaks, referred to figure 17-22

Time [min]	FA
21.50	C18:1
22.24	C18:0
23.60	C18:3n3
23.94	C19:0
24.49	CLA
24.91	C18:2
25.43	C18:3
26.42	C20:0
26.50	C20:1
27.46	C20:2n6
27.81	C20:3n6
28.00	C20:4n6
28.72	C21:0
28.91	C20:4n3
29.07	C20:5n3
31.02	C22:0
32.78	C22:2n6
33.23	C23:0
33.85	C22:5n3
34.00	C22:6n3
35.46	C24:0

5.2.2 Urea adduct formation | OI

Urea adduct formation is a method to separate and eliminate SFAs from unsaturated FAs. When the mixture is allowed to slowly cool down, urea crystals are formed again. These crystals enclose the SFAs. PUFAs remain in the solution.

Since butter consists mainly of milk fat, it is appropriate for conducting the pre- tests. In addition, one does not have to extract the milk fat. It is easier and faster to take the raw butter fat. The FA profile is nearly the same and can be represented as milk FAs. Pure butter FAs were used for with urea adduct formation. The first experiment with methyl- esters overnight did not work properly. The mixture built one big crystal and no solution which should have been for analysis was left. It was tried to dissolve the crystal again. Both GC-MS chromatograms were not satisfying, because they were too overloaded. After a further dilution step, the problem of unidentifiable was still present. As there are still many SFAs, the method did not work. The second attempt was successful. In this trial, the mixture was not stirred over the whole night and got covered. Therefore, the solution could not evaporate. Both chromatograms (two dilutions) show well-separated and narrow peaks, also nearly no SFAs have been detected. The separation with urea adduct overnight formation is useful for identification purposes, in contrary to the 4 h method. The 4 h method is indeed less time consuming, but the chromatograms could not really be used for identification purposes, because the peaks were not as nicely separated as from the overnight method. Moreover, some SFAs were released and did not bind to urea. In both methods solutions had to be diluted with hexane prior to the GC-MS injection. Otherwise no separation would have been possible. The best separation could be achieved with urea adduct formation overnight (see chromatogram a09, Figure 24). As the advice of Christie suggests, the overnight method is a proper method if only small amounts of esters are available (Christie, 2011).

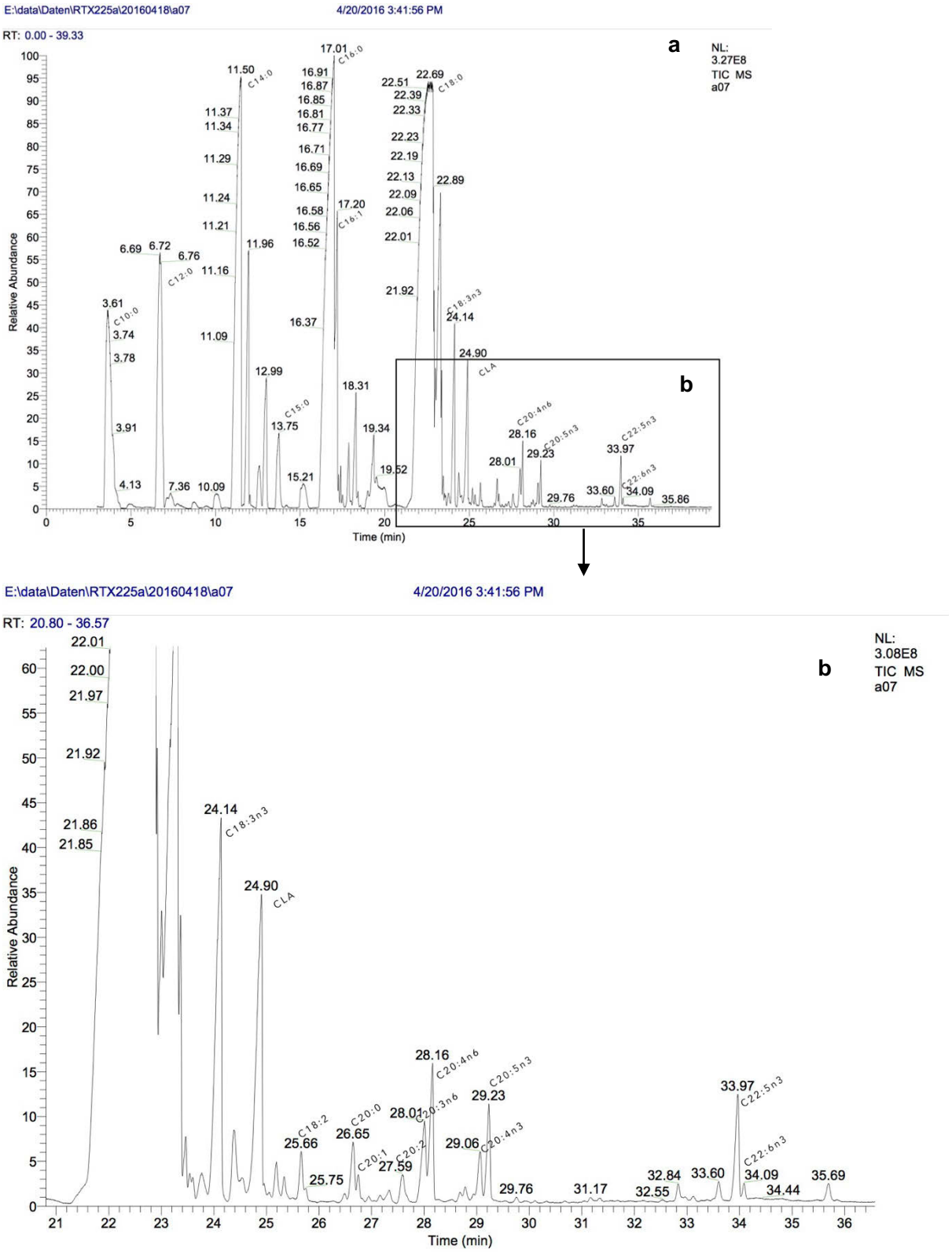

Figure 23: Chromatogram of the 1st attempt of urea adduct formation; overnight method; diluted once

The chromatogram above (Figure 23) shows the urea adduct formation from the first attempt. The solution got one time diluted with hexane. It is obvious, that the separation

improved by one further dilution step, but the chromatogram is still too overloaded. With the dilution step, the assumed SFAs could even be set.

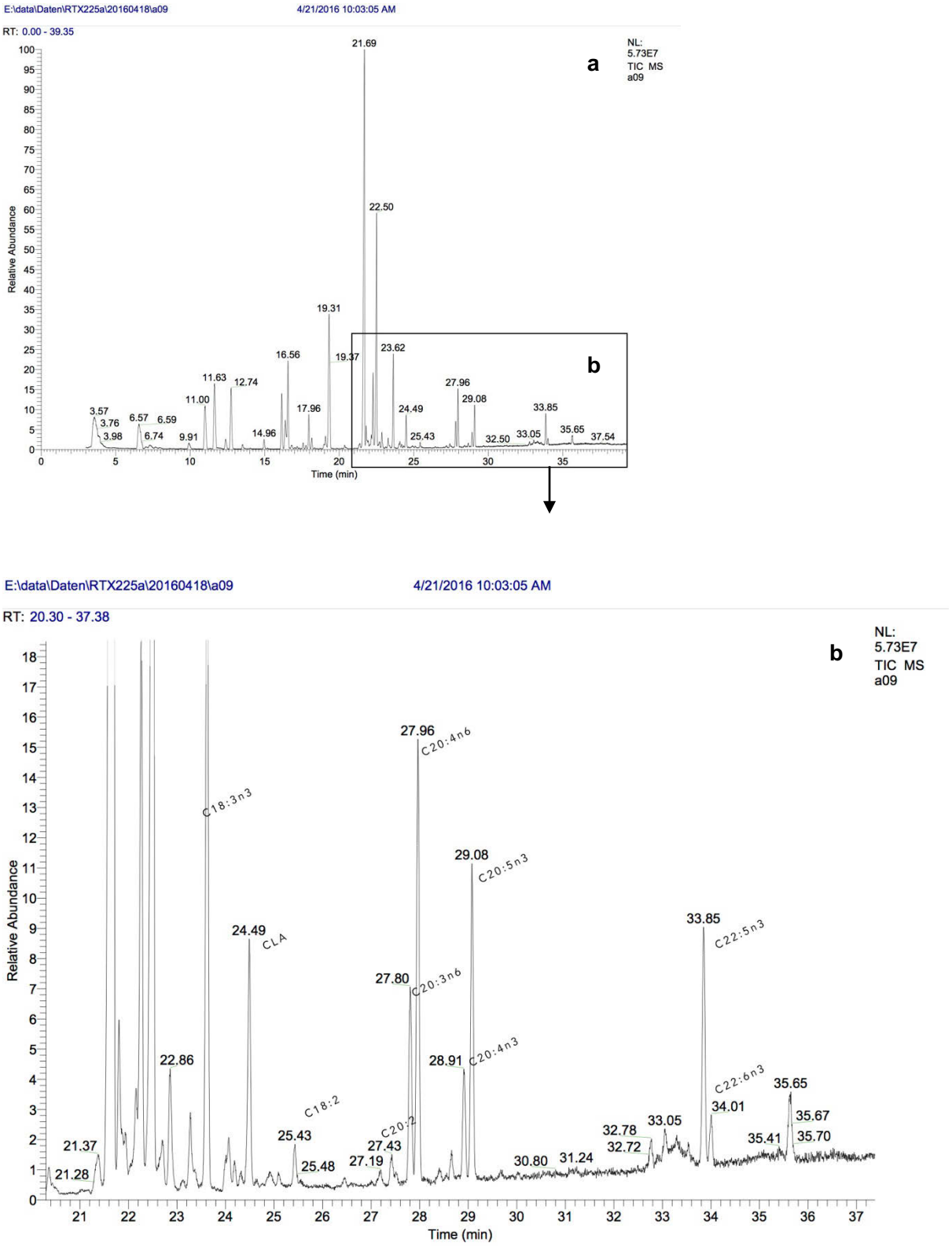

Figure 24: Chromatogram of the 2nd attempt of urea adduct formation; overnight method, diluted once

Figure 24 shows the chromatogram of the second attempt of the urea adduct formation with the overnight method. The chromatogram shows no SFAs which confirms that the complexation worked properly.

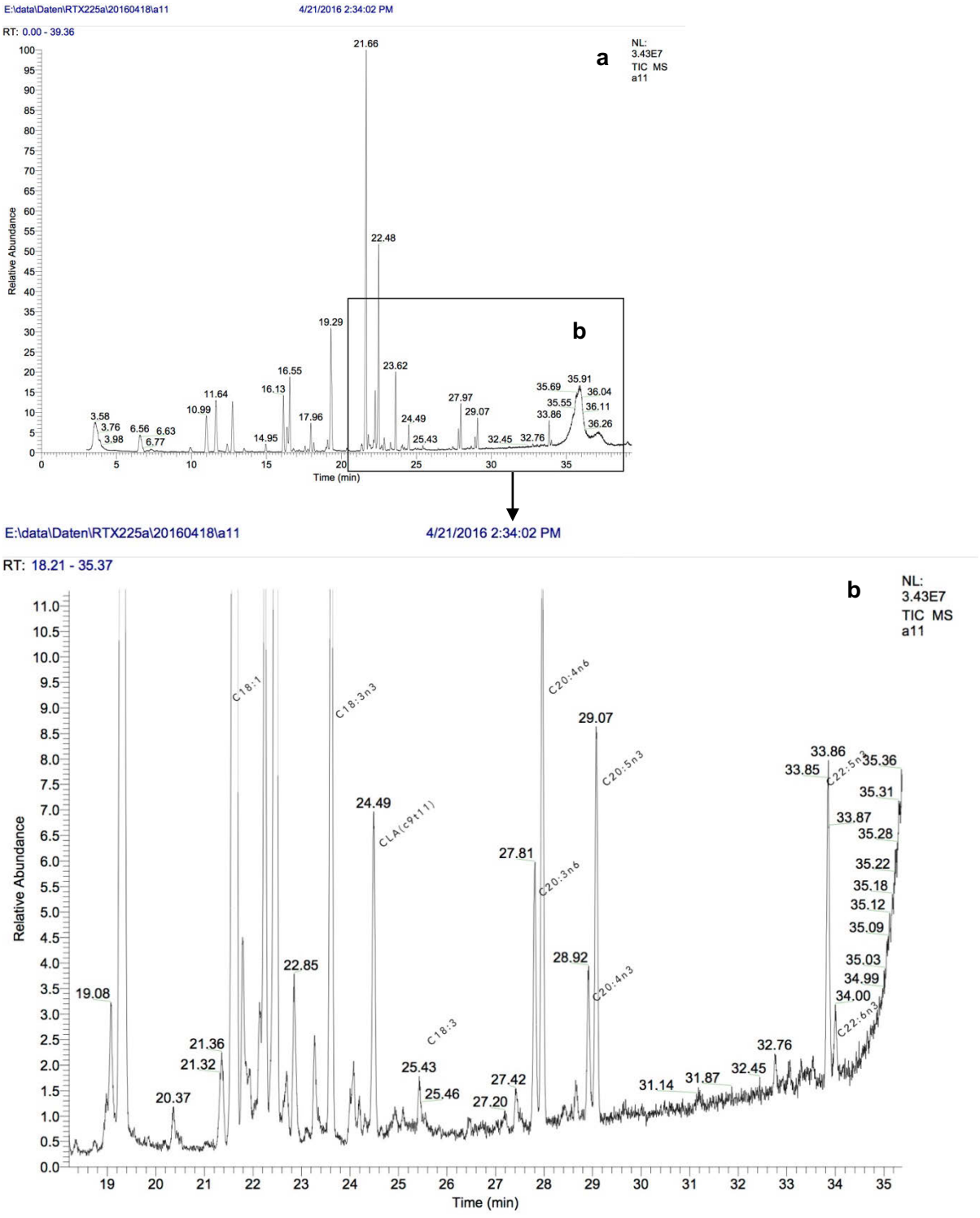

Figure 25: Chromatogram of the 2nd attempt of urea adduct formation; overnight method, 2 times diluted

The urea adduct formation was performed a second time with the overnight method. The remaining solution got diluted two times with hexane prior to injection. The fractioning worked out in an accurate way, so that the peaks are thin, narrow and well-separated. With the results of the second trial an identification of the peaks was possible. As for this study minor LCFAs are of special interest, it is sufficient to consider particularly the LCFAs, starting at minute 22. No SFAs could be observed at this time. Table 16 shows the retention times with the corresponding FAs. In Figure 23 a time-shift could be detected.

Table 16: Retention times of urea adduct formation(GC-MS) and shift of the retention time of Figure 23

Time [min]	Time [min] Urea a07	FA
23.60	24.14	C18:3n3
23.94		C19:0
24.49	24.90	CLA
24.91	25.66	C18:2
25.43		C18:3
26.42	26.65	C20:0
26.50	26.73	C20:1
27.46	27.59	C20:2
27.81	28.01	C20:3n6
28.00	28.16	C20:4n6
28.72		C21:0
28.91	29.06	C20:4n3
29.07	29.23	C20:5n3
31.02		C22:0
32.78		C22:2n6
33.23		C23:0
33.85	33.97	C22:5n3
34.00	34.09	C22:6n3
35.46		C24:0

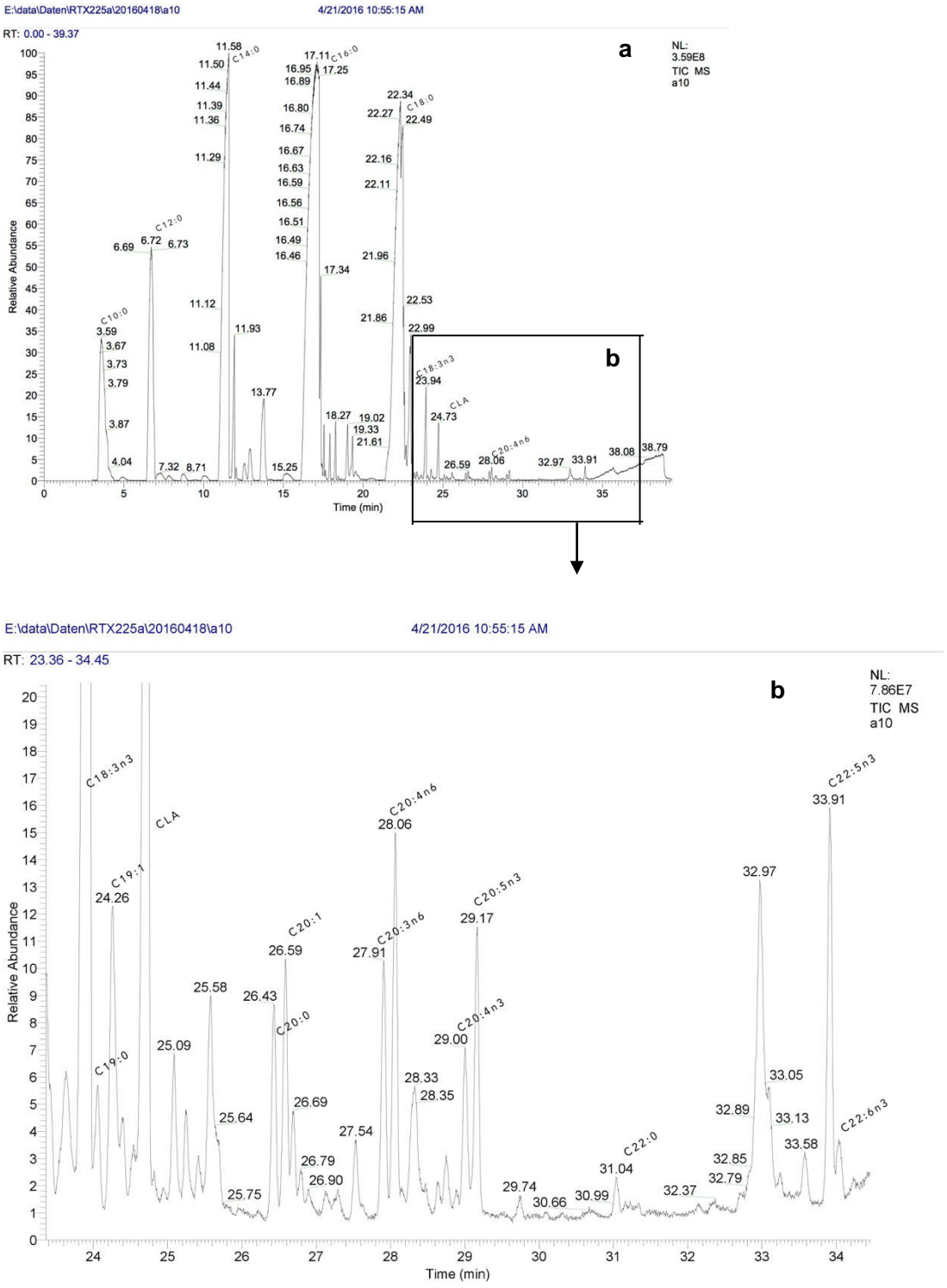

Figure 26: Chromatogram of urea adduct formation, 4h-method; diluted once

The results of the urea adduct formation with the 4 h method is shown in Figure 26. The chromatogram is too overloaded, no good separation of single peaks could be achieved thus, a proper qualification was not possible. Obviously the complexation did not work properly; many SFAs remained in solution, e.g. C16:0 and C18:0 could be detected. Additionally, long-chain, branched-chain PUFAs do not seem concentrated and they do not show higher peaks than in a not-enriched sample. Saturated and short-chain FAs seem to be enriched instead of long-chain FAs. It can be assumed that complexation failed, because the complex formation was too intense with stable complexes of a relative big diameter. One way to reduce/ avoid this massive formation would be to shorten the resistance time and continuing earlier with the filtration process and an increased amount of sample (approx. 10 g butter) could be suggested. On figure 24 and 25 one can see the interaction of DBs with the resulting chromatogram. CLA has a smaller peak than C18:3n3, because CLA has only one *cis*- DB. Also, C20:3n6 is smaller than C20:4n6, because with one *cis*- DB more the FA has a bigger volume. *Cis*- DBs introduce an ankle and prevent the tight packaging of FAs. *Trans*- DBs look like single bonds and maintain the rigid structure. That explains the smaller peak of CLA. This pattern can also be seen with C20:4n3 and C20.5n3.

Summary:

Table 17: Retention times and corresponding peaks Urea adduct formation o/n (GC-MS), referred to figure 23-26

Time [min]	**FA**
3.61	C10:0
6.72	C12:0
11.50	C14:0
17.01	C16:0
22.69	C18:0
23.60	C18:3n3
24.49	CLA
24.91	C18:2
25.43	C18:3
27.81	C20:3n6
28.00	C20:4n6
28.16	C20:0
28.91	C20:4n3
29.07	C20:5n3
32.78	C20:2n6
33.85	C22:5n3
33.97	C22:0
34.00	C22:6n3

5.2.3 Phospholipids | KJ

Fractioning of PLs was performed with SPE with the intention to consider all LCPUFAs or to detect unknown/ unidentified FAs. No further FAs could be detected by extraction of PLs of milk fat in this experiment.

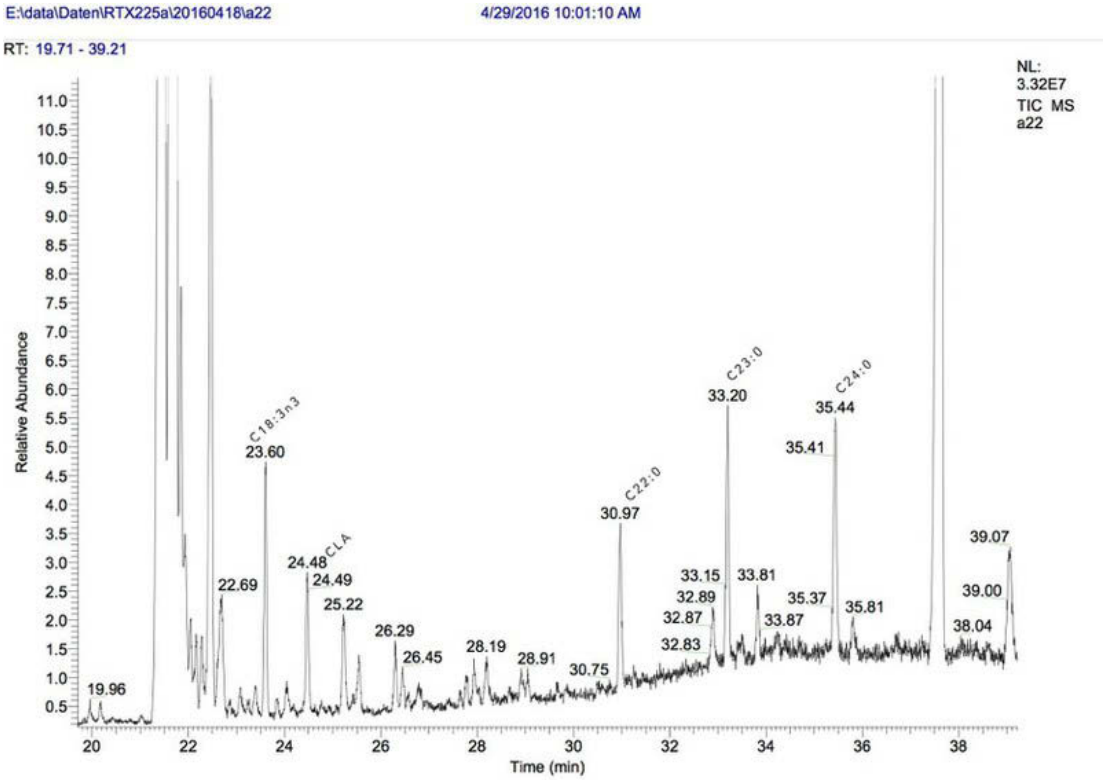

Figure 27: Chromatogram showing the section of FAs starting at 18:3n3, fractionation of phospholipids by solid phase

5.3 GC-MS of the developmental methods

For the structural analysis and identification of FAs in milk GC- MS was used.

One trial was performed with FAMEs of a raw milk sample. The FA pattern is comparable with the ones from the GC-FID. By using data of the website of the “lipid library” many peaks could be identified and determined for all other samples. Some peaks could still not be identified and remain unknown/ unidentified. Moreover, peaks could be deleted because by GC-MS examination they turned out to be contamination residues of previous sample analysis or other contaminants.

5.3.1 Comparison Split and Splitless injection | KJ

For preliminary experiments a comparison of Split and Splitless injection was performed with the aim of finding a proper or the most suitable method for the sample analysis. Therefore, prepared milk sample was first injected with split and a second time Splitless method, see (Figure 53 and Figure 54). The comparison of the two chromatograms demonstrated, that peaks are sharper, narrow, symmetric and a better separation could be achieved. So overall the identification of more FA was possible by using the Splitless injection. As visible in the chromatogram below, split injection resulted in completely overloaded peaks. Therefore, the decision was on the Splitless injection for all of the samples.

5.3.2 Comparison of different GC-MS chromatograms | OI

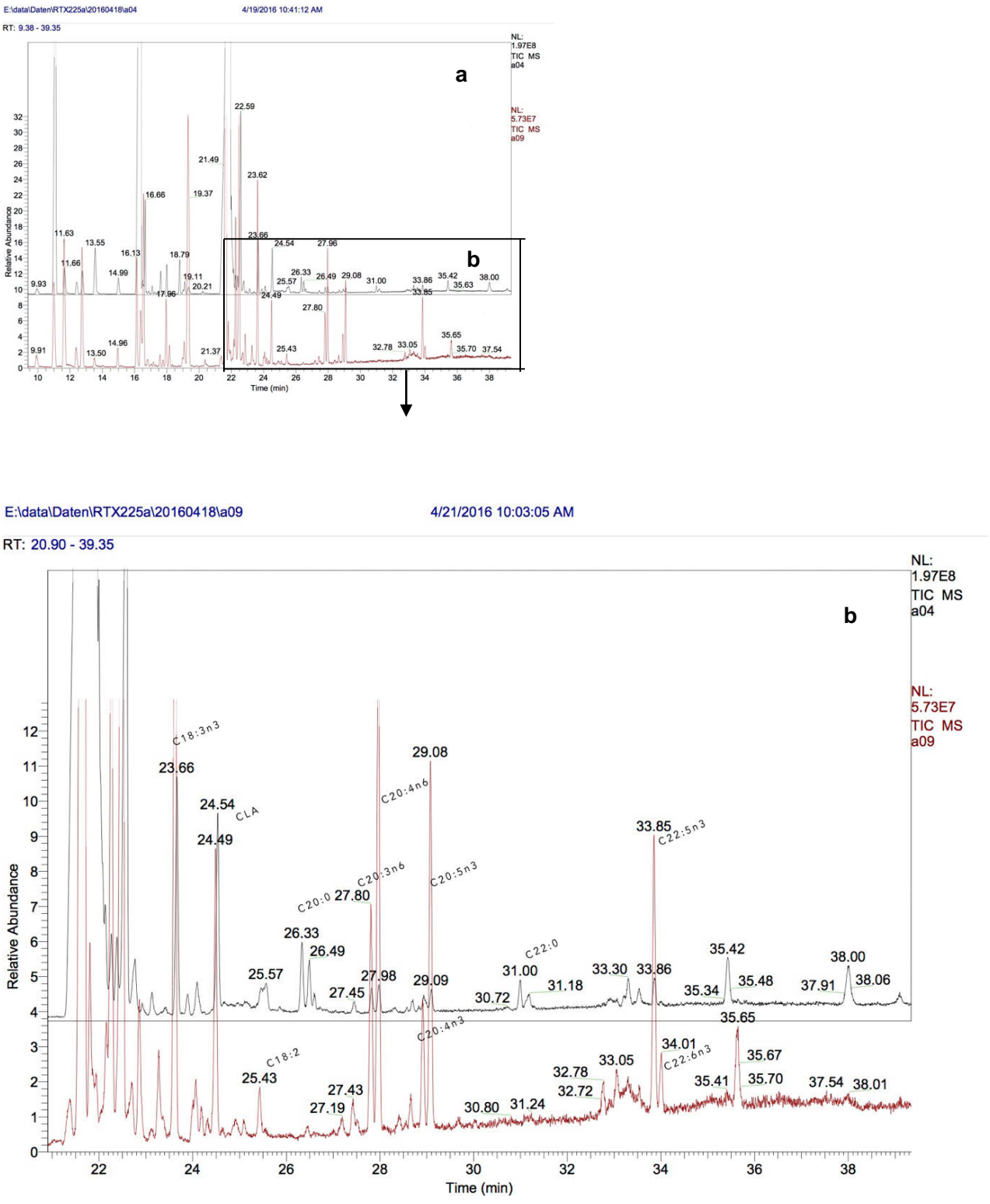

Figure 28: Chromatogram of conventional raw milk vs. urea adduct formation

A raw milk sample without urea adduct formation (a04, see black line) is compared to the urea complexion sample (a09, see red line) in Figure 28. Although C20:0 and C22:0 seem to

be missing in the urea sample they are not, because through urea complexation SFAs are filtered out. This comparison shows that the complexion worked properly and the two SFA are missing. Looking at the relative abundance levels, it appears, that the urea samples contain more. This is due to the concentration procedure. The noise to signal ratio is an important indicator to see if the concentration worked out properly. In this case, the relation noise to signal proportionally decreased whereas LCPUFA peaks were higher and could be differentiated with better clarity. In Figure 28 it can be seen clearly, that the peaks of C22:5n3 and C22:6n3 are separate and detectable. This figure further demonstrates, how the concentration methods helped for peak identification. By using urea complexation, especially C22:6n3 and C20:4n3 could be verified precisely.

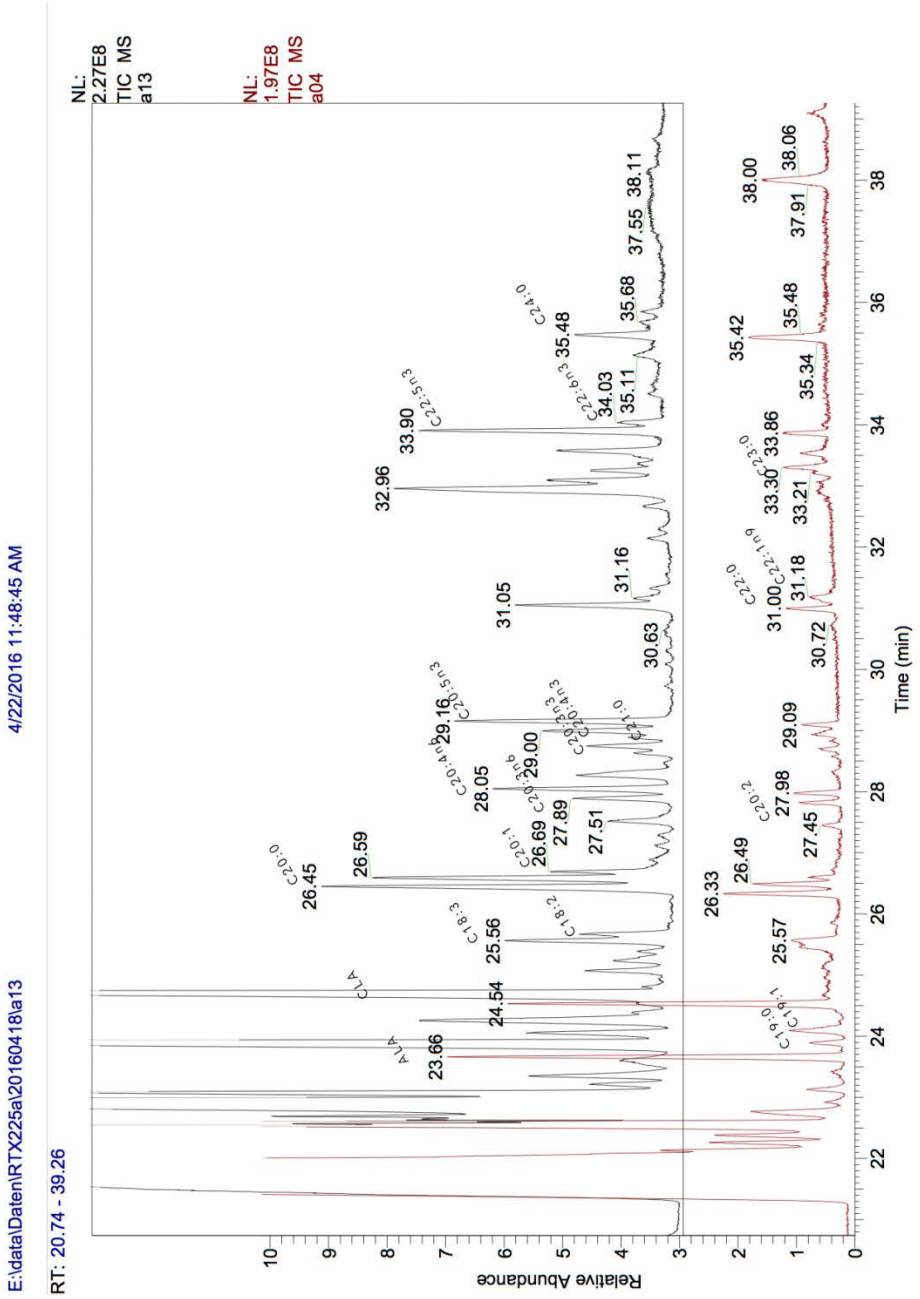

Figure 29: Chromatogram of raw silage milk vs. raw milk unknown; starting at min. 22

Comparing the chromatograms of two raw milk samples (see Figure 29) the same FA profile could be observed. Sample number a013 is a raw milk sample from silage fed cows. The origin of the a04 sample is unknown. C22:6n3 is nearly absent in sample a04. All other n-3 FA did not give a satisfying result as Gaussian peaks. But C22:1n9 seems to be present in a higher concentration.

5.4 Characterization of the fatty acid profile in milk samples

For the calculation of the mass percentages of the single FAs, the results from the FAs eluted before C18:3n3 were used to receive the values of total FAs. The results, which were examined for this thesis, start at C18:3n3.

Quarter 1 and 4 (Q1, Q4) represent the winter period and Q2 and Q3 the summer period.

Table 18: Seasonal division of quaters

Quarter	Months
1	January- March
2	April- June
3	July- September
4	October- December

5.4.1 Statistical analysis

5.4.1.1 ANOVA

An analysis of variance (ANOVA) was run to investigate whether the season or the farming system had an effect.

Table shows the results of the t-test (see appendix, Table 35;Table 36).

5.4.1.2 t-test

For the determination of the difference between HAY and CON milk and seasons, all the results were taken together in a paired sample t-test. With the help of the test it is possible to see, if the groups statistically significant differ or not.

5.4.1.3 Outlier test

Looking at evaluated CLA data there is one sample, in each feeding regime, representing the minimum value (see Table 26 and 28), which can be considered as outlier by just a visible view. These values were also integrated in the study, because via the outlier test (according to Grubbs) they were not classified as outlier (see Table 29). Additionally, CLA values are generally within a higher variation rage.

5.4.2 Overview of all fatty acids in milk samples

The following section gives a comprehensive overview in all FAs present in HAY and CON milk samples.

Table 19: Mean values and p- values of all FAs in milk fat

FA	CON	HAY	p-value	significance
C4:0	3.780	3.734	0.456	
unk.	0.014	0.010	0.162	
C6:0	2.206	2.182	0.445	
unk.	0.016	0.011	**0.028**	*
C8:0	1.275	1.259	0.430	
unk.	0.025	0.014	**0.001**	**
C10:0	2.845	2.796	0.390	
unk.	0.394	0.376	0.862	
C11:0	0.063	0.053	0.323	
C12:0	3.346	3.308	0.576	
unk.	0.035	0.036	0.858	
C13:0	0.089	0.065	**0.004**	**
C14:0i	0.128	0.137	0.159	
C14:0	11.466	11.619	0.314	
C15:0i	0.391	0.479	**0.001**	**
C15:0ai	0.143	0.140	0.956	
C14:1	0.842	0.893	**0.007**	**
C15:0	1.203	1.259	**0.000**	***
(C16:0i)	0.284	0.286	0.876	
C16:0	30.846	30.479	0.467	
unk.	0.388	0.445	**0.000**	***
unk.	0.141	0.144	0.818	
C16:1	1.954	1.971	0.608	
C17:0ai	0.104	0.089	0.193	
C17:0	0.592	0.652	**< 0.0001**	***
C18:0i	0.236	0.245	0.491	
C18:0	9.449	9.061	0.140	
C18:1 t11	2.317	2.780	**0.017**	*
C18:1t- is	0.083	0.040	0.113	
unk.	0.442	0.355	**0.007**	**
C18:1n9c	18.318	17.704	0.125	
C18:1n7c is	0.397	0.393	0.931	
unk.	0.123	0.051	**< 0.0001**	***
C18:1 12-is	0.286	0.254	0.233	
unk.	0.021	0.028	0.630	
C18:2n6t	0.111	0.141	0.103	
C18:2t-is	0.313	0.301	0.703	
C18:2n6c	1.269	1.284	0.624	

C18:3n6	0.125	0.112	0.208	
C18:3n3	0.720	0.979	**< 0.0001**	***
unk.	0.078	0.089	**0.001**	**
unk.	0.143	0.169	**0.000**	***
unk.	0.026	0.016	**< 0.0001**	***
CLAc9t11	0.872	1.137	**0.002**	**
unk.	0.014	0.010	0.082	
unk.	0.059	0.052	0.188	
unk.	0.028	0.031	0.180	
unk.	0.012	0.013	0.179	
unk.	0.060	0.063	0.283	
unk.	0.090	0.098	0.147	
C20:0	0.187	0.187	0.999	
unk.	0.145	0.152	0.081	
C20:1	0.057	0.055	0.195	
unk.	0.012	0.012	0.881	
unk.	0.013	0.011	**0.035**	*
C20:2n6	0.045	0.052	**0.000**	***
C20:3n6	0.068	0.065	0.482	
C20:4n6	0.095	0.099	0.518	
C20:3n3	0.013	0.017	**< 0.0001**	***
unk.	0.016	0.023	**< 0.0001**	***
C21:0	0.082	0.100	**< 0.0001**	***
C20:5n3	0.070	0.091	**< 0.0001**	***
C22:0	0.074	0.080	0.151	
C22:1n9	0.009	0.010	0.251	
unk.	0.006	0.017	**< 0.0001**	***
unk.	0.037	0.038	0.711	
C23:0	0.027	0.034	**< 0.0001**	***
unk.	0.028	0.034	**0.001**	**
C22:5n3	0.066	0.083	**0.001**	**
C22:6n3	0.006	0.009	0.061	
C24:0	0.021	0.025	0.118	
C24:1	0.008	0.010	**0.003**	**

	S (CON)	**W (CON)**	**S (HAY)**	**W (HAY)**
∑ SFA	66.1 ± 0.30	70.2 ± 0.08	64.8 ± 0.21	68.5 ± 0.29
∑ MUFA	25.6 ± 0.27	22.4 ± 0.15	26.4 ± 0.27	22.2 ± 0.23
∑ PUFA	3.9 ± 0.08	3.5 ±0.05	4.7 ± 0.07	4.1± 0.06
∑ branched	3.2 ± 0.07	3.0 ± 0.02	3.1 ± 0.07	3.5 ± 0.11
∑ n-3	0.9 ± 0.05	0.8 ± 0.04	1.2 ± 0.03	1.2 ± 0.04
∑ n-6	1.7 ± 0.05	1.6 ± 0.04	1.6 ± 0.06	1.8 ± 0.07
n-6/ n-3	1.8	2.1	1.4	1.5

*S= summer; W= winter; Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1*

The overall SFA content in HAY and CON milk show lower values in HAY than in the CON milk samples. In both compared groups, the SFA content is slightly higher in winter than is summer.

BCFAs show nearly conformity in comparison of HAY and CON milk and additionally there is no difference in seasons. Only in HAY, BCFAs are slightly and insignificantly higher during the winter period. The same pattern is visible in the total MUFA content, but no remarkably differences at all.

KJ

The amount of BCFAs in milk can be increased with feed that is high in fibre and low in energy, like starch. Moreover, BCFA can be an indicator of the cow's ruminal status. Also in organic farming regimes, BCFAs are usually higher (increased pasture feed) than in CON (Jahreis *et al.,* 1997; Shingfield *et al.,* 2005; Vlaeminck et al., 2005). In this study no differences of BCFA could be detected comparing HAY and CON samples and also there was no measured effect according to seasons. BCFAs in milk are not necessarily long-chain FAs and also mostly saturated, therefore only a side consideration in context of this study. These FAs are influenced by the cows feed to a major extent, therefore it was suggested to allow a conclusion on the cows feeding regime and were also included in this thesis. The overall BCFA content in this study (see also Table 19) did not differ in HAY and CON milk samples. Very probably some BCFAs could not be identified, hence were not included in the evaluation, which is a lack of the study. Looking at the results of this study, the evaluation of BCFA content is not a suitable parameter for differentiation between HAY and CON milk. Probably there are some BCFA connected to certain ruminal bacteria that are associated with either silage- or pasture- based feed and leading to differences of single BFCAs in milk.

OI

The PUFA content is significantly higher ($p \leq 0.05$) in HAY than in CON milk and both groups show increased levels in summer. Comparing the PUFA content of winter HAY milk to summer CON milk samples the difference is nearly vanishing. It can be stated, that the effect of seasons influences the PUFA content to a higher extent than the HAY or CON feeding regime. In other words: summer CON milk and winter HAY milk are qualitatively comparable considering their PUFA pattern. Hence, n-3 FAs are also constantly higher ($p \leq 0.05$) in HAY samples than in CON, the n-6 content HAY and CON are more contiguous. This leads to a nutritional higher valuable n-6 to n-3 ratio for hay milk (see detailed description in ratio n-6/ n-3, chapter 5.4.5.3, p.105).

Interestingly the C18:0 content is slightly lower in CON milk in summer than in hay milk (Table 20). In winter CON is about 10% higher in C18:0 than hay milk. C18:0 is an indicator for the biohydrogenation process of PUFAs. This suggests that there is a more sufficient biohydrogenation in HAY milk during summer and less during winter. But the smaller amount can also be linked to a higher supply of PUFAs from feed. Because there was a higher amount of PUFAs found in HAY milk, the higher C18:0 level is supposed to originate from there and not from a more intensive biohydrogenation. The effect of higher SFA contents in winter and a lower ratio between summer and winter represents the feeding with silage in CON.

Table 20: Seasonal variation of C18:0 in CON and HAY

	CON	Hay
summer	9.8 ± 1.35	10.3 ± 0.60
winter	9.1± 0.28	8.1 ± 0.64

5.4.3 Identification of long chain fatty acids | OI

Many FAs still remained unknown after the GC- FID analysis, because they are not included in the 37- component STD, which was used for identification of the FAMEs by GC-FID. It was possible to identify some by pre- concentration techniques, like urea adduct formation or a clean-up. However, the most important method for identification was Ag^+- TLC followed by an analysis of the fractions with GC-MS. Due to the Ag^+- TLC the milk FAs could be separated into their corresponding fractions. Their retention differed according to the number and configuration of DBs. PUFAs remained on the starting line, SFAs migrated towards solvent front. By pre- concentration it was possible to enrich and furthermore to analyse the minor LCPUFAs, like C22:6n3. Table 17 shows the peak number and retention times in the way the peaks came out of the GC-FID. Some of them could be matched to FAs by comparison of the retention times from the 37 component STD. But still, many remained unknown (unk.).

In the appendix (see Table 30, Table 31) there is an overview on the FAMEs which could be detected in the milk samples. HAY and CON milk samples are regarded separately. Figure 30 shows an example of a GC- FID chromatogram with the separated and identified peaks.

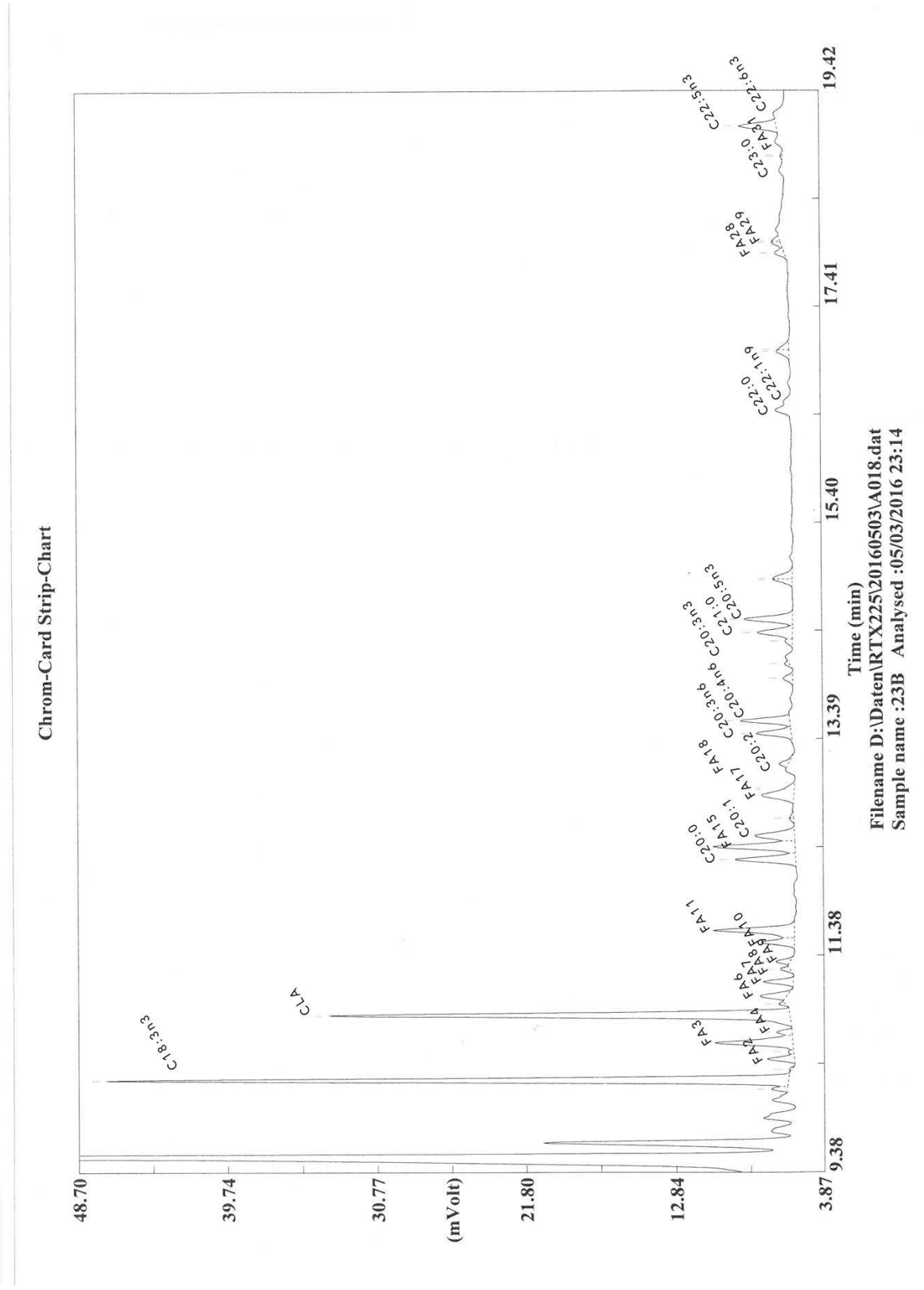

Figure 30: Chromatogram of GC-FID showing fatty acid spectra of identified and unidentified fatty acids

Table 21: Detected peaks and way of identification; starting at C18:3n3

PEAK NUMBER	RETENTION TIME [MIN]	FA	NAMED	IDENTIFIED BY
1	**10.22**	**C18:3n3**	**C18:3n3**	**STD**
2	**10.42**	**unk.**	**FA2**	**-**
3	**10.57**	**unk.**	**FA3**	**-**
4	**10.68**	**unk.**	**FA4**	**-**
5	**10.84**	**CLAc9t11**	**CLAc9t11**	**STD**
6	**10.92**	**unk.**	**FA6**	**-**
7	**11.13**	**unk.**	**FA7**	**-**
8	**11.24**	**unk.**	**FA8**	**-**
9	**11.31**	**unk.**	**FA9**	**-**
10	**11.49**	**unk.**	**FA10**	**-**
11	**11.65**	**unk.**	**FA11**	**-**
12	**12.29**	**C20:0**	**C20:0**	**STD**
13	**12.39**	**unk.**	**FA15**	**-**
14	**12.49**	**C20:1**	**C20:1**	**STD**
15	**12.66**	**unk.**	**FA17**	**-**
16	**12.94**	**unk.**	**FA18**	**-**
17	**13.17**	**C20:2n6**	**C20:2n6**	**STD**
18	**13.43**	**C20:3n6**	**C20:3n6**	**STD**
19	**13.59**	**C20:4n6**	**C20:4n6**	**STD**
20	**14.08**	**C20:3n3**	**C20:3n3**	**STD**
21	**14.16**	**unk.**	**FA23**	**-**
22	**14.37**	**C21:0**	**C21:0**	**STD**
23	**14.55**	**C20:5n3**	**C20:5n3**	**GC-MS**
24	**16.47**	**C22:0**	**C22:0**	**STD**
25	**16.71**	**C22:1n9**	**C22:1n9**	**GC-MS**
26	**17.89**	**unk.**	**FA28**	**-**
27	**18.05**	**unk.**	**FA29**	**-**
28	**18.70**	**C23:0**	**C23:0**	**GC-MS**
29	**18.96**	**unk.**	**FA31**	**-**
30	**19.08**	**C22:5n3**	**C22:5n3**	**GC-MS**
31	**19.21**	**C22:6n3**	**C22:6n3**	**STD/ GC-MS**
32	**20.94**	**C24:0**	**C24:0**	**STD**
33	**21.29**	**C24:1**	**C24:1**	**STD/ GC-MS**

Figure 31 shows the mean percentage of FAs obtained by GC-FID, starting at C18:3n3. Milk samples derived from hay-fed cows almost always contain a higher amount of the FA of interest.

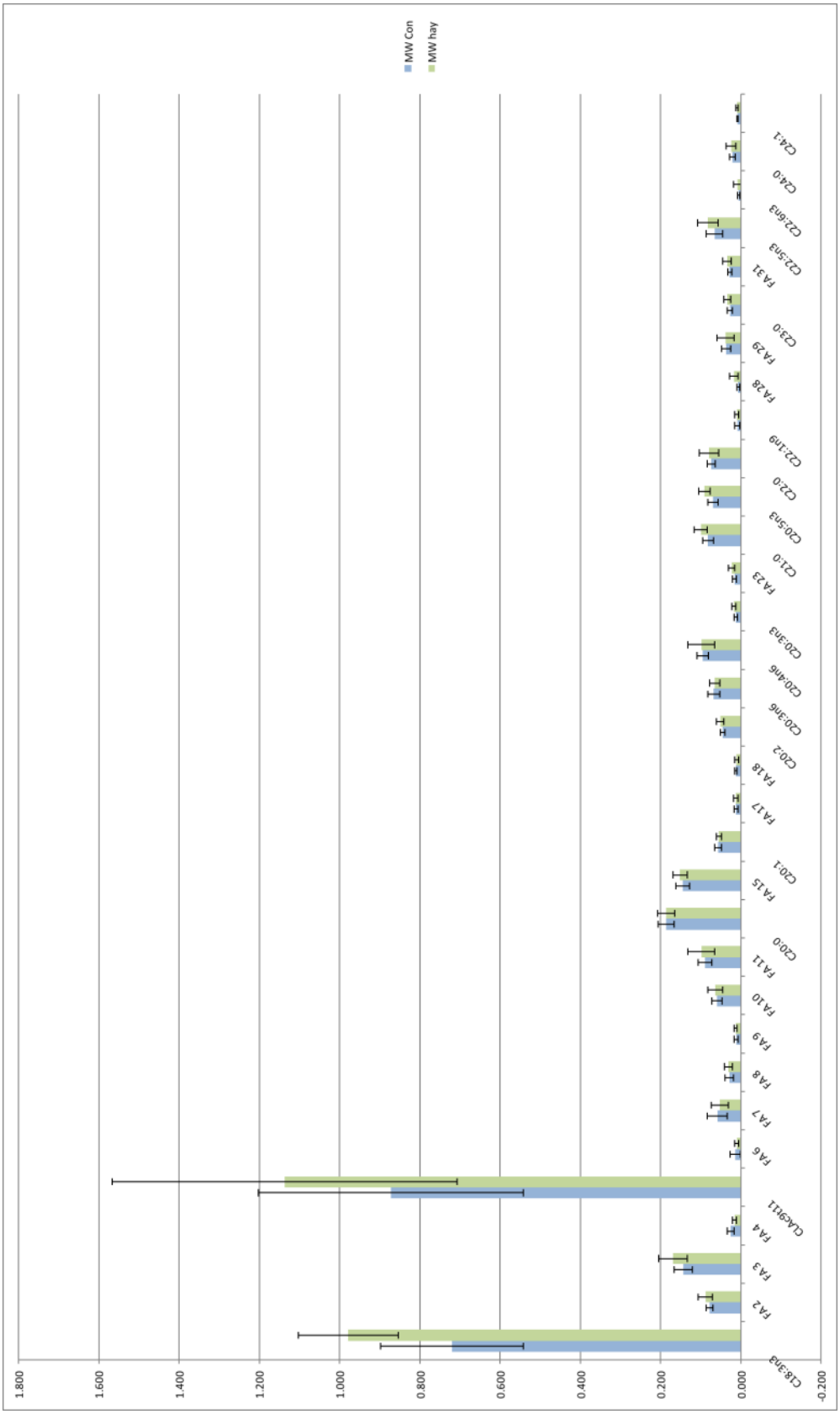

Figure 31: Overview of the whole FA composition (seasonal variation of the mean values and STD); starting at C18:3n3

5.4.4 Mono- and polyunsaturated long chain fatty acids

The main focus of this study is on the (minor) LCFAs of milk fat. The detected and identified FAs are presented in this section. Error bars in the figures showed below, represent the standard error.

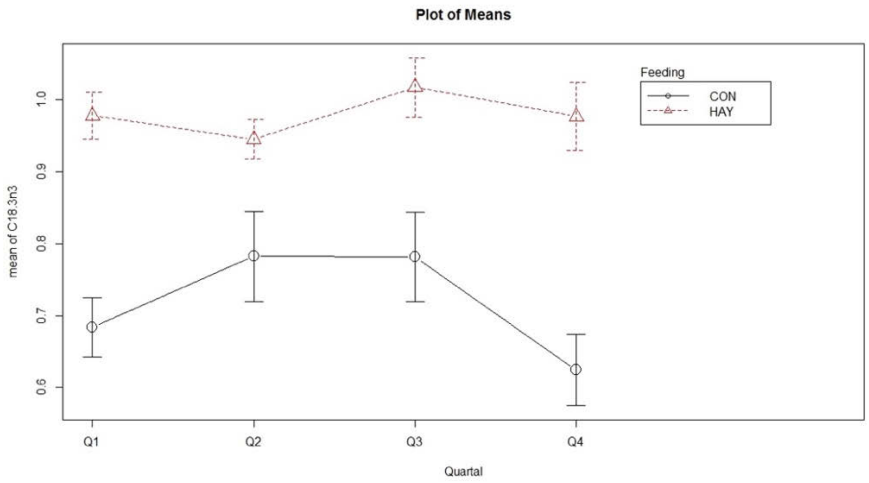

Figure 32: Seasonal variation of ALA content, comparing HAY and CON

The amount of C18:3n3 is always higher in hay milk, but there is no marginal ($p > 0.05$) rise during the year like in CON milk. There the amount is highest during summer for both feeding systems.

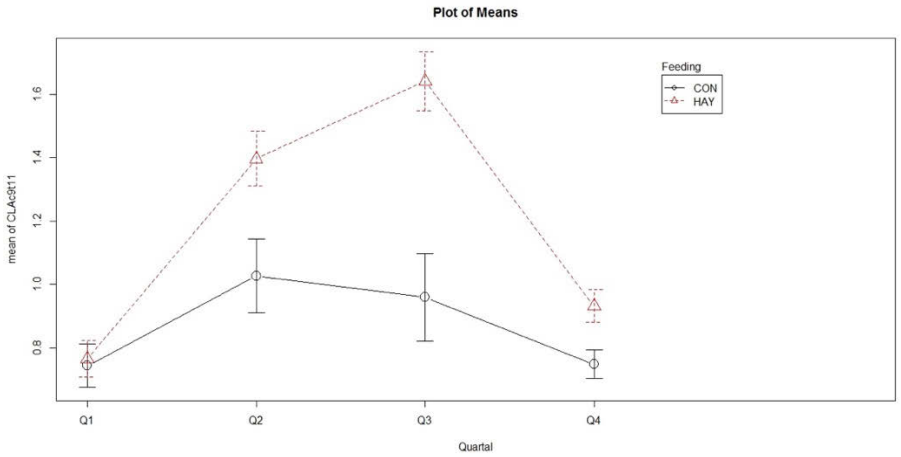

Figure 33: Seasonal variation of CLA(c9t11) content, comparing HAY and CON

The amount of CLA, is higher in HAY during summer. In the winter period (Q1 and Q4), the values of both feeding regimes are nearly the same.

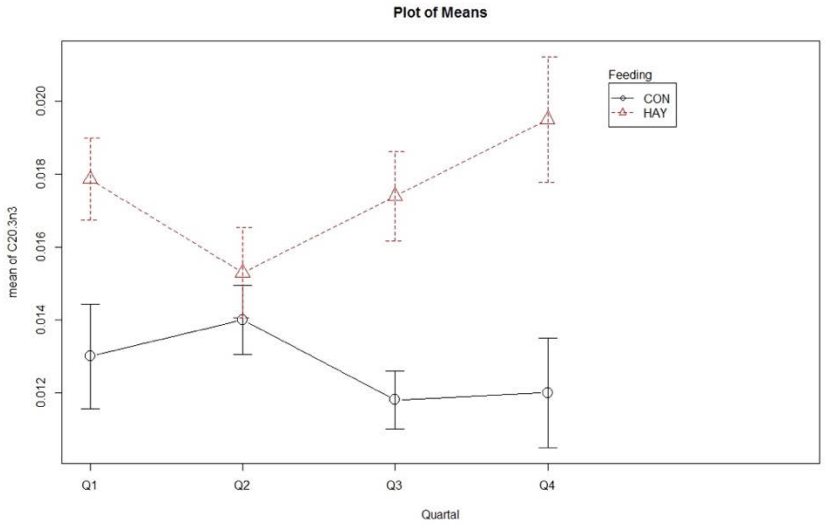

Figure 34: Seasonal variation of C20:3n3 content, comparing HAY and CON

The values of C20:3n3 in HAY declines in Q2 but rises again during Q3 and Q4, where it reaches its maximum. In Q4 there is the biggest gap between the two groups. Looking at the mean values, there is not a big difference during the whole year. CON milk has a mean value of 0.013. HAY milk only got a slightly higher amount with 0.0019. The difference between the two groups is significant during the whole year ($p \leq 0.05$).

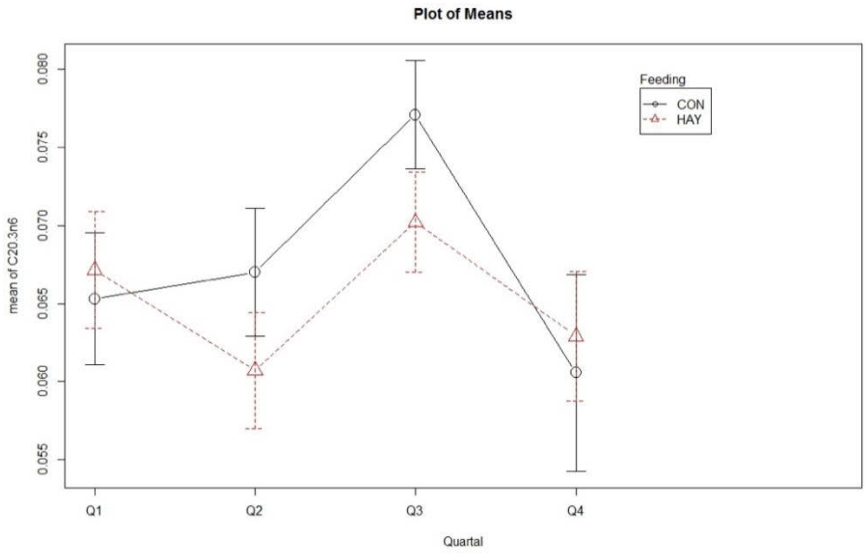

Figure 35: Seasonal variation of C20:3n6 content, comparing HAY and CON

The course over the year of C20:3n6 shows an increase in summer, especially in Q3, and lower levels during winter. The values are higher ($p > 0.05$) during the summer months in CON milk. During winter, the HAY samples show slightly higher values. C20:3n6 is the only FA where the CON samples have higher values in summer than HAY.

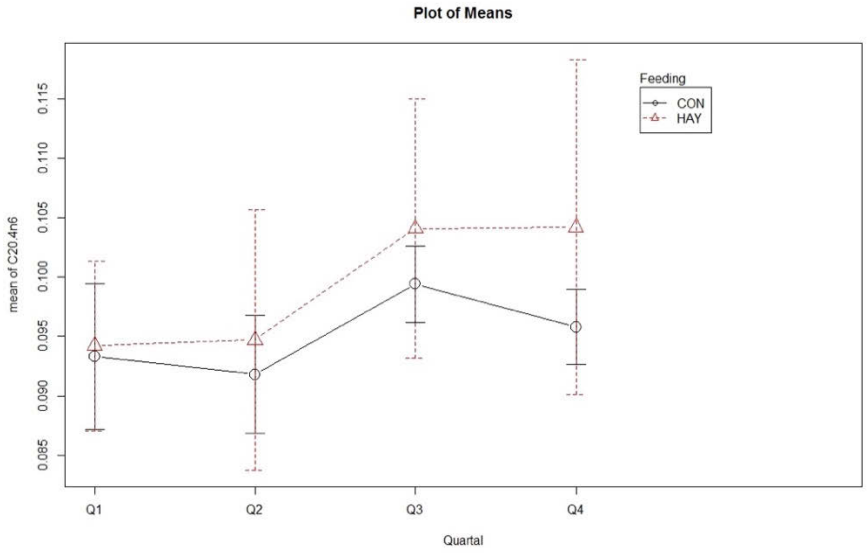

Figure 36: Seasonal variation of C20:4n6 content, comparing HAY and CON

The values of C20:4n6 of both feeding regimes do not statistically differ ($p > 0.05$). Also the trend throughout the year is for both groups the same. Values are highest in summer and lowest during winter.

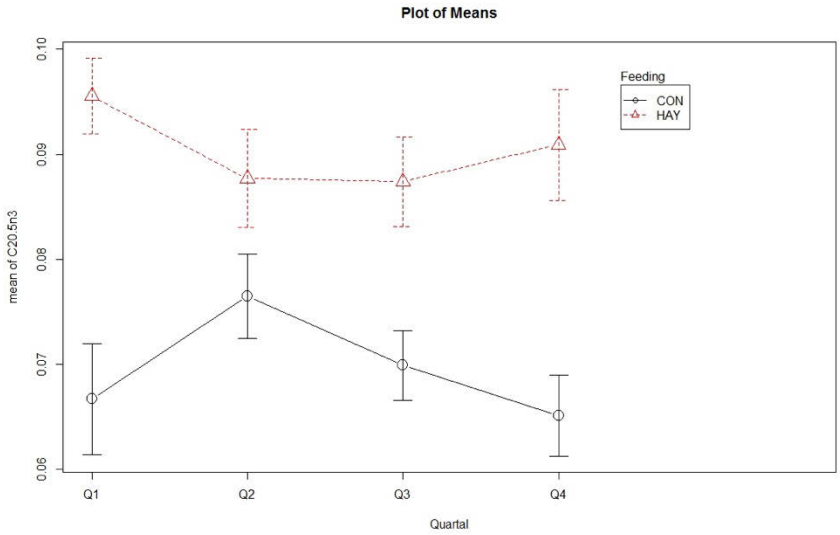

Figure 37: Seasonal variation of C20:5n3 content, comparing HAY and CON

The amount of C20:5n3 in HAY slightly decreases during summer and rises again slightly in winter. Contrary to CON milk, where C20:5n3 is highest in spring and decreases in summer/ autumn. The lowest values can be detected in winter.

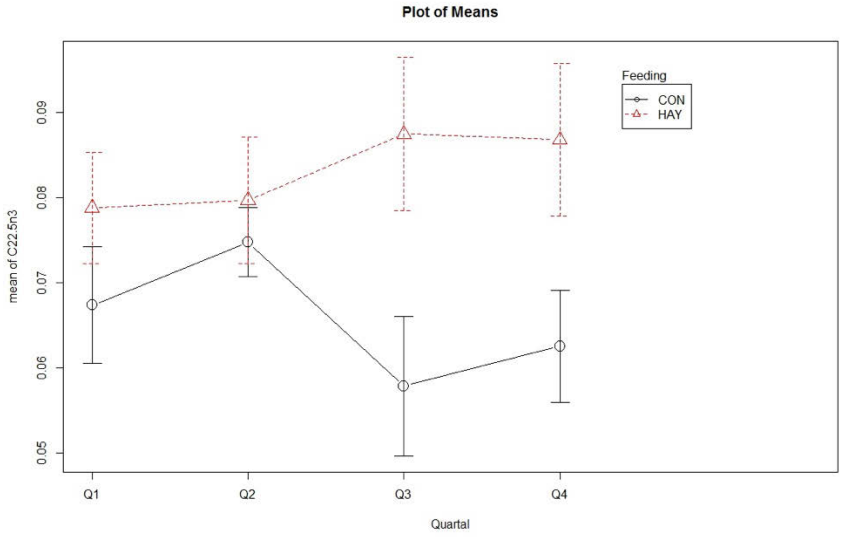

Figure 38: Seasonal variation of C22:5n3 content, comparing HAY and CON

C22:5n3 is built from elongation of C20:5n3, which in turn comes from C18:3n3. There is a minor rise in HAY during summer. Overall the values stay constant. In Figure 38 it can also be seen, that there is a huge decrease of C22:5n3 in Q3 but a slight rise again from Q3 to Q4 and Q1.

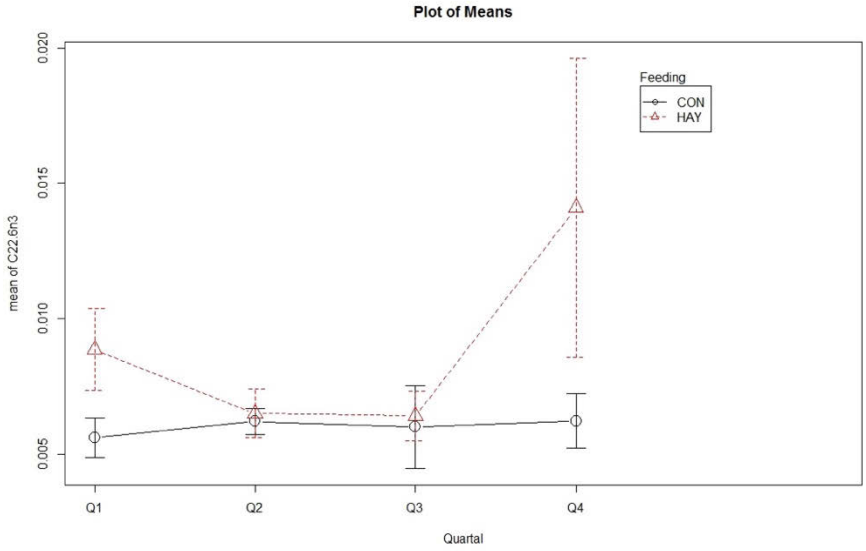

Figure 39: Seasonal variation of C22:6n3 content, comparing HAY and CON

C22:6n3 is a FA which is a structural component of the brain and is the most abundant n-3 FA in the brain and retina. It is synthesised from C22:5n3. The levels in both milk groups are not diverse ($p > 0.05$). Only in Q4 the amount is higher in hay milk but also with a great error.

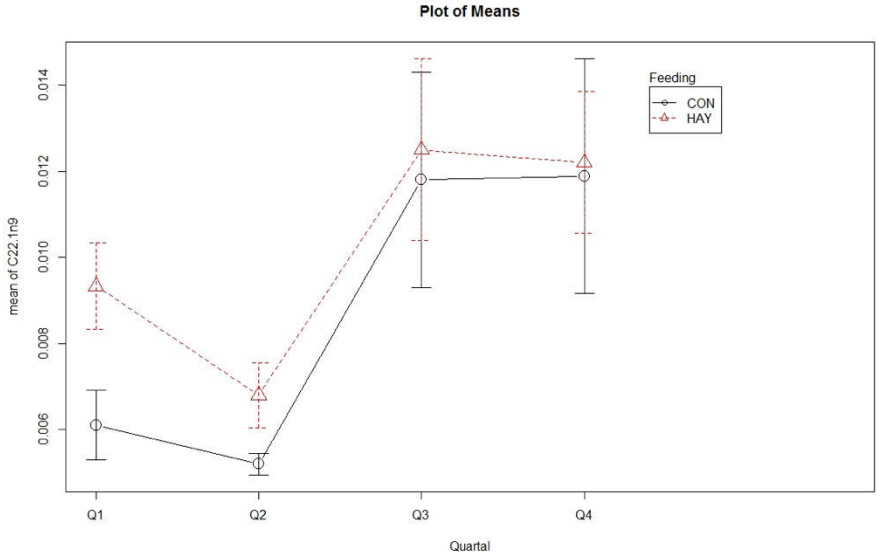

Figure 40: Seasonal variation of C22:1n9 content, comparing HAY and CON

The mean values of both groups show the same graph and do have comparable values. Hay milk samples contain slightly more C22:1n9. They both have a big step between Q2 and Q3. In hay milk this can be due to the start of the grazing period. As it is unknown how the cows of the CON regime were fed exactly, a clear statement cannot be made. The farmers might have fed oil concentrates/supplements, which contain a higher amount of UFAs.

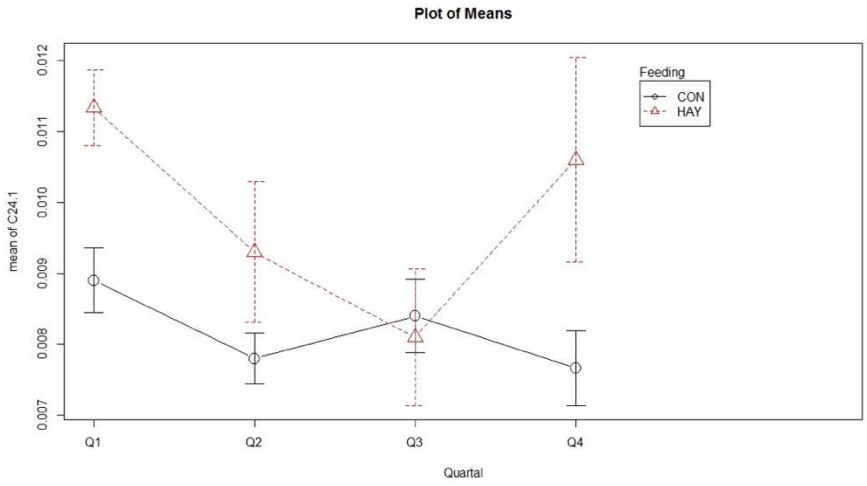

Figure 41: Seasonal variation of C24:1 content, comparing HAY and CON

Looking at the hay milk values of C24:1, they decrease during summer and are highest in winter. The CON samples do not show that pattern and they stay nearly constant over the year. The decline during summer can be in connection with fresh grass and less concentrated feed from the hay feeding regime. The feed composition from cow being conventionally fed, does not change a lot during the year and has therefore less fluctuations.

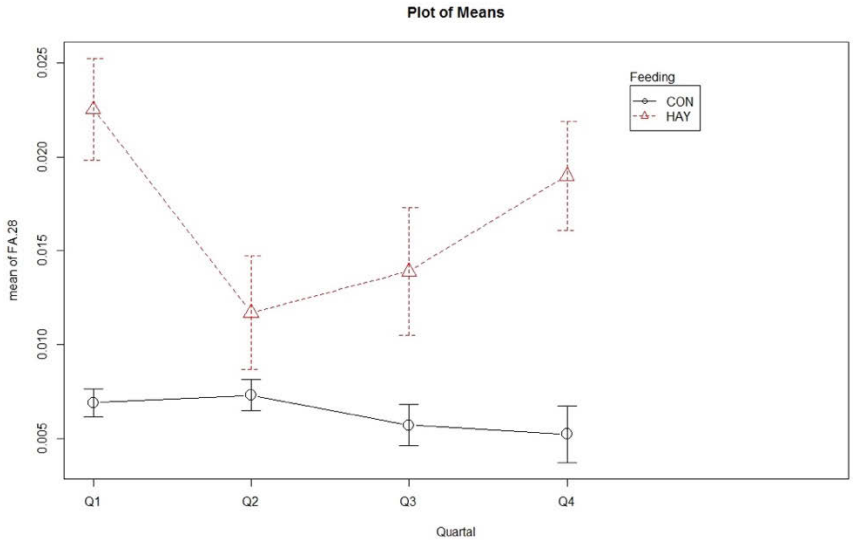

Figure 42: Seasonal variation of peak FA28 content, comparing HAY and CON

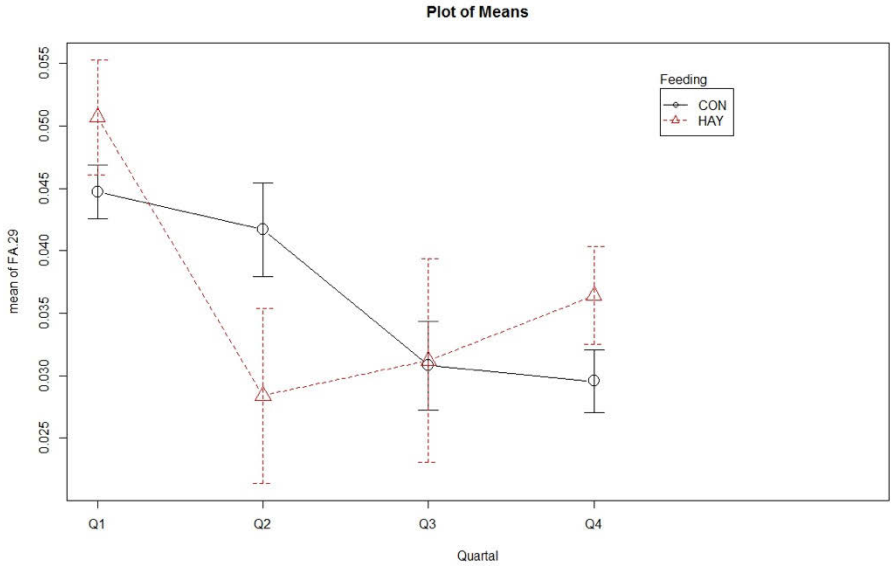

Figure 43: Seasonal variation of peak FA29 content, comparing HAY and CON

The peak number 26 and 27 (= FA28/ FA29) remain unidentified, but show a statistical significance. Looking at FA28, the values for CON do not change, whereas the values from

the HAY samples change during the year. They are highest in winter and lowest in summer. This phenomenon indicates a change in feeding regime. This can be due to a higher intake of concentrated feed in winter. The same pattern is seen in the HAY milk samples of FA29. The values decrease during summer and are highest in the winter period. CON milk also shows the highest amount in winter until spring, but decreases during summer and autumn. FA29 shows higher values in CON than HAY in Q2, this tendency was also observed in 20:3n6. This indicates again a change in the feed. The decrease in CON starting from Q1 on can be in connection with matured silage.

KJ

PUFAs seem to be insignificant in milk with a proportion of approx. 2,3% (by weight) of the total milk fat content (Månsson *et al.,* 2008). But in an average Austrian diet dairy products are consumed regularly and depending on the amount, quality, fat content of the product, milk contributes to dietary PUFA supply. Studies already confirmed, that feeding and animal keeping influences the PUFA content in milk (Collomb *et al.,* 2002; Dewhurst *et al.,* 2006; Kraft *et al.,* 2003; Dannenberger *et al.,* 2004; Bauman and Griinari, 2003; Bauman *et al.,* 2008). Most of the studies that analyse the alteration of FAs though feed focus on CLA (mainly referring to rumenic acid) and C18:3n3. In consideration of the variation in HAY and CON milk, this study tried to identify and analyse additional long chain FAs also besides CLA and C18:3n3. The reason for higher PUFAs in HAY vs. CON milk is, that an exclusion or reduction of silage and concentrated feed results in higher trans-Vaccenic acid (tVA), CLA, C18:3n3 and n-3 FAs. This effect fits to the results of this study, also because these above mentioned PUFAs increase independently of mature status or origin of the grass/HAY and the constitution of pasture (Leiber *et al.,* 2005). So the mixing of different hay milk suppliers in the dairy does not restrict the effect of higher PUFAs in total. A higher amount of PUFAs is also found in alpine cow milk. This observation can firstly be explained by a different botanical composition in height and secondly with fresh/immature grass. In alpine regions vegetation regenerates in higher frequency, so grass has shorter time to mature. Additionally, lower temperature in height leads to a higher content of C18:3n3 in plants and from C18:3n3 long chain n-3 FAs can be synthesized (Hawke, 1973 in Kraft *et al.,* 2003). It is also suggested that the Δ^9-desaturase works with higher efficiency in alpine cows (Kraft *et al.,* 2003).

Two known strategies for CLA increase are applied for milk fat adulteration: once the direct consumption of CLA-rich feed (e.g. trough enriching cows feed) or through decreasing ruminal activity (biohydrogenation) (Jahreis *et al.,* 1997). Contrary, a silo- and concentrated feed – rich diet in cows, causes a lower pH-value in the rumen. This results in reduced

mammary milk fat synthesis and in lower CLA synthesis from tVA (tissue) (Choi *et al.,* 2000). There are already several experiments showing that CLA is significantly higher in organic milk or generally in cows that mainly graze on pastures (Jahreis *et al.,* 1997 ;Morales *et al.,* 2015). The results of this study respectively the study confirms that the existing findings on CLA is influenced by feed. CLA values are significantly higher in the hay milk samples than in the CON milk. Also the dependency on seasons is clear. CLA content in the CON milk is more constant, which can be explained by less variation in feed seasonal dependency. Cows producing CON milk might constantly receive a certain amount of concentrated feed, contrary to hay milk cows. The difference of CLA content in the 1st and the 4th quarter is lower by trend, whereas in Q3 the difference of HAY and CON peaks. Schreiber and Ginzinger observed as well a steady increase of CLA from May on and a maximum concentration in October/ November (Schreiber, 2002; Ginzinger, 2012) The reason why hay milk has a relatively high content of CLA in the 3rd quarter with a maximum is obvious. During the summer months' cows are (nearly) exclusively grazing on pastures, they are likely to only consume fresh grass. The remaining time of the year the content of dried or matured grass in feed increases and relatively to that, CLA decreases. Also Kraft stated in their study, that the CLA content of pastures (where cows graze) correlates with the CLA amount in their milk (Kraft *et al.,* 2003). To accomplish a maximal CLA content in milk the combination of grazing going along with animal keeping and season respectively young and leafy grass supply is required (Jahreis *et al.,* 1997). Alternatively the supplementation with plant rich in C18:2n6 and C18:3n3 like soybean, linseed oil or fish oils in cows feed can increase CLAs to a certain extent (Chouinard *et al.,* 2001; Shingfield *et al.,* 2003; Kairenius *et al.,* 2015). CLA composition (distribution of isomers) or amount can vary in fermented milk products dependent on the used starter culture (Kim and Liu 2002; Bisig, 2008). It has been discussed, if external parameters might change the FA pattern in milk (-products) and most studies confirm that milk processing does not or to a negligible extent influence the FA pattern (Herzallah *et al.,* 2005; Bergamo *et al.,* 2003; Velik *et al.,* 2010).

5.4.5 Saturated long chain fatty acids

Error bars in the figures showed below, represent the standard error.

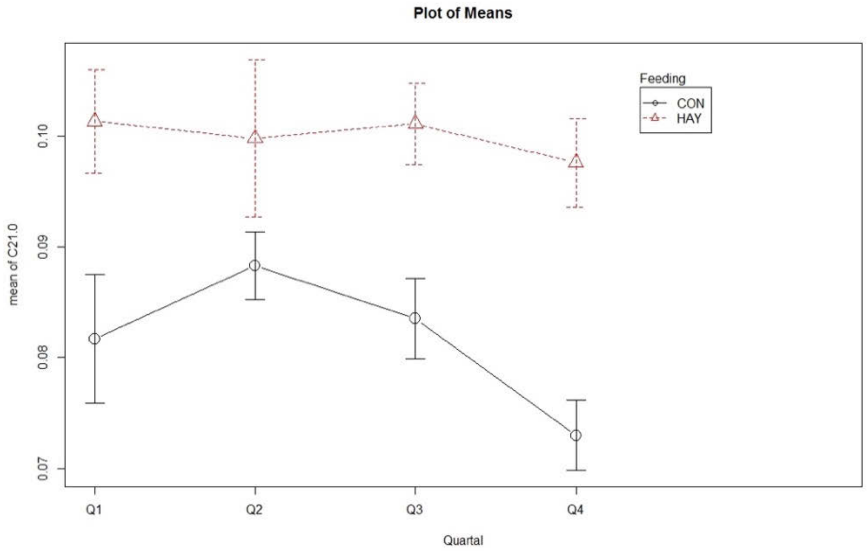

Figure 44: Seasonal variation of C21:0 content, comparing HAY and CON

The mean values of C21:0 stay constant in hay milk throughout the year. Looking at the CON milk values the amount of C21:0 rises in spring but declines afterwards in Q2 and 3.

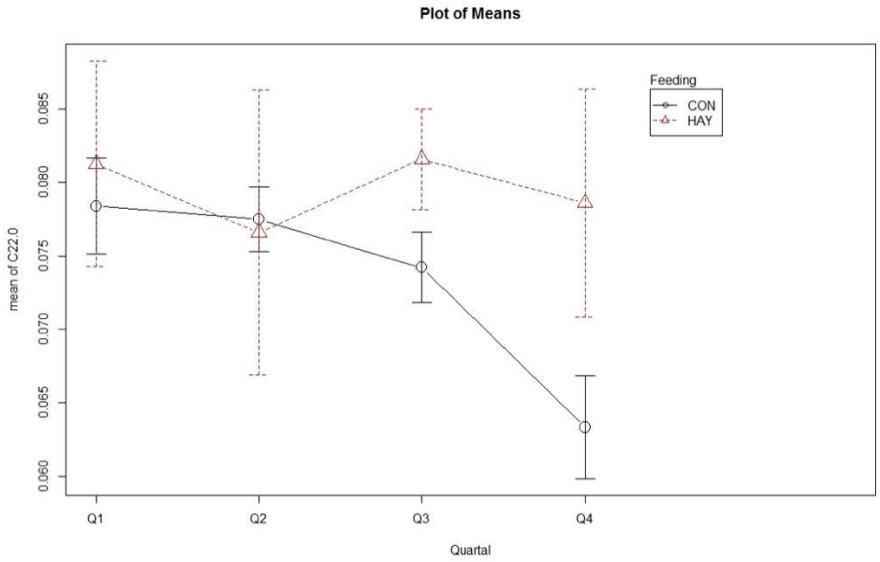

Figure 45: Seasonal variation of C22:0 content, comparing HAY and CON

The values of C22:0 show the same pattern like C21:0. The content of C22:0 in CON decreases from spring to autumn and the results of HAY nearly stay constant.

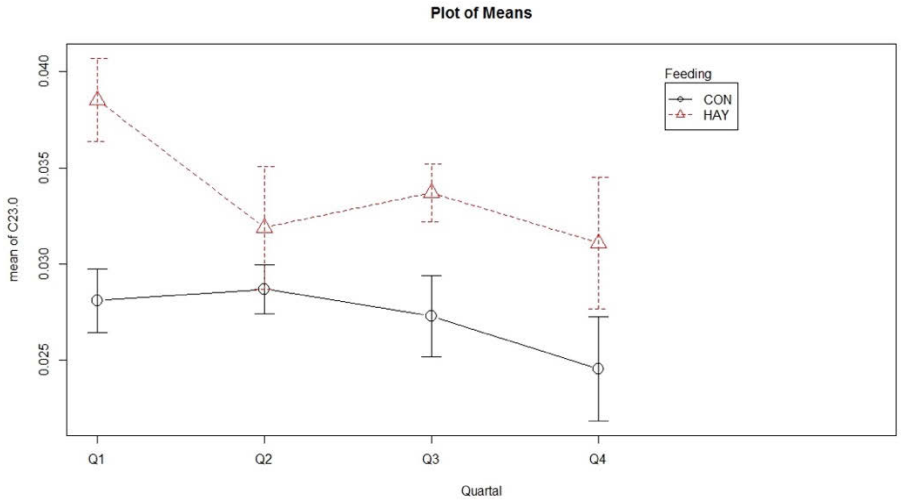

Figure 46: Seasonal variation of C23:0 content, comparing HAY and CON

C23:0 decreases in spring and also from summer to winter in HAY. The reason for that is that fresh grass contributes to a higher amount of PUFAs. Regarding CON milk, the amount stays the same. The reason for that is, that the amount of SFAs/ PUFAs added by feed does not change in silage (nearly always the same composition).

Considering the SFAs in CON milk, the values in Q1 are often higher than in Q4. This is probably due to the age of the silage. In Q1, the new silage from autumn might be fed, whereas in Q4 the “old” silage from the year before has been still fed. As there is no such difference in hay milk, it indicates, that HAY has the same quality as fresh grass in terms of SFAs.

KJ

Generally, SFAs in milk are higher in cows that receive more fermented feed or silage, hence for cows that are not able to graze on pastures. If cows consume less fresh grass, C18:3n3 uptake is usually reduced which causes increasing *de novo* synthesis of C16:0 (Bauman *et al.,* 1998). The microbial composition is effected by uptake of concentrated feed or silage due to lower ruminal pH value. Literature is explicit in finding higher SFAs in milk of cows kept in a CON regime respectively with cows that get less amounts of fresh grass (Kalač and Samková, 2010). This is why the expectation of this study could seem surprising, because there is hardly a difference in SFAs of HAY and CON milk. Sampling once each quarter highlighted seasonal variation in the milk fat composition. SFA concentrations were highest

in winter and lower during summer in both groups. Only a minimal, insignificant difference of overall SFA content can be observed during the Q3. Overall the SFA content occurs congruent in the comparison of HAY and CON milk. This observation goes along with the results of Velik *et al.,* who compared different feeding regimes of cows to the FA profile in milk. It was found, that HAY and silage fed cows resulted in approx. the same amount of SFAs in milk, whereas alpine or exclusively grazing cows had a lower content of SFA (Velik *et al.,* 2013). So the difference in SFA would very likely be more divergent if only pasture grazing cow milk would be compared to CON milk. The reason for the importance of the SFA content in milk products is, that the SFA amount in milk can be suggested as parameter for feeding. Controversy, as it is shown in this experiment, one cannot conclude only by the SFA content if milk comes from HAY or CON feeding regime. An observation of single specific SFAs, BCFAs like PHY gives more information about feed interference. In Austria and many other counties dietary SFAs intake is associated with adverse health effects and nutritional recommendations advice to generally reduce saturated fat uptake (DGE, 2015). But this recommendation might be outdated or too generalized, because it is unsure if SFAs really cause harm in the healthy human organism (DeSouza *et al.,* 2015). Contrary, some SFAs can positively influence human health e.g. some BCFAs like PHY (Hellgren, 2010).

	Management		Season				P- value				
	HAY	CON	HAY summer	CON summer	HAY winter	CON winter	Main factor			Interaction	
FA	n= 45	n= 39	n= 20	n= 20	n= 25	n= 19	M	HAY	CON	MxS	MxW
C18:3n3	0.979	0.720	0.981	0.782	0.977	0.656	***	NS	*	***	***
FA2	0.089	0.078	0.089	0.080	0.088	0.075	**	NS	NS	NS	**
FA3	0.169	0.143	0.164	0.153	0.173	0.134	***	NS	**	NS	***
FA4	0.016	0.026	0.017	0.026	0.015	0.025	***	NS	NS	***	***
CLAc9t11	1.137	0.872	1.519	0.993	0.831	0.745	**	***	*	***	NS
FA6	0.010	0.014	0.011	0.011	0.010	0.016	NS	NS	NS	NS	NS
FA7	0.052	0.059	0.070	0.061	0.038	0.057	NS	***	NS	NS	**
FA8	0.031	0.028	0.037	0.030	0.027	0.027	NS	***	NS	**	NS
FA9	0.013	0.012	0.015	0.012	0.012	0.011	NS	**	NS	NS	NS
FA10	0.063	0.060	0.076	0.062	0.053	0.057	NS	***	NS	**	NS
FA11	0.098	0.090	0.112	0.090	0.087	0.089	NS	*	NS	**	NS
C20:0	0.187	0.187	0.189	0.187	0.185	0.186	NS	NS	NS	NS	NS
FA15	0.152	0.145	0.151	0.151	0.152	0.139	NS	NS	*	NS	**
C20:1	0.055	0.057	0.055	0.059	0.055	0.056	NS	NS	NS	NS	NS
FA17	0.012	0.012	0.014	0.014	0.011	0.011	NS	*	*	NS	NS
FA18	0.011	0.013	0.012	0.012	0.010	0.013	*	NS	NS	NS	*
C20:2n6	0.052	0.045	0.050	0.045	0.053	0.046	***	NS	NS	NS	**
C20:3n6	0.065	0.068	0.065	0.072	0.065	0.063	NS	NS	NS	NS	NS
C20:4n6	0.099	0.095	0.099	0.095	0.098	0.094	NS	NS	NS	NS	NS
C20:3n3	0.017	0.013	0.016	0.013	0.018	0.012	***	NS	NS	**	***
FA23	0.023	0.016	0.025	0.017	0.022	0.016	***	NS	NS	***	*
C21:0	0.100	0.082	0.100	0.086	0.100	0.077	***	NS	NS	**	***
C20:5n3	0.091	0.070	0.087	0.073	0.094	0.066	***	NS	NS	**	***
C22:0	0.080	0.074	0.079	0.076	0.080	0.071	NS	NS	NS	NS	NS
C22:1n9	0.010	0.009	0.010	0.009	0.010	0.009	NS	NS	NS	NS	NS
FA28	0.017	0.006	0.013	0.006	0.021	0.006	***	**	NS	**	***
FA29	0.038	0.037	0.030	0.036	0.045	0.037	NS	*	NS	NS	NS
C23:0	0.034	0.027	0.033	0.028	0.036	0.026	***	NS	NS	*	**
FA31	0.034	0.028	0.031	0.028	0.037	0.027	**	*	NS	NS	**
C22:5n3	0.083	0.066	0.084	0.066	0.082	0.065	**	NS	NS	*	*
C22:6n3	0.009	0.006	0.006	0.006	0.011	0.006	NS	NS	NS	NS	NS
C24:0	0.025	0.021	0.024	0.021	0.026	0.021	NS	NS	NS	NS	NS
C24:1	0.010	0.008	0.009	0.008	0.011	0.008	**	*	NS	NS	**

*Table 22: Distribution of minor FAs; starting at C18:3n3 (summer= Q2 and Q3; winter= Q1 and Q4;M= Management); Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1*

OI

Contrary to the pattern of SFAs, PUFAs do not show a difference looking at the single quarters in both groups HAY and CON. The values rise in summer and decline during winter. Except for 22:1n9. The values are higher in Q3 and Q4 than in Q1 and Q2 in both regimes. For hay milk, this is clearly connected to feeding of fresh grass. Maybe the CON fed cows also receive fresh grass or other supplements which could explain these higher values. C20:5n3 and C22:5n3 stay constant in hay milk. They originate from enzymatic synthesis from C18:3n3. This effect can also be observed in C18:3n3. The values do not differ a lot between summer and winter. This indicates again, that the HAY feeding has the same quality as fresh grass.

Q2 contains the highest values of C20:5n3 and C22:5n3 in CON milk (see Table 32). A possible explanation could also be the lactation stage. The values of C18:3n3 are significantly ($p \leq 0.05$) lower in winter, whereas the values of C20:5n3 and C22:5n3 stay constant. This indicates a higher turnover of C18:3n3.

As already seen in trend Figure 42 and Figure 43 of the means, FA28 and FA29 display differences during the year. Considering the values, these differences are statically significant ($p \leq 0.05$). FA28 shows these among the different feeding regimes also in between the seasons. FA29 has only differences among the HAY making regime over the year. Other unknown FAs are FA23 and FA3. These FAs are of special interest, because they also show huge statistically significances ($p \leq 0.05$) between the two feeding regimes and seasons. Combining the results of the Ag^{+}- TLC and GC-FID one can assume, that FA3 might be C19:0. In CON milk, the values stay nearly constant over the year, only Q4 show a slightly lower value (0.023, Table 32). The hay milk samples show the same pattern, but the values in Q3 and Q4 are lower than in Q1 and Q2 (Table 32). These values also indicate that FA3 might be C19:0. Milk from cows, receiving fresh grass during summer period contains less SFAs. In HAY the content of this FAs decreases in Q3 and Q4. In CON, the value stays nearly constant over the whole year, expect in Q4.

Regarding the GC-FID chromatograms some of the identified peaks are not as nicely separated as they should (C21:0, FA28, FA29). They contain little shoulders and it appears, that there are two peaks which haven't been separated by the column. Comparing the mean values of C21:0 of every quarter, it can be assumed, that C20:4n3 might be enclosed in this peak and representing the shoulder. The higher value in Q2 of CON milk indicates that there is another FA hidden in the peak. Normally, the SFA content is lower during the summer

months and rises in winter. In CON milk, the values of SFAs do not change dramatically over the year. This fluctuation exposes the other FAs. In HAY, this phenomenon is not that distinct. The reason for that might be that the lower level of the saturated C21:0 gets compensated by the other, probably C20:4n3, FA. There is a pattern seen by comparing all figures from n-3 FAs (Figure 34, Figure 37, Figure 38, Figure 39). The values of CON and HAY milk are distant in Q1 and approach in Q2. Then, in Q3 and Q4 the two curves separate. The HAY milk curve rises and the CON milk curve decreases. The curves of n-6 FAs behave differently. The two curves look alike, the only difference is, that the values of HAY are higher. The pattern from the n-3 FAs is also observed in FA28. This indicates, that FA28 might be an n-3 FA. As none of the two patterns can be monitored in FA29, FA29 might be dirt or not a FAME.

Comparing the mean values of CON and HAY independent from seasons, some FAs indicate a significance ($p \leq 0.05$). Highly statistical differences ($p \leq 0.001$) are evaluated for: C18:3n3, FA4, C20:3n3, FA23, C25:5n3, FA28, C23:0.

Table 22 shows also the p-values of CON and HAY depending on the season. There are more significances during the winter, which means that there is a real difference in the FA pattern. Here, again, C18:3n3, FA4, C20:3n3, FA23, C20:5n3, FA28, C23:0 indicate a difference ($p \leq 0.05$). In the summer period, CLA also has a huge ($p \leq 0.001$) significance; which represents an expected result. During summer, the cows are fed with fresh grass and are allowed to graze on pastures. This way they can choose on their own what they seek to digest and also have the opportunity to eat some fresh herbage, which contain higher amounts of PUFAs. The ratio between leaf and stem is also higher in the summertime. Leafs contain the most FAs. This explains the higher amount in summer period. Comparing mean values of HAY and CON between the summer and winter period, one could assume that the values are always lower in winter than in summer. But the graphs of the mean values of every quarter show different results. Sampling every quarter is a good method to see more explicit seasonal variations. The changes in the feeding procedure can be monitored more accurate than sampling two times a year (summer and winter).

There are still several unidentified FAs. Some of them show significant differences in between the feeding regime as well as in between seasons. FA28 and FA29 for example show differences but as it can be seen in Figure 42 and Figure 43 the peaks do not have an optimal shape and therefore it is hard to identify them. Also by comparing the GC-FID chromatograms with the GC-MS chromatograms (urea adduct formation and TLC) the FAs stay unknown. Since all peaks are at a very low range, these FAs are present only in traces

and the identification is even harder. Another suggestion might be that these peaks might not be FAs. There could be some dirt, septum residues or other organic compounds present.

For identification and a better separation of the peaks other columns/ other settings in the GC- programme or further developmental methods can be chosen. Using a TLC helps to get rid of the SFAs. Also, the urea adduct formation helps to focus on the PUFAs. After achieving the knowledge about where to focus on, these methods might help for further identifications.

KJ

According to literature findings the effect of season could overweigh feeding regime. For instance, Wendlinger *et al.,* found this in their study when comparing FFAs of CON to organic butter. In their results, organic butter showed constantly a higher content of FFAs. But if concentrations of FFA in summer butter of CON regime was compared to FFA content in organic winter butter, the concentrations were comparable in some samples (Wendlinger and Vetter, 2014). There are no results for FFA in this study, a proper analysis would require special concentration or enrichment techniques. Isolation, identification and evaluation of FFA can be complex, because there is also a lack of appropriate standards. However, a comparison of FFA could be relevant and interesting in context of PUFA analysis in milk, because FFA usually occur (as minor components) in presence of PUFAs. Moreover, FFA can work as radical scavengers, thus protect lipid peroxidation (Vetter and Wendlinger, 2013; Vetter, 2016)

5.4.6 Comparison of specific fatty acids

5.4.5.1 Comparison of fatty acids by groups | OI

Table 23: FA comparison by groups

	CON	HAY
season	**S | W**	**S | W**
∑ short chain	7.1 ± 0.07 | 7.7 ± 0.03	5.8 ± 0.09 | 6.1 ± 0.14
∑ medium chain	7.8 ± 0.18 | 8.6 ± 0.04	7.2 ± 0.13 | 8.4 ± 0.11
∑ long chain	85.7 ± 0.15 | 84.4 ± 0.06	86.5 ± 0.12 | 84.2 ± 0.13
∑ C18	38.3 ± 0.12 | 33.9 ± 0.06	40.5 ± 0.10 | 33.1 ± 0.09

S= summer; W= winter

It can be seen, that during summer, the short chain and medium chain FAs are lower than in winter (see Table 23). Contrary, the amount the long chain FAs is higher in the summer. Looking at all C18 FAs the same pattern is seen as for the long chain FAs. Especially HAY fed cows show a huge difference between summer and winter. Fresh grass influences the C18 FA intake. Nevertheless, the amount of long chain FAs is nearly the same for both groups during the year. Lower levels in winter can be linked to less fresh grass feeding and supply. As seen in Table 20 the C18:0 values are not as much varying as all C18 FAs compared together. That highlights an obvious reduction of the content of unsaturated C18 FAs during the winter period. Due to the fact, that the reduction in unsaturated C18 FAs does not lead to a higher amount of C18:0, the fodder and thus the supply von PUFAs might be a clear reason for this. The higher short and medium chain levels in CON fed cows can be explained by the concentrate feeding which results in more short chain FAs.

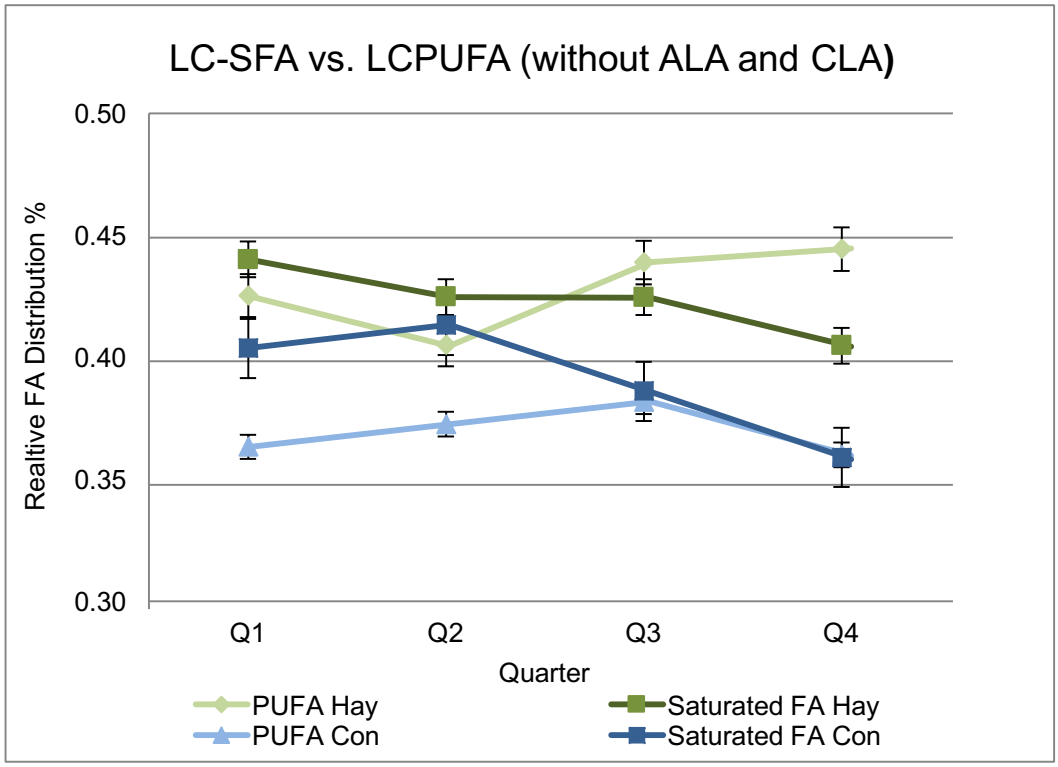

Figure 47: Seasonal comparison of PUFAs vs. SFAs of HAY and CON milk

Comparing the total amounts of PUFAs and SFAs in minor LCFAs over the year, no big difference can be detected in SFAs. The differences are slightly higher in Quarter 3 and 4. One thing to mention is that the samples were taken out of ready-processed milk cartons. Therefore, it is not possible to make a clear statement about the real feeding regime and the HAY treatment, which would have an influence on the FA pattern.

Looking at the PUFAs amounts, the hay milk samples show a greater difference. The highest gap is obtained in Q3. That is the milk, which is produced in summer to late summer, in which the levels of PUFAs in plants are very high. The rise from Q1 to Q2 as well as the rise from Q2 to Q3 in CON milk is quite remarkable. Here, the enclosure of fresh grass into indoor feeding can be seen. The hay milk samples have a huge rise in Q2 to Q3. This can be associated with fresh grass and herbs on the pasture. This increase from Q2 to Q3 in both feeding regimes can also be related to the maturation stage of the leafs.

Further, it is visible in Figure 47, that hay milk contains a higher amount of minor LCPUFA. But, contrary, the amount of SFAs is also higher in the hay milk samples.

5.4.5.2 Comparison of minor omega-6 and omega-3 - fatty acids | OI

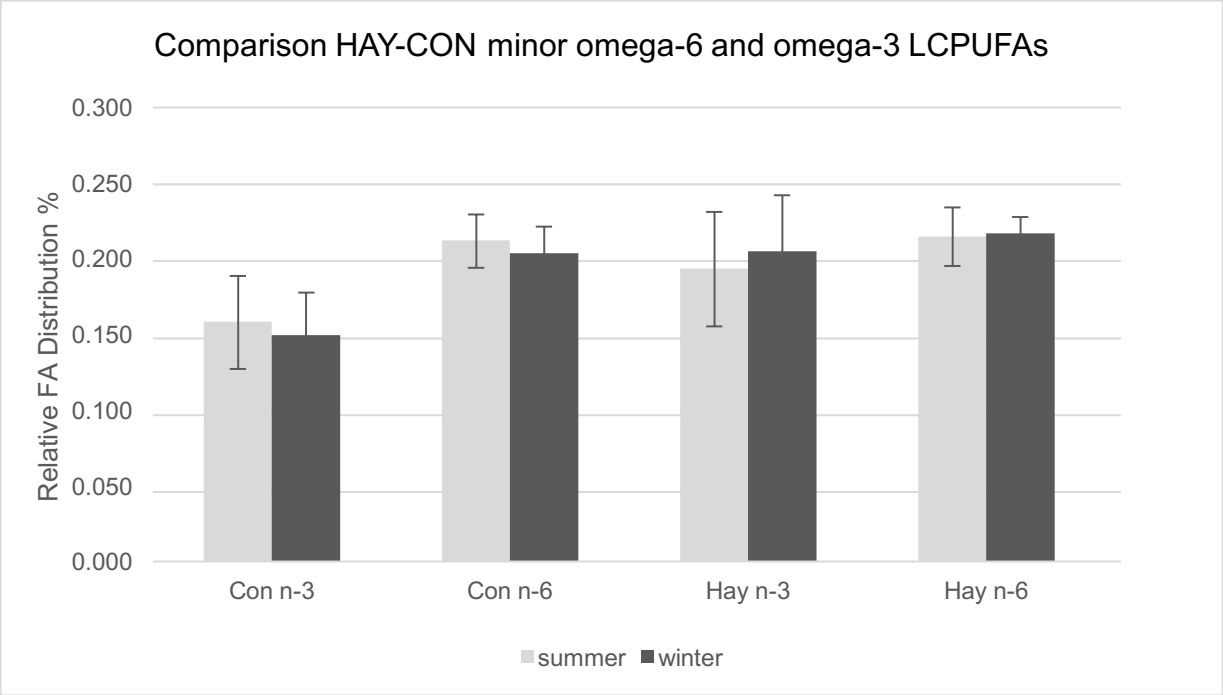

Figure 48:Seasonal comparison of minor omega-6 and omega-3 LCPUFAs of HAY and CON

Comparing only the n- FAs (of the minor LCFAs) in both groups, the n-6 FA have nearly the same percentage. There is also no big difference between summer and winter. The HAY samples contain more n-3 FA than the CON samples. There is a decline of the n-3 FAs during winter period in the CON samples, but so do not the n-3 FAs from hay milk. They are even slightly higher. C22:6n3 and C22:5n3 are the most valuable n-3 FAs. They come from the conversion of C18:3n3. It is remarkable, that the contents of n- FAs decline from summer to winter in CON milk, whereas they show a slight rise in hay milk. That indicates, that the feeding with HAY during winter can improve the FA spectra.

Comparing the CLA levels in both groups, they show again a higher percentage in HAY fed cows than in conventionally fed cows. The CLA levels rise during summer, when the cows are allowed to graze, and are lower during winter. In Q1 the amounts are nearly the same. This indicates a minor difference in HAY vs. CON feeding. During the whole year, the CLA level of conventionally fed cows, does not remarkably change. Looking at the means of hay milk, the difference from Q1 to Q2 is double than the difference from Q2 to Q3. Comparing the CLA levels of CON summer and HAY winter, the levels are almost the same. This means, that summer CON milk is of comparable quality as HAY winter milk. Apart from this, extensive fed cows show the same level as intensive fed cows in winter, which get a high

ratio of concentrate. Through the intake of fresh grass, the PUFA intake is also enhanced. As PUFAs form the base for the development of CLA, extensive fed cows can build much of it. This correlates with the results in summer.

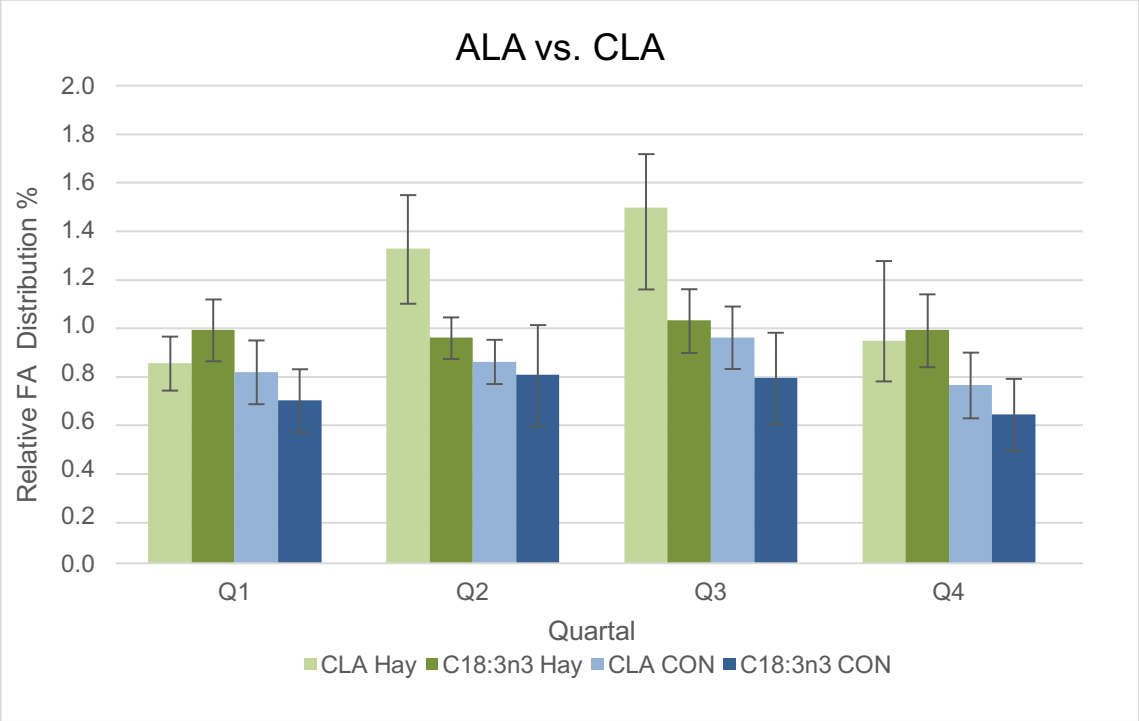

Figure 49: Seasonal comparison of ALA and CLA ratio in HAY and CON

Figure 49 clearly demonstrates the differences between the two feeding regimes by the comparison of CLA and C18:3n3, which could be already detected in Table 22. During summer, both FAs show differences ($p \leq 0.05$), whereas in winter only C18:3n3 shows a differences ($p \leq 0.05$) between the two feeding regimes. Looking at the HAY samples in winter, C18:3n3 has a greater value than CLA, but this changes during summer. Not so for the CON samples. C18:3n3 is always lower than CLA. The ratio between the two FAs is highest in Q3. CLA prevails here. C18:3n3 is directly incorporated into the milk from the diet, whereas CLA originates from both sources. It is possible to see, that fresh grass results in a higher CLA level ($p \leq 0.05$) in HAY. The CLA from CON milk does not change a lot. This phenomenon can therefore be linked to the incorporation of fresh grass. Further can be seen, that feeding HAY in winter does make a difference. It leads to a higher amount of C18:3n3. Since C18:3n3 comes directly from the feeding regime, it is evident that HAY is a better source of PUFAs than silage.

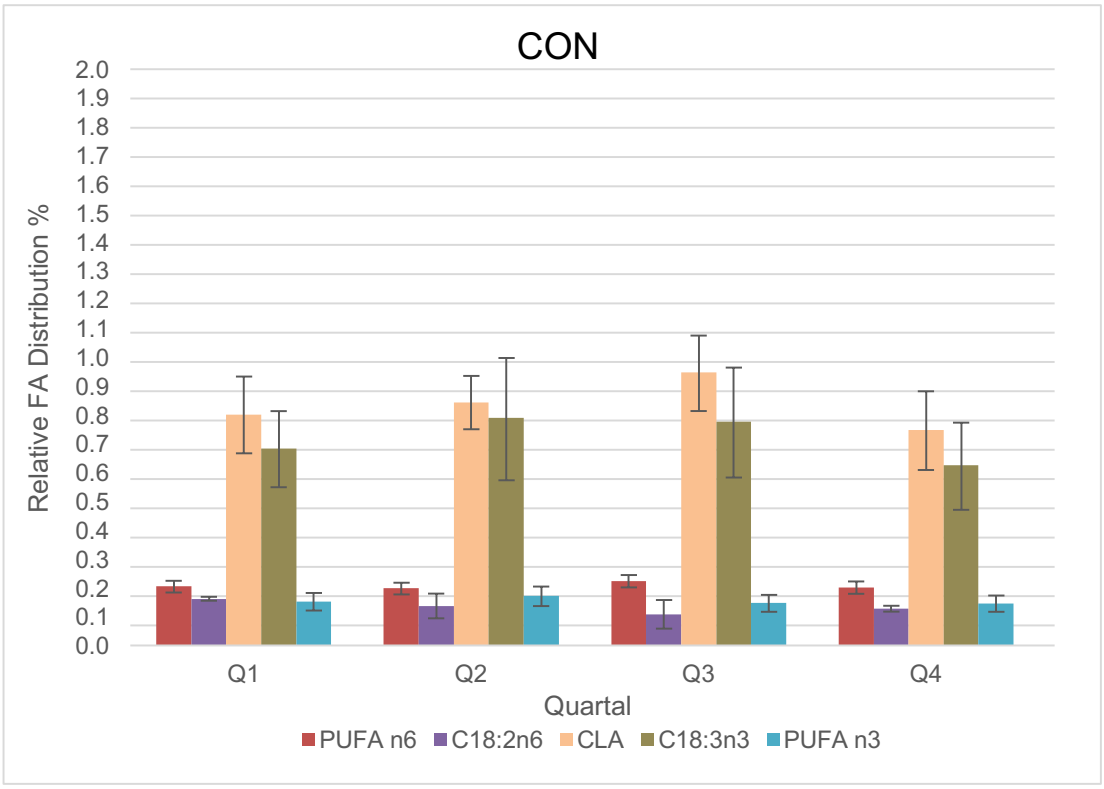

Figure 50: Seasonal variation of PUFAs, CLA, ALA and LA in CON

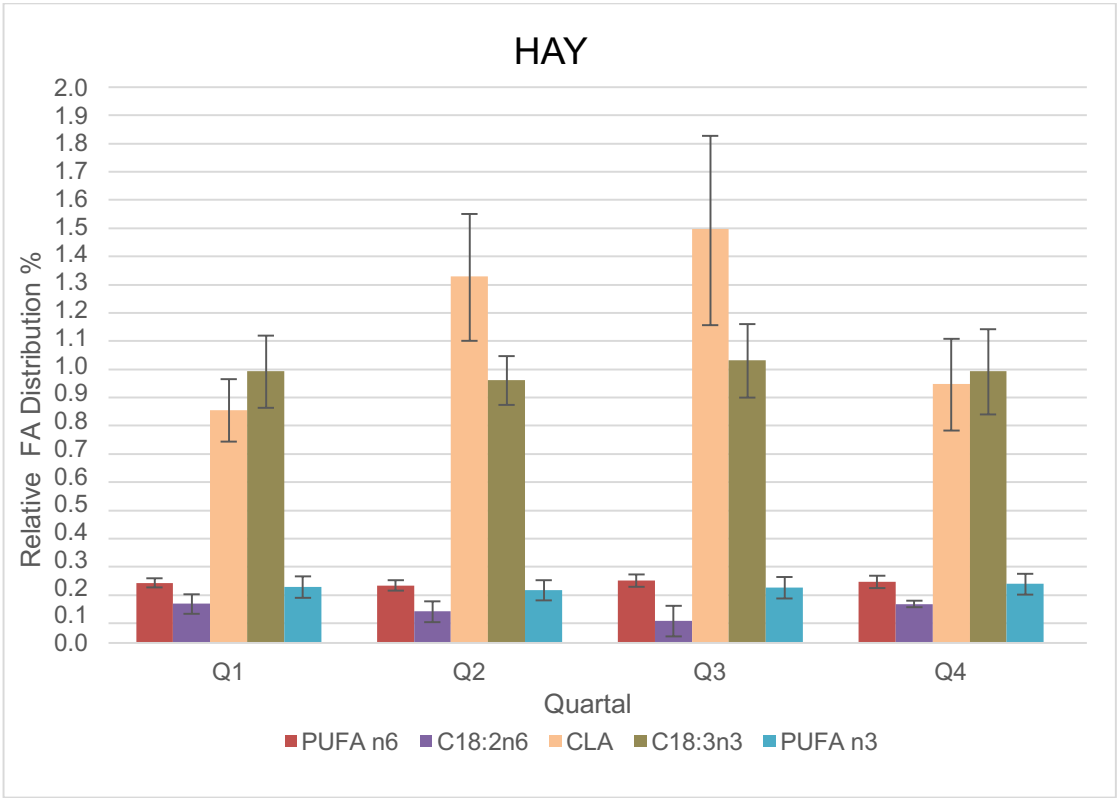

Figure 51: Seasonal variation of PUFAs, CLA, ALA and LA in HAY

Figure 50 and 51 demonstrate the proportions between C18:2n6, C18:3n3, CLA and the remaining minor n- LCPUFAs. Again, it is nice to see, that the CLA content is highest in hay milk, with a peak in Q3. The values are lower than C18:3n3 in Q1 and Q4, unlike in CON milk. The ratio between all FAs is lower in CON milk, than in the hay milk samples. During summer, the C18:2n6 levels decrease, while the n-6 PUFAs do not change. The ratio between them is highest in Q3. This phenomenon is not obtained for the n-3 FAs. The ratio stays the same over the whole year. In CON milk, there is a slight change during the winter seen. The ratio is lower there.

KJ

Although several study results are discussed controversially and the question of an optimum in LCPUFA supply and appropriate biomarkers is not answered clearly, the ratio of C18:2n6 to C18:3n3 seems particularly crucial to infants (Gibson *et al.,* 1994). Besides potential health benefits of an optimized LA/ ALA ratio the purposes of this work was, to see if that ratio could be a potential parameter for differentiation between the two feeding regimes HAY and CON. Additional to the direct FA intake, there are (mostly biochemical) factors besides an efficient biohydrogenation influencing the transfer of C18:2n6 and C18:3n3 to the milk, which are not fully explored. It is suggested that hay milk would contain higher concentrations of C18:3n3 than CON milk, which goes along with recent findings (Khiaosa-Ard et al. 2010). Whereas the inclusion of silage feed, especially maize-based silage by tendency increases C18:2n6 in milk (Walker et al. 2004) while decreasing C18:3n3 (Slots et al. 2009). Dhiman *et al.*, and Chilliard *et al.,* found also that LAs were more than twice as high in milk when cows could graze on pasture compared to mainly indoor kept cows. The higher level of C18:3n3 in pasture-fed cow's milk was explained by an increased uptake and a higher ruminal escape of these FAs (Chillard *et al.,* 2000; Dhimand *et al.,*1999). This phenomenon could partly be transferred to this experiment. Firstly, there were no ($P > 0.05$) differences by comparing the ratios throughout the quarters of either HAY or CON samples. As it is visible on Figure 51 HAY samples show a more constant level of LA/ ALA over the year, but overall the visible increased variation in CON samples is not significant. Haymilk samples have higher ($P < 0.05$) levels of C18:3n3 in all quarters compared to CON. The greatest difference of HAY and CON could be observed in winter season, meaning Q1 and Q4. During Q2 and Q3 the LA/ ALA content in HAY and CON is still significant but is slightly more drawn together. The increased difference in Q1 and Q4 can be explained by higher silage content in feed in the CON regime during the colder months, but the slight increase of C18:3n3 also in the CON regime due to fresh grass supply in the warmer months. Although differences of the ratio in HAY and CON regime in mixed consumer milk might get blurred;

there is a measurable difference. In respect to this, hay milk showed a more desirable FA profile than CON milk.

5.4.5.3 Ratio of omega-6 to omega-3 fatty acids | OI

The importance of the ratio has already been explained. The important n-6 FAs are starting before this study. Here, the n-6 values eluting before C18:3n3 are included in the calculation.

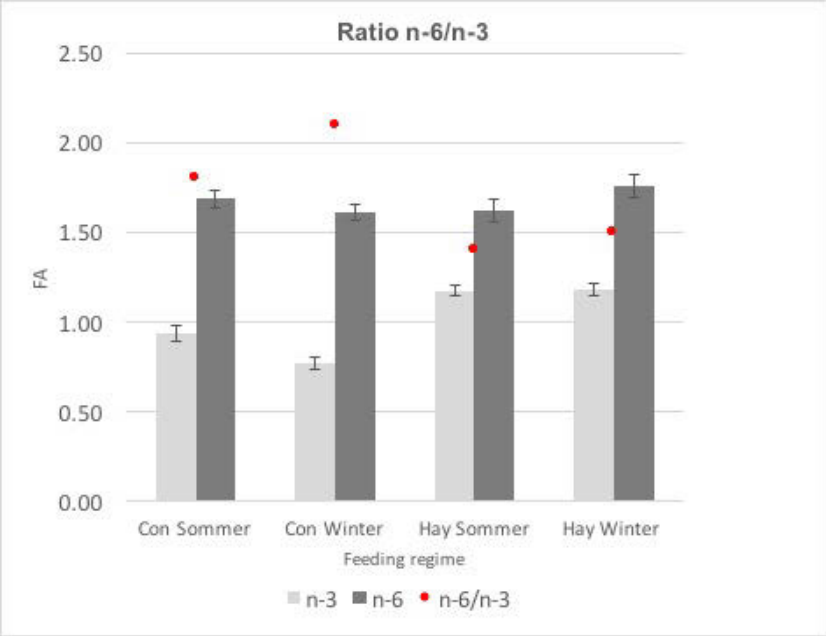

Figure 52: Seasonal variation of omega-6 to omega-3 FA ratio in HAY and CON

In this trial the ratio for CON milk in summer is 1.8 and 2.1 in winter. Compared to the results of HAY milk, the results are higher. The ratio in HAY milk is not varying between the seasons, whereas in CON milk is varies. Looking at the n-3 values you can see, that the values of hay milk does not vary. That means, that there are as much n-3 FAs in summer HAY-milk than in winter. In both groups, the n-6 FAs stay constant. The higher the n-3 FAs, the narrower is the ratio. It can be concluded, that fresh grass has a positive effect on the n-3 level. Due to the fact, that the n-3 level does not change in winter (HAY feeding), the feed composition may not influence the level. This indicates that the energy balance of the cow may influence the content. Both, DeWit and Leiber found higher levels as the energy balance decreased (DeWit *et al.,* 2006, Leiber *et al.,* 2005). Overall, both groups have a very narrow ratio (< 5:1) and are in the optimal area, that's why all samples can be ranked positively.

5.4.5.4 Technological aspects | KJ

An increase of PUFAs in dairy products should be in a combination with an increase of antioxidants. Otherwise dairy products might contain off-flavours e.g. especially if n-3 Content in milk is increased with fish oils in feed. Additionally, dairy products with higher PUFAs are more susceptible for oxidation processes which go along with a shorter shelf life and rancidity (Nelson and Martini, 2009; Silva-Kazama *et al.,* 2010). An increase of PUFAs by the animal keeping regime like organic or alpine kept (more grazing) cows, that produce milk with relatively higher PUFAs, naturally contains more antioxidants like beta-carotene or tocopherols (Kristensen *et al.,* 2004).

5.5 Factors influencing the fatty acid pattern | OI

5.5.1 The effect of organic vs. conventional farming regime

There are only few literature sources on winter studies with HAY fed cows only. Most literature refers to organic/ecological produced milk, but in this case, cows are often fed with grass silage during the winter. Most studies compare pasture feeding in summer vs. no access to pasture. Hay milk is often declared as organic milk, but in this research the focus is on the definition hay milk itself. Nevertheless, the processing restrictions are very similar. Organic farming has stricter guidelines on concentrated feed. Thus, it is possible to compare literature results of organic milk to hay milk. Conventionally fed cows are often also offered fresh grass during the summer season. This may explain the higher values in certain FAs here.

In this research, some significances ($p \leq 0.05$) have been found between the two different farming styles. The mean contents of C18:3n3, CLA, C20:2, C20:3n3, C21:0, C20:5n3, C23:0, C22:5n3, C24:1 and of some unidentified FAs over the whole year were all significantly higher in hay milk. Except for C21:0 and C23:0, the PUFAs are causing the difference here. For both SFAs (C23:0, C21:0) the significance is more severe (C21:0; $p \leq 0.001$ and C23:0; $p \leq 0.01$) in winter than in summer ($p \leq 0.01$(C21:0) and $p \leq 0.05$ (C23:0)) which might influence the mean level of the whole year. Also Dhiham, found higher concentrations of CLA in HAY-fed cows compared to corn silage (Dhiman *et al.,* 1999). At the beginning of the experiment a proportional increase of fresh grass in the diet lead to a linearly raise of CLA levels. On pasture, the content even increased up to 500%. The same results could be determined by Bernadini and Staszak. Staszak found a higher CLA content in milk from cows fed on a HAY based diet compared to maize-silage-fed cows (Bernardini *et al.,* 2010; Staszak, 2005). Every month showed higher CLA levels. The mean content of CLA from maize-silage was 3.9 mg/g milk FAs and 7.9 mg/g milk FAs with a 98% hay- grass diet. Comparing these values, the CLA level is twice as high. This phenomenon could not be detected in CON milk in the present study, but the CLA level between fresh grass feeding in summer and HAY-feeding in winter level doubled (from 0.831 to 1.516). The same pattern could be detected by organic farming from Bellof. Bellof found, that through fresh grass the CLA content had doubled (Bellof *et al.,* 2013). Equally Wyss found out, that feed based on fresh grass can increase the amounts of n-3 and CLA compared to CON feeding with grass- and maize-silage and concentrate (Wyss, 2007). The study of Bloksma also demonstrated, that organic raw bulk milk was richer in CLA and n-3 FA than CON milk in February - although the cows of the organic regime were fed with silage in winter as well (Bloksma *et*

al., 2008). These findings indicate, that a lower ratio of concentrate and a higher level in grass- based conserved feed (red- clover silage) influences the level of PUFA. The levels of CLA in our study do not show a difference between the two feeding regimes. The value in HAY- milk is 0.831 and 0.745 in CON- milk ($p > 0.05$). This result indicates on the one hand, that the conventionally fed cows did probably not get much concentrate and on the other hand that feed based on HAY in winter is as good as the CON system.

A seasonal variation is already a known factor, which has an influence on the milk composition and has been reported, on farm level, by Rego and Lock and Bauman 2004. Due to the influence of fresh grass, which contain many PUFAs, seasonal differences occur (Rego *et al.,* 2008). In the present study, these varieties could be seen in hay milk but not for conventionally fed cows. The effect was also higher for the unknown FAs than for known PUFAs. For a high PUFA content, the supply of C18 PUFAs is of interest. A high intake, for example through grazing, can lower the SFAs level and increases the C18:3n3 and the CLA level because of incomplete biohydrogenation in the rumen and a decrease in the *de novo* synthesis (Chilliard *et al.,* 2007). Chilliard also found out, that feeding concentrate did not influence the fat content, but had an impact on the FA composition. The experiment showed, that feeding HAY and concentrate results in higher *trans* oleic acid and lower C14:0, C16:0 and C18:0 levels. Like other studies already stated, Butler found, that summer milk has a lower concentration of SFAs and higher concentrations of PUFAs. They also examined greater differences in between seasons than in between years. The weather might influence the forage availability, quality and intake in between years (Butler *et al.,* 2011). 2015, the year where the samples for the present study were taken, was an extremely hot and dry year with only little rainfall. In the alpine region, it was the warmest year since the first weather recording in 1786. The results might not be comparable to samples from other years, because the fodder might be changed due to the dryness.

During winter, the diet represents conserved forage and concentrates to meet the nutritional requirements. The wilting procedure can destroy some PUFAs through oxidative stress. The process of silage production and also lipolysis causes a decline of PUFAs content (Dewhurst *et al.,* 2006). Another point is the height above sea level, which possibly also has an impact on the n-3 FA. This may be due to the high amount of herbage on alpine pasture (Collomb *et al.,* 2002 ; Frelich *et al.,* 2009). Frelich found differences in between the season applied to the whole FA content in between mountain farms. So did Collomb find differences in the FAs from geographical sites, especially in the CLA levels (Collomb *et al.,* 2002). Austria is a mountainous country at different levels of height. As the temperature in height is lower, plants contain more PUFAs, because of the lower melting point. The milk samples for this study are assumed to be collected from all over the country. CON milk samples might also

origin from intensive farming, where the sea level has no impact, because cows have to stay in stables. On the other hand, feed meant for intensive farming is collected and conserved from surroundings all over the country. Thus, feed may also origin from higher sea levels. Nevertheless, the influence might be more significant for the hay milk samples. Again, intensive farming is done mostly in the low-land provinces of Austria. The hay milk samples originate from the alpine region, because this is the area, where intensive farming is not really possible.

5.5.2 The effect of the lactation stage

The exact origins of the milk samples used in this experiments are unknown. There is the possibility of certain calving cows that contributed milk, especially in spring. This fact is also responsible for lowering SFA levels (for both groups). At the beginning of the lactation stage, cows are often fed supplemental fodder to maintain the milk yield in high performance cows, due to higher energy requirements. High performance cows represent a negative energy balance at the early lactation stage. Thus, they are fed additionally with extra cereals. This feed consists of a starch to a major extent, which leads to a decrease of ruminal pH, that negatively influence the digestion of fibre and the milk fat content (Dohme, 2005). During the early lactation stage SFA levels are lower. Higher energy needs result in FA synthesis from adipose tissues, which decreases *de novo* synthesis (Palmquist *et al.,* 1993). Wyss examined the impact of the lactation stage on the n-3 level. They did not find any influences, so the n-3 level was unaffected by the lactation stage (Wyss, 2007).

5.5.3 The effect of lipids in plants- seasonal effects: regional effects

In the present study, the production process of hay as feed is not defined and very likely to differ from farm to farm. Further it is unknown, how the fed silage was produced or conserved and what the exact composition was. Generally, the grass for hay is harvested later than for silage (Chilliard *et al.,* 2007) which may explain the lower levels of PUFAs in HAY than in silage. In contrast, the efficiency transfer is higher in HAY than in grass silage (Shingfield *et al.,* 2005). *Lock* and *Bauman* found a higher Δ^9 -desaturase activity in summer than in winter. In terms of activity determination, the ratio of C14:0 and C14:1 is important and considered as indicator for enzymatic activity. C14:1 is built only *de novo* by desaturation. This in return represents the activity (Lock and Bauman, 2004). Borreani found out, that milk from fresh

herbage/ grass and legume silage had higher contents of C18:3n3 and a lower n-6/ n-3 ratio. In their study all diets were based on a corn silage feed, which is not applicable to the hay feeding regime considered in this study, but to the CON regime. Borreani also investigated more BCFAs in the supplemented herbage diet due to greater proportion of roughage (Borreani *et al.,* 2013). Another suggestion for increasing C18:3n3, is to feed cows certain plants containing secondary metabolites (tannins, terpenoids) that could influence the microbial constitution. Secondary plant metabolites could cause an inhibitory effect of specific hydrating ruminal bacteria and this can finally lead to increased release of C18:3n3 in milk (Leiber *et al.,* 2005)**.** *Kraft* mentioned in their experiment three specific sorts of plants (*Leontodon hispidus, Lotus corniculatus, and Trifolium pratense)* that are associated with a higher PUFA content (Kraft *et al.,* 2003). Plants store energy either in the form of starch or oil. Thus, a supplementation with oils can contribute to the same levels of CLA as in hay milk. An interesting study from Collomb on supplementation with oilseeds found the highest concentrations of unsaturated FAs and CLAs with supplementation of sunflower seeds. The high amount is referred to the sunflower's high content of linoleic acid. Since it is allowed to feed and supplement with seeds in the HAY feeding system, this study can explain the higher contents of FAs and help further enhancements of milk quality according to this feeding regime. Collomb's study compared three different kind of seedlings (linseed, rapeseed and sunflower seeds). Linseed has the highest content of C18:3n3 and n-3. Rapeseed has the highest of oleic acid and sunflower has the highest of C18:2n6. Depending on the diet, the highest values of these FAs could be figured out. Further, C20:5n3 and 22:6n3 were present at very low concentrations in all milks (Collomb *et al.,* 2004). Linseed showed an increase of 20:5n3, C22:6n3 and C20:3n3 (Kraft *et al.,* 2003). Higher levels can be achieved with fish oil supplementation (Chilliard *et al.,* 2001; Mansbridge and Blake, 1997) but they are not allowed for hay milk and have an influence on the taste of milk. Hence, it is not an alternative. Dhiham studied the effect of soybean oil supplementation, which resulted in higher CLA levels. The supplementation may cause incomplete biohydrogenation of CLA and its escape from the rumen to the lower digestive tract (Dhiman *et al.,* 1999). Also Dohme figured out, that feeding oil supplements can influence the FA pattern. Therefore, oil can be an alternative to starch rich diets to enhance the FA pattern in milk (Dohme, 2005). A higher C18:1n9 content could be detected by Grummer through the feeding of extra oil. Resumed, feeding extra oilseeds can improve the nutritional quality of milk (Grummer, 1991). Considering the preservation methods for hay, the drying step is another potential influencing factor. Drying in the barn results in higher C18:3n3 levels in both, the milk and the plants (Chilliard *et al.,* 2007). Dewhurst found species and cutting interaction effects, which show that management factors are as important as breeding effects. FA concentrations were highest here in early and late season.

The study also stated like other studies that the leaf proportion is very important. For example, a decrease in the leaf content means a decrease of FAs. Prolonged maturation of the leaves results in an increase of fibre and grain and thus a lower FA content (Dewhurst *et al.,* 2001). Elgersma conducted a study on the effect of feeding fresh grass followed by a change to grass silage diet. The FA composition of the milk altered within a few days. The CLA level decreased, and similarly, the VA level decreased (Elgersma *et al.,* 2004). In another study of Elgersma the FA concentrations in plants declined within the regrowth stage in fresh grass the FA are present as esterified FAs, in silage as free FAs. Elergsma *et al.,* detected lower levels for C18:1n9 and C18:3n3 in pre-wilted grass (Elgersma *et al.,* 2003). Glasser *et al.,* did a meta-analysis on the effect of hay and silage diets. The major FAs in plants were C18:3 species and the main factor influencing FA composition is the vegetation stage at harvest. Also wilting and drying altered the FA profile. In contrast, ensiling additives had minor effects. During hay production there was a decrease in total fat, as well as a decrease in C18:3n3 acid through an increase of C16:0. This effect was even worse when drying conditions were bad (like wet weather or barn dried). Ensiling of corn silage had no effect on total FA, but showed a decrease in C17:0, C22:0 and all C18:0 isomers, expect C18:1n9. Ensiling of grass showed a slight increase in total fat. Without prior wilting, the linolenic acid level stayed constant (Glasser *et al.,* 2013). Khan *et al.,* found, the exposure of silage to air leads to lower contents of C18:2n6, C18:3n3 and a decreased total FA content. Again this is compensated by an increase of C16:0. The free FAs in the silage are further oxidised by the exposure to air (light, oxygen, microbes). Vegetation differences in plants can also be factor influencing the FAs in plants (Khan *et al.,* 2009). Boufaid *et al.,* found decreasing concentrations of C16:0, C18:2n6 and C18:3n3 during stem elongation and flowering. At that time, the leaves are less important to the plant. The study also stated, in accordance to the previous mentioned study, that wilting and drying reduced the levels of C18:2n6 and C18:3n3. They were higher in summer regrowth and in spring (Boufaïed *et al.,* 2003). Similar results were concluded by Morel. Ensiling reduced the C18.3n3 (Morel *et al.,* 2005).

Shingfield *et al.,* discovered that hay production has an influence on the plants FAs. The conservation method lowers the C18:2n6 and C18:3n3 content in the plant tissue. Contrary, the content of the two FAs is higher in milk. It can be assumed, that the transfer is better than with grass silage. Also the odd-chain FAs and BCFAs content is higher in hay milk. Actually, silages had higher concentrations of FAs, but there was a 7- day delay in harvesting hay. Because of this delay, the FAs in plants may have already changed (Shingfield *et al.,* 2005).

5.5.4 The effect of feeding different silages

In this study the CON milk packages were bought without knowing the precise feeding of the milk within. Cows could have been fed with different silages (legume, maize, grass). All of them have different impacts on the FA pattern. Maize silage is rich in C18:2n6 and C18:1n9 but poor in C18:3n3. (A diet with silage decreased the BCFAs.) In the organic farming regimes, it is allowed to feed (grass-) silage. Studies have shown that milk form organic farming leads to higher contents of C18:3n3 and CLA and this can also be explained by the higher use of legume silage (Chilliard *et al.,* 2007). On the other hand, the results of the literature research on the effect of ensiling are contradictory. Some of them report a decrease (Dewhurst, 1998; Elgersma *et al.,* 2003; Arvidsson *et al.,* 2009), some an increase (Boufaïed *et al.,* 2003; Alves *et al.,* 2011). Alves described, that ensiling of fresh grass had no effect on the C18:3n3 and C18:2n6. Their content was even higher. Other results showed the procedure of ensiling corn. The proportions of C18:2n6 and C18:3n3 decreased. But this study was not carried out on field. Studies, which were carried out on field showed a decrease in both crops. These losses are assumed to be caused by the field manipulations in general. Arvidsson *et al.,* did not find an effect caused by wilting the grass. This study was conducted with a shorter wilting time (< 24h) than all other studies (Arvidsson *et al.,* 2009). Therefore, the effect resulting from the experiments of Dewhurst/ Elgersma are associated with prolonged wilting. There was more reaction time for enzymatic processes (Dewhurst, 1998; Elgersma *et al.,* 2003). It can be possibly linked to botanical reasons and the developmental stage of the plant.

5.5.5 The effect of breed: individual cows

Another lack of the present study is, that the cow breed is unknown and likely to be diverse. Sasanti *et al.,* found differences in the total n-6 and CLA content between two different breeds. Also other researchers found some differences within different breeds. As it is unknown for both groups, the factor of breed can be excluded in this experiment. Also because this trial was carried out with retail milk, where all collected milk from the farmers is mixed together. A differentiation could not be made afterwards in both groups (Sasanti *et al.,* 2015). Baars *et al.,* could not detect any differences between two breeds (Holstein and brown Swiss). They also compared different feeding strategies (hay and maize silage) which resulted in more obvious differences. The comparison of individual animals showed big differences in the Δ^9-desaturase activity (Baars *et al.,* 2015).

5.5.6 The effect of ruminal biohydrogenation

The level of the C18:0 reflects the ruminal biohydrogenation activity. C18- PUFAs are ingested by feed and metabolized until C18:0 saturation. The reaction is more emphasised from PUFAs to monounsaturated FAs, than from monounsaturated FAs to SFAs. That is reason why often many monounsaturated FAs can be found. Intermediates vary depending on the feed composition. The results of this experiment show higher levels of C18:0 in summer and a lower concentration in winter in both feeding regimes. The difference in between the seasons is higher for hay. Less biohydrogenation, results in a higher the PUFA content.

5.5.7 The effect of energy restriction

The PUFA content in milk is often associated with the cow's total energy uptake and does not depend on the feed composition. DeWit *et al.,* stated, that the total feed consumption is more influential than its components. An energy deficit in cows lead to higher n-3 concentrations in milk in their study (DeWit *et al.,* 2006). Also others found, that a reduced supply of feed would result in higher n-3. The higher PUFA content in undernourished ruminants is explained by an increased release in adipose tissue which consists mainly of C18 PUFAs (Leiber *et al.,* 2005).

6 Conclusion | OI

It is not clear, if a differentiation between HAY and CON milk is possible by minor LCFAs. Both feedings regimes have higher values during summer. A differentiation does therefore not make sense. Further it is not clear, how the cows are fed by the intensive farming regime. They might get fresh grass and supplements which increase the PUFA content as well. Comparing the results of the winter period, some differences ($p \leq 0.05$) are seen e.g. for C18:3n3, C20:2n6, C20:3n3, C21:0, C20:5n3, C23:0, C22:5n3 between the two feeding regimes.

The question of this study was, if the analysis of minor FAs does give an additional benefit about the differentiation and does it make sense to quantify them. It was possible to find some differences, despite the fact of the mixed consumer milk. The distinctions might be more severe, when tank milk gets examined. The greater the amount of green fodder is incorporated into the feed, the higher the levels of n-3 FAs are. C18:3n3 is a pre-stage of C20:5n3, C22:5n3 and C22:6n3. Except C22:6n3, all values of these FAs differ ($p \leq 0.05$) between CON and HAY during summer and winter. In general, HAY has higher values, but C18:3n3 stays constant over the year compared to CON ($p \leq 0.05$). Contrary, n-6 FAs do not show a difference between the farming regimes, except C20:2n6, which differs ($p \leq 0.05$) during winter. In conclusion, milk from pasture- based feeding had a more beneficial FA pattern.

The hypothesis that a detailed observation of minor FAs in cow's milk could allow a way of verification and reliable differentiation between HAY and CON milk is mainly confirmed. Of course it depends on which specific FA is used for comparison.

Although the experiment was conducted with consumer milk, where potential effects of different feeding/ animal keeping regimes could interfere or get counterbalanced, a differentiation according LCPUFAs was possible throughout seasons. Very likely the evaluation of LCPUFAs in milk fat might not be an applicable method for industrial purposes e.g. rapid analysis for discrimination of HAY and CON milk.

7 Summary | KJ

The milk industry, which is an important economic and commercial sector in Austria (see chapter “milk production in Austria”), is interested in a “healthy” image of milk. There are several reasons to reconsider milk as a nutritionally valuable and even functional food. Due to the fact, that milk and dairy products mainly consist of SFAs, a negative connotation for human health seems obvious. Science on the other hand indicates that the positive effects of a moderate milk consumption (approx. 0.5 l/ d) overweigh the negative findings (Haug *et al.,* 2007). During the last couple of years, the discussion about dairy products has been controversial. Some studies and experts are convinced that dietary milk is very likely to cause harm in the human organism (Wiley, 2012). These possible adverse effects of milk consumption reach from a certain allergic potential (Høst, 1994) to an increased risk for some types of cancer, amongst others (Song *et al.,* 2013).This study presents an emphasis on milk fat composition, and won’t discuss on other milk components (proteins, vitamins) influencing milk quality or its effect on the human organism. According to the broadly accepted nutritional recommendations for fat, the intake of saturated fat - respectively fat originating from animals - should be kept at a minimum (DGE, 2015). Most studies found in literature research were conducted under controlled conditions. Mostly, extreme feeding regimes in cows were compared. Farm studies are hard to find and if, they are the topic of mountain farming systems (Collomb *et al.,* 2002; Ferlay *et al.,* 2008) and organic farming (Butler *et al.,* 2008) of those cases, diets based on pasture and silage were compared. There is only little knowledge on cows in intensive farming regimes or farming with HAY-based diets. Furthermore, a wide range of farms contribute to the supply of retail milk. No statement can be made on single or individual farms in this study.

The aim of this study was the comparison of the FA pattern of HAY versus CON cow’s milk that is available in Austrian supermarkets. The emphasis of this comparison is on LCPUFAs influenced by the feeding regime. Pre-tests and concentration techniques have been performed to enable a proper identification of these minor FAs via gas chromatography, respectively GC coupled with mass spectrometry. The results of this analyses are discussed with those of other relatable studies giving an overview of the current state of research.

Concluding on the outcomes of scientific literature, FAs in milk are influenced by various parameters interacting to different extent with each other. Namely those parameters are: farming regime, lactation state, ruminal biohydrogenation, animal breed/genetics, age, specific plant (-components) in feed, season, region and energy restriction in feeding.

Considering the results of the comparison in HAY and CON milk samples, some findings are clear: hay milk shows higher values of some nutritional valuable FAs like CLA, n-3 and n-6

FAs. The biggest gap is in the n-3 levels, HAY has a higher content than CON milk, although the level is not fluctuating much over the year. And both preservation methods (HAY/ silage) for grass result in a decrease of total FA content. This study shows again that although farmers can influence the FA pattern with supplements, including fresh grass in the diet, is still the most important way for achieving high milk quality. The selection of the right grass species/ breeds can improve the profile naturally and is on a low- cost level.

The main limitation of this study is the rather unprecise differentiation of the two compared groups: HAY and CON milk. HAY and CON produced milk can be of diverse quality, additionally the analysed samples are a mixture originating of different farms. For a precise interpretation of results or clear correlations of specific parameters alerting milk fat, a sample analysis of single farms under controlled circumstances would be required.

In a consumer's point of view, the difference in HAY and CON milk is relatively marginal considering the FA composition. Especially during summer HAY and CON milk are of similar quality, because in this period cows of both regimes receive more fresh grass.

After all the declaration is of economic importance, supermarkets sell hay milk for higher prices and consumers are by trend willing to pay more for increased quality. Until now there are no precisely defined parameters to determine the authenticity of hay milk or reliable methods for distinguishing HAY from CON milk. Besides those slight differences in FAs of HAY and CON milk the idea and the demand of a more sustainable farming could defend higher prices for hay milk. Because hay milk production is often connection to organic farming and a feeding regime that relates better to the cows natural eating behaviour.

8 Outlook | KJ

The need for methods that allow clear discrimination of different milk qualities is reasonable for mostly economic factors like ensuring product quality meaning prevention or recognition of fraud (correct labelling) as well as traceability reasons. In Austria the parameters for hay milk production are regulated at EU-level and defined to a certain extent (compare regulation (EU) 2016/304). These regulations do not include (standardised) verification methods for hay milk quality (European Commission, 2016).

There is an ongoing process of developing and improving methods for practicable, accurate, low-cost and high-throughput FA analysis in foods. Until today GC is still the method of choice when it comes to qualitative FA analysis and also for quantification. Although CG is a well-tried, established and precise method, it can be costly and time consuming when used on big scale like for industrial purposes. Due to this conditions, GC analysis is industrially used for spot tests for which single samples are examined that should represent one batch.

A faster and explicit chemical analysis is possible with Infrared spectroscopy (IR) (wavelength 800 nm- 1 mm), a method that is often used for rapid screening and routine analysis of FA on food including different kind of oils, shortenings, lards, pig fat. By using IR single FAs can be quantified with calibration but also a comprehensive FA profile can be achieved. When milk and dairy products are analysed with IR its major application is for quantification of *trans*-FAs (Afseth *et al.,* 2010). Determination of *trans*-FA with GC-FID often requires time-consuming preparation or pre-fractionation like the silver-ion TLC or silver-ion solid phase extraction or by application of highly polar columns like the SP-2560 that was used for sample analysis in this study (Mjøs and Pettersen, 2001). The first known application of IR in milk reaches back to 1950 and since then IR experienced several improvements. Especially the so called “Fourier transform infrared spectroscopy” (FTIR) is common for different analysis. FTIR is a specific kind of spectrometer that is used in IR analysis. Companies developed different IR analysers, one is for instance the “MilkoScan™”. With such technologies it is possible to perform precise, fully automated, fast and relatively cheap analysis in a standardized way, but some difficulties/uncertainties remain. Thus, a proper calibrations allows the evaluation of total USFAs, SFA, PUFAs and MUFAs (Anon., 2011).

The identification and quantification of single minor FAs in milk are still challenging (Uvenbeck, 2008). A study was conducted to tested a modified version (“dried film”) of FTIR and compared it to standard FTIR for FA analysis of cow’s milk. For the modified FTIR method a pre-concentration of the FAs was done by using dried thin films. Hence, a feasible

calibration for PUFA and *trans*-FAs could be achieved as well as overall errors were reduced with the modified FTIR (Afseth *et al.,* 2010).

Soyeur *et al.,* demonstrated in their small-scale study that FTIR has the potential for relatively specific analysis of FA in cow's milk. It was shown that FTIR analysis is mainly applicable in satisfying way for FAs that are present in a higher amount in milk fat. It has to be mentioned that a proper calibration and statistical evaluation model is important and the basis for predictability of milk fat analysis by FTIR (Soyeurt *et al.,* 2006). Aiming for increasing predictability Rutten *et al.,* conducted a study which considered a higher amount of samples, different evaluation models and also the influence of season on the FA profile of milk. Rutten *et al.,* confirmed that FTIR is a rapid and applicable method for the main FAs in milk fat or for grouped FAs e.g.: for comparison of total SFAs to USFAs. Contrary it can be concluded, that with a lower concentration of certain FAs in milk bias increased, so minor FAs could not be predicted accurately. Although an improved data analysis including more data and influence factors could improve prediction of milk FA analysis with FTIR, it is currently not appropriate for a detailed evaluation of LCPUFAs in milk (Rutten et al., 2009). Coppa *et al.,* also used a form om IR for milk fat prediction called "near-infrared reflectance spectroscopy" (NIRS) (wavelength 700- 2500 nm). Using NIRS also minor FAs und single FAs could not be analysed in a reliable way. When dried milk samples were analysed NIRS showed clearer results, because a higher water/liquid content leads to higher interference in spectra observation (Coppa *et a*l., 2010). The identification and quantification of single minor FAs in milk are still challenging (Uvenbeck, 2008). A study was conducted that tested a modified version ("dried film") of FTIR and compared it to standard FTIR for FA analysis of cow's milk. For the modified FTIR method a pre-concentration of the FAs was done by using dried thin films. Hence, a feasible calibration for PUFA and *trans*-FAs could be achieved as well as overall errors were reduced with the modified FTIR (Afseth *et al.,* 2010). Obviously development of IR respectively FTIR shows potential for a fast, cheap and high-throughput milk analysis, also considering PUFAs. Overall IR and related methods seem at its current state of development inappropriate for specific analysis of minor FAs respectively LCPUFAs in milk. Probably there is a realistic chance for achieving more precise results with IR by improving statistic models or/and sample preparation methods (e.g.: concentration method).

Another method for evaluation different milk qualities is isotope-ratio mass spectrometry (IRMS) and it has already found use in food authenticity testing's. With that method the potential is given to trace back to milk production respectively feeding strategy by analysing the isotope ratio of organic material (carbon, hydrogen, sulphur) (Rossmann, 2001).

Studies have already been conducted using IRMS in milk evaluation concluding on the cows' diet. A differentiation of HAY and CON could be done with isotope analysis by considering the origin of feeding plants. Silage is often made out of maize or partly contain maize plants, which are categorized as C4-plants whereas grass that cows graze on pastures are C3 plants. So if milk contains a relatively higher content of ^{13}C, evaluated as a ratio of $^{12}C/^{13}C$, it can be assumed that cows received silage. Of course carbon-isotope differentiation does not work if silage is also produced from grass or other C_3 plants, hence it is inappropriate to generally discriminate HAY from CON milk (Molkentin and Giesemann, 2007).

Furthermore, carotenoid (mainly beta-carotene also known as pro-vitamin A) or vitamin A (retinol) content could be inspected as differentiation criterion of HAY vs. silage-fed cows. The transfer of carotenoid and vitamin A from feed into milk is rather low. However, it has been proven that cows receiving greater amounts of fresh grass, the increased carotenoid content is measureable in milk. It has to be taken into account, that measurement of carotenoid and vitamin A is also potentially influenced by other factors as lactation stage, genetics, region. In addition, heat treatment, as in this study pasteurized samples were examined, could influence vitamin A contents or its molecular composition (cis to trans isomerisation). Although these changes trough processing are variable, sometimes negligible and depend on heat treatment they have to be considered (Nozière *et al.*, 2017).

Another idea to proof the quality and authenticity of hay milk is to define specific parameters for certain components. E.g. define a certain ratio of n-6 to n3 (LA to ALA), a minimum content of ALA or other PUFAs al well as the ratio of isomers of PHY (relation of diastereomers RRR/SRR). Besides the determination of certain FAs also vitamins (tocopherols), carotenoids, could be included in a model for defining hay milk quality. Of course this specific requirement profile does not prevent convergence of CON and hay milk during summer months. And the hypothetical question arises, of how to consider conventionally produced milk that achieves hay milk quality. In a consumer's point of view an increased overall quality of CON milk during summer is very probably desired, but there is also room for complaints from hay milk production. Especially for summer months a valid differentiation method of HAY and CON milk is required and other parameters than the FA spectrum needs to be considered.

In addition, the microbial population could give information about different milk qualities. Until today there are no clear or large-scaled examinations on HAY vs. CON milk regime concerning their probable difference in present bacteria, especially regarding clostridia. Maybe new techniques as the development of next generation sequencing could not only

help for microbial monitoring of milk, but also for analysis or determination of diverse milk qualities.

After all, it would probably be a feasible and manifest way to control certified hay milk farms for compliance of the defined standards. That means, execution of unheralded on-site controls and check, if the farmer keeps silage feed and controlling the overall feeding system.

Appendix

Table 24: Values of corrected FA peak areas (CON Q1- Q2)

sample	Probe 19	Probe 30	Probe 31	Probe 32	Probe 33	Probe 34	Probe 35	Probe 36	Probe 38	Probe 39	Probe 56	Probe 57	Probe 60	Probe 61	Probe 64	Probe 65	Probe 68	Probe 69
Feeding.Quartal	Con. Q1	Con. Q1	Con. Q1	Con. Q1	Con. Q1	Con. Q1	Con. Q1	Con. Q1	Con. Q1	Con. Q1	Con. Q2	Con. Q2	Con. Q2	Con. Q2	Con. Q2	Con. Q2	Con. Q2	Con. Q2
C18:3n3	0.543	0.869	0.893	0.702	0.704	0.795	0.583	0.573	0.597	0.580	0.660	0.691	0.593	0.708	0.723	0.683	1.141	1.155
2	0.085	0.084	0.085	0.091	0.092	0.076	0.067	0.066	0.066	0.079	0.065	0.082	0.078	0.085	0.084	0.079	0.089	0.081
3	0.152	0.166	0.163	0.171	0.177	0.140	0.136	0.114	0.129	0.151	0.123	0.148	0.140	0.164	0.162	0.156	0.160	0.166
4	0.039	0.014	0.017	0.033	0.035	0.009	0.026	0.017	0.027	0.040	0.005	0.030	0.027	0.038	0.038	0.034	0.026	0.026
CLAc9t11	0.258	0.868	0.936	0.859	0.868	0.980	0.618	0.589	0.712	0.747	0.898	0.678	0.838	0.912	0.820	0.768	1.634	1.779
6	0.049	0.006	0.009	0.009	0.022	0.009	0.012	0.021	0.005	0.006	0.006	0.036	0.007	0.005	0.015	0.006	0.009	0.014
7	0.012	0.088	0.095	0.043	0.052	0.101	0.030	0.025	0.050	0.049	0.040	0.028	0.070	0.072	0.055	0.048	0.115	0.063
8	0.011	0.037	0.038	0.022	0.028	0.040	0.011	0.009	0.021	0.022	0.022	0.017	0.026	0.035	0.026	0.027	0.054	0.023
9	0.027	0.010	0.013	0.009	0.012	0.014	0.006	0.003	0.007	0.008	0.011	0.017	0.007	0.013	0.013	0.008	0.020	0.012
10	0.060	0.060	0.065	0.054	0.056	0.070	0.048	0.044	0.048	0.053	0.049	0.030	0.060	0.061	0.061	0.054	0.091	0.076
11	0.090	0.087	0.103	0.093	0.102	0.126	0.104	0.102	0.078	0.075	0.074	0.128	0.083	0.101	0.077	0.071	0.104	0.090
C20:0	0.218	0.194	0.198	0.220	0.219	0.193	0.167	0.168	0.171	0.188	0.149	0.221	0.187	0.191	0.193	0.185	0.212	0.207
15	0.162	0.146	0.147	0.176	0.175	0.142	0.118	0.128	0.132	0.146	0.113	0.160	0.146	0.149	0.149	0.145	0.156	0.155
C20:1	0.063	0.051	0.053	0.070	0.068	0.052	0.049	0.053	0.053	0.057	0.057	0.067	0.067	0.061	0.057	0.054	0.065	0.071
17	0.013	0.009	0.013	0.008	0.005	0.016	0.010	0.009	0.007	0.014	0.007	0.008	0.021	0.013	0.014	0.013	0.020	0.018
18	0.013	0.012	0.011	0.016	0.014	0.006	0.020	0.021	0.012	0.016	0.004	0.013	0.014	0.014	0.014	0.011	0.012	0.009
C20:2n6	0.050	0.039	0.041	0.048	0.050	0.040	0.051	0.060	0.043	0.045	0.035	0.045	0.042	0.043	0.042	0.044	0.042	0.038
C20:3n6	0.081	0.049	0.049	0.068	0.076	0.048	0.062	0.074	0.063	0.083	0.043	0.074	0.079	0.075	0.072	0.075	0.048	0.056
C20:4n6	0.136	0.075	0.074	0.096	0.107	0.071	0.085	0.103	0.090	0.096	0.062	0.112	0.094	0.096	0.100	0.094	0.071	0.081
C20:3n3	0.011	0.020	0.020	0.014	0.010	0.015	0.012	0.005	0.011	0.012	0.012	0.014	0.011	0.012	0.014	0.011	0.019	0.018
23	0.006	0.021	0.013	0.007	0.016	0.014	0.017	0.018	0.008	0.013	0.012	0.016	0.014	0.016	0.017	0.015	0.020	0.029
C21:0	0.082	0.108	0.099	0.072	0.093	0.095	0.076	0.049	0.059	0.084	0.077	0.083	0.082	0.087	0.094	0.086	0.111	0.083
C20:5n3	0.055	0.093	0.087	0.058	0.072	0.081	0.062	0.040	0.052	0.067	0.065	0.063	0.067	0.073	0.080	0.072	0.096	0.101
C22:0	0.082	0.085	0.086	0.089	0.090	0.083	0.067	0.062	0.066	0.074	0.066	0.086	0.069	0.076	0.076	0.074	0.088	0.080
C22:1n9	0.006	0.007	0.005	0.006	0.005	0.005	0.004	0.013	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.006	0.007
28	0.005	0.007	0.012	0.006	0.005	0.009	0.006	0.007	0.008	0.004	0.002	0.006	0.009	0.005	0.011	0.009	0.007	0.008
29	0.046	0.044	0.060	0.049	0.042	0.048	0.037	0.037	0.045	0.039	0.013	0.047	0.045	0.042	0.053	0.047	0.032	0.039
C23:0	0.020	0.034	0.034	0.030	0.032	0.032	0.022	0.027	0.022	0.028	0.027	0.031	0.027	0.028	0.025	0.028	0.038	0.032
31	0.031	0.035	0.042	0.029	0.033	0.031	0.026	0.019	0.026	0.029	0.026	0.017	0.031	0.031	0.028	0.029	0.037	0.034
C22:5n3	0.063	0.086	0.093	0.080	0.077	0.076	0.055	0.016	0.060	0.068	0.069	0.050	0.068	0.073	0.079	0.074	0.092	0.096
C22:6n3	0.010	0.007	0.008	0.006	0.003	0.003	0.004	0.006	0.004	0.005	0.005	0.008	0.005	0.006	0.008	0.005	0.006	0.009
C24:0	0.019	0.028	0.024	0.032	0.027	0.022	0.025	0.005	0.019	0.022	0.022	0.019	0.022	0.026	0.025	0.025	0.028	0.020
C24:1	0.009	0.009	0.009	0.011	0.011	0.009	0.009	0.008	0.006	0.008	0.009	0.010	0.008	0.007	0.008	0.008	0.008	0.007

Table 25: Values of corrected FA peak areas (CON Q2- Q4)

sample	Probe 72	Probe 73	Probe 76	Probe 77	Probe 80	Probe 81	Probe 84	Probe 85	Probe 88	Probe 89	Probe 92	Probe 93	Probe 96	Probe 97	Probe 100	Probe 101	Probe 104
Feeding.Quartal	Con. Q2	Con. Q2	Con. Q3	Con. Q3	Con. Q3	Con. Q3	Con. Q3	Con. Q3	Con. Q3	Con. Q3	Con. Q3	Con. Q3	Con. Q4	Con. Q4	Con. Q4	Con. Q4	Con. Q4
C18:3n3	0.747	0.722	0.644	0.681	0.688	0.637	0.672	0.715	1.027	1.244	0.775	0.733	0.530	0.518	0.554	0.537	0.547
2	0.088	0.084	0.073	0.081	0.075	0.074	0.078	0.058	0.082	0.096	0.087	0.085	0.071	0.072	0.068	0.066	0.078
3	0.169	0.160	0.145	0.158	0.150	0.151	0.156	0.126	0.145	0.149	0.167	0.156	0.132	0.141	0.131	0.115	0.079
4	0.029	0.026	0.031	0.032	0.025	0.030	0.025	0.019	0.010	0.026	0.023	0.031	0.027	0.035	0.027	0.016	0.008
CLAc9t11	1.002	0.940	0.928	0.833	1.036	0.894	0.924	0.843	1.784	0.008	1.232	1.105	0.619	0.647	0.734	0.728	0.609
6	0.008	0.008	0.008	0.008	0.008	0.020	0.009	0.023	0.010	0.014	0.008	0.008	0.007	0.005	0.006	0.024	0.051
7	0.052	0.048	0.052	0.037	0.076	0.065	0.051	0.047	0.115	0.069	0.066	0.049	0.035	0.031	0.056	0.042	0.069
8	0.028	0.027	0.031	0.022	0.036	0.032	0.027	0.024	0.058	0.021	0.038	0.029	0.020	0.014	0.028	0.043	0.025
9	0.009	0.011	0.012	0.009	0.010	0.010	0.011	0.022	0.020	0.012	0.012	0.012	0.008	0.002	0.007	0.012	0.016
10	0.058	0.056	0.054	0.053	0.067	0.056	0.059	0.061	0.096	0.061	0.073	0.068	0.045	0.045	0.053	0.074	0.050
11	0.077	0.086	0.079	0.078	0.092	0.081	0.087	0.099	0.128	0.092	0.085	0.082	0.068	0.046	0.062	0.089	0.101
C20:0	0.203	0.191	0.176	0.200	0.171	0.163	0.184	0.168	0.179	0.153	0.206	0.205	0.172	0.209	0.157	0.186	0.186
15	0.163	0.157	0.148	0.165	0.142	0.142	0.155	0.140	0.141	0.148	0.177	0.166	0.133	0.143	0.127	0.105	0.145
C20:1	0.068	0.059	0.066	0.063	0.057	0.055	0.060	0.053	0.055	0.019	0.057	0.060	0.052	0.061	0.055	0.060	0.057
17	0.018	0.006	0.020	0.015	0.011	0.012	0.013	0.010	0.012	0.009	0.026	0.013	0.011	0.013	0.017	0.012	0.005
18	0.012	0.012	0.013	0.019	0.012	0.012	0.012	0.013	0.009	0.013	0.014	0.015	0.012	0.013	0.012	0.013	0.013
C20:2n6	0.046	0.045	0.042	0.052	0.041	0.045	0.048	0.050	0.040	0.046	0.048	0.059	0.049	0.042	0.047	0.045	0.045
C20:3n6	0.072	0.076	0.081	0.079	0.074	0.072	0.074	0.065	0.065	0.095	0.070	0.096	0.071	0.080	0.080	0.060	0.028
C20:4n6	0.105	0.103	0.101	0.111	0.093	0.093	0.097	0.094	0.084	0.097	0.104	0.120	0.096	0.104	0.092	0.111	0.095
C20:3n3	0.012	0.017	0.012	0.012	0.010	0.010	0.010	0.010	0.018	0.013	0.013	0.010	0.008	0.009	0.010	0.013	0.011
23	0.021	0.007	0.014	0.011	0.019	0.013	0.014	0.016	0.021	0.016	0.018	0.021	0.023	0.011	0.016	0.016	0.011
C21:0	0.096	0.084	0.083	0.086	0.080	0.072	0.078	0.077	0.099	0.104	0.089	0.067	0.061	0.067	0.062	0.081	0.081
C20:5n3	0.077	0.071	0.063	0.068	0.069	0.061	0.065	0.072	0.083	0.089	0.075	0.054	0.053	0.058	0.058	0.069	0.056
C22:0	0.081	0.079	0.073	0.079	0.068	0.064	0.072	0.063	0.081	0.075	0.084	0.083	0.064	0.074	0.055	0.073	0.042
C22:1n9	0.005	0.004	0.006	0.010	0.009	0.013	0.020	0.024	0.003	0.003	0.007	0.023	0.028	0.006	0.017	0.009	0.011
28	0.006	0.010	0.006	0.005	0.007	0.004	0.003	0.014	0.004	0.006	0.001	0.007	0.006	0.003	0.002	0.006	0.005
29	0.046	0.053	0.036	0.031	0.033	0.029	0.029	0.037	0.027	0.046	0.003	0.037	0.037	0.029	0.027	0.037	0.030
C23:0	0.026	0.025	0.025	0.027	0.023	0.021	0.022	0.022	0.032	0.041	0.035	0.025	0.022	0.022	0.017	0.027	0.011
31	0.027	0.027	0.027	0.028	0.029	0.023	0.028	0.028	0.030	0.028	0.031	0.025	0.023	0.026	0.015	0.028	0.015
C22:5n3	0.075	0.072	0.071	0.068	0.071	0.068	0.069	0.024	0.095	0.013	0.031	0.069	0.056	0.066	0.053	0.066	0.058
C22:6n3	0.005	0.005	0.007	0.002	0.006	0.004	0.005	0.018	0.008	0.000	0.006	0.004	0.005	0.004	0.002	0.006	0.005
C24:0	0.026	0.021	0.019	0.024	0.020	0.022	0.031	0.007	0.021	0.031	0.003	0.016	0.022	0.023	0.030	0.021	0.013
C24:1	0.006	0.007	0.008	0.010	0.008	0.009	0.012	0.008	0.007	0.008	0.008	0.006	0.010	0.007	0.008	0.008	0.004

Table 26: Values of corrected FA peak areas (CON Q4- HAY Q1)

sample	Probe 108	Probe 109	Probe 112	Probe 113	Probe 20	Probe 21	Probe 22	Probe 23	Probe 24	Probe 25	Probe 26	Probe 27	Probe 28	Probe 29	Probe 3797	probe 3750	Probe 0956	Probe 2760
Feeding.Quartal	Con. Q4	Con. Q4	Con. Q4	Con. Q4	Hay Q1	Hay Q1	Hay Q1	Hay Q1	Hay Q1	Hay Q1	Hay Q1	Hay Q1	Hay Q1	Hay Q1	Hay Q1	Hay Q1	Hay Q1	Hay Q1
C18:3n3	0.895	0.873	0.573	0.594	0.950	0.833	0.811	1.006	0.734	1.053	1.010	1.040	1.046	1.293	0.928	1.009	0.989	0.970
2	0.078	0.073	0.067	0.069	0.091	0.081	0.081	0.038	0.086	0.101	0.104	0.087	0.087	0.098	0.091	0.089	0.102	0.088
3	0.078	0.116	0.123	0.127	0.205	0.171	0.170	0.153	0.153	0.211	0.192	0.173	0.181	0.209	0.177	0.159	0.208	0.164
4	0.026	0.026	0.022	0.023	0.016	0.016	0.016	0.021	0.028	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.010	0.005
CLAc9t11	0.838	1.048	0.771	0.732	0.713	0.012	0.700	0.804	0.758	0.807	0.812	0.800	0.802	0.915	0.858	0.815	0.862	0.773
6	0.050	0.008	0.005	0.008	0.019	0.017	0.010	0.009	0.009	0.008	0.009	0.009	0.009	0.009	0.019	0.005	0.015	0.013
7	0.088	0.117	0.053	0.045	0.031	0.014	0.020	0.053	0.037	0.036	0.044	0.032	0.030	0.031	0.033	0.033	0.049	0.030
8	0.034	0.047	0.029	0.025	0.024	0.018	0.018	0.049	0.020	0.022	0.028	0.023	0.020	0.024	0.030	0.027	0.033	0.029
9	0.012	0.018	0.008	0.019	0.013	0.013	0.018	0.012	0.016	0.010	0.016	0.014	0.014	0.013	0.017	0.010	0.009	0.011
10	0.065	0.085	0.055	0.052	0.034	0.064	0.041	0.027	0.047	0.044	0.040	0.039	0.038	0.053	0.046	0.045	0.044	0.042
11	0.090	0.118	0.064	0.100	0.178	0.192	0.094	0.068	0.103	0.086	0.095	0.088	0.079	0.087	0.084	0.059	0.058	0.063
C20:0	0.189	0.178	0.157	0.168	0.195	0.164	0.187	0.192	0.208	0.185	0.188	0.198	0.196	0.246	0.177	0.177	0.181	0.169
15	0.131	0.130	0.125	0.134	0.165	0.153	0.159	0.168	0.155	0.153	0.155	0.168	0.172	0.187	0.150	0.148	0.149	0.141
C20:1	0.052	0.051	0.049	0.053	0.060	0.055	0.058	0.064	0.054	0.055	0.055	0.052	0.056	0.050	0.070	0.051	0.060	0.056
17	0.015	0.003	0.005	0.015	0.007	0.010	0.015	0.008	0.014	0.016	0.006	0.016	0.012	0.007	0.003	0.004	0.007	0.011
18	0.008	0.016	0.009	0.012	0.008	0.011	0.008	0.011	0.011	0.009	0.009	0.007	0.019	0.011	0.011	0.005	0.010	0.008
C20:2n6	0.050	0.047	0.046	0.040	0.049	0.070	0.047	0.053	0.054	0.044	0.043	0.048	0.046	0.063	0.071	0.053	0.048	0.069
C20:3n6	0.042	0.043	0.077	0.064	0.064	0.105	0.074	0.064	0.077	0.058	0.046	0.065	0.064	0.080	0.062	0.067	0.054	0.078
C20:4n6	0.083	0.082	0.096	0.103	0.101	0.014	0.103	0.089	0.100	0.085	0.076	0.096	0.094	0.124	0.099	0.094	0.088	0.104
C20:3n3	0.020	0.019	0.008	0.010	0.018	0.011	0.011	0.012	0.014	0.019	0.021	0.021	0.021	0.026	0.016	0.019	0.017	0.021
23	0.023	0.024	0.025	0.015	0.013	0.023	0.023	0.015	0.009	0.016	0.015	0.016	0.016	0.022	0.039	0.018	0.018	0.023
C21:0	0.084	0.084	0.067	0.070	0.113	0.083	0.100	0.093	0.095	0.126	0.121	0.119	0.123	0.100	0.095	0.101	0.106	0.088
C20:5n3	0.083	0.084	0.059	0.066	0.097	0.088	0.080	0.100	0.076	0.110	0.103	0.101	0.101	0.130	0.090	0.095	0.100	0.086
C22:0	0.073	0.069	0.059	0.061	0.090	0.007	0.086	0.037	0.087	0.094	0.094	0.097	0.100	0.123	0.083	0.082	0.085	0.075
C22:1n9	0.016	0.015	0.003	0.002	0.007	0.018	0.007	0.011	0.006	0.007	0.006	0.008	0.009	0.006	0.018	0.011	0.007	0.009
28	0.003	0.016	0.006	0.000	0.019	0.050	0.017	0.014	0.012	0.020	0.021	0.016	0.018	0.027	0.033	0.010	0.034	0.030
29	0.023	0.032	0.037	0.014	0.057	0.037	0.051	0.018	0.056	0.060	0.065	0.055	0.056	0.077	0.038	0.032	0.085	0.035
C23:0	0.034	0.033	0.021	0.034	0.039	0.037	0.034	0.016	0.031	0.044	0.041	0.045	0.044	0.048	0.051	0.036	0.041	0.037
31	0.035	0.031	0.025	0.022	0.044	0.035	0.032	0.013	0.030	0.057	0.051	0.042	0.037	0.051	0.034	0.027	0.056	0.038
C22:5n3	0.089	0.086	0.067	0.022	0.087	0.006	0.077	0.085	0.078	0.097	0.086	0.092	0.091	0.113	0.042	0.075	0.095	0.075
C22:6n3	0.012	0.010	0.006	0.006	0.007	0.028	0.004	0.009	0.005	0.009	0.007	0.007	0.004	0.005	0.009	0.006	0.013	0.011
C24:0	0.024	0.021	0.021	0.007	0.025	0.009	0.029	0.025	0.029	0.028	0.028	0.032	0.034	0.040	0.012	0.056	0.016	0.017
C24:1	0.008	0.008	0.008	0.008	0.014	0.010	0.011	0.010	0.009	0.012	0.013	0.011	0.012	0.017	0.010	0.010	0.011	0.010

Table 27: Values of correctedFA peak areas (HAY Q1- Q3)

sample	Probe 1111	Probe 58	Probe 59	Probe 62	Probe 63	Probe 66	Probe 67	Probe 70	Probe 71	Probe 74	Probe 75	Probe 78	Probe 79	Probe 82	Probe 83	Probe 86	Probe 87	Probe 90
Feeding.Quartal	Hay Q1	Hay Q2	Hay Q2	Hay Q2	Hay Q2	Hay Q2	Hay Q2	Hay Q2	Hay Q2	Hay Q2	Hay Q2	Hay Q3	Hay Q3	Hay Q3	Hay Q3	Hay Q3	Hay Q3	Hay Q3
C18:3n3	0.993	0.957	1.004	0.846	1.045	1.029	0.993	0.766	0.902	0.934	0.971	1.052	1.113	1.110	1.162	1.069	1.106	0.910
2	0.104	0.101	0.110	0.046	0.102	0.133	0.095	0.088	0.089	0.088	0.061	0.069	0.079	0.126	0.092	0.076	0.081	0.084
3	0.210	0.203	0.216	0.127	0.170	0.253	0.161	0.153	0.151	0.150	0.147	0.117	0.133	0.269	0.156	0.130	0.137	0.141
4	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.036	0.015	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.017
CLAc9t11	1.039	1.399	1.463	1.363	1.578	1.145	1.368	0.940	1.164	1.654	1.889	1.635	1.862	1.357	2.124	1.782	1.868	1.350
6	0.010	0.009	0.014	0.000	0.010	0.011	0.008	0.010	0.022	0.010	0.010	0.020	0.011	0.005	0.024	0.010	0.010	0.009
7	0.029	0.071	0.067	0.066	0.073	0.052	0.060	0.039	0.054	0.057	0.073	0.088	0.109	0.072	0.100	0.083	0.088	0.067
8	0.019	0.038	0.024	0.030	0.036	0.031	0.034	0.021	0.032	0.035	0.051	0.045	0.051	0.044	0.031	0.049	0.051	0.039
9	0.013	0.018	0.012	0.007	0.013	0.014	0.013	0.011	0.016	0.017	0.024	0.012	0.018	0.013	0.013	0.020	0.014	0.015
10	0.059	0.065	0.068	0.063	0.065	0.073	0.078	0.057	0.065	0.074	0.109	0.084	0.087	0.101	0.096	0.063	0.063	0.078
11	0.122	0.111	0.165	0.043	0.098	0.117	0.096	0.093	0.119	0.123	0.077	0.111	0.148	0.110	0.134	0.148	0.134	0.115
C20:0	0.220	0.184	0.210	0.148	0.205	0.187	0.217	0.200	0.188	0.202	0.201	0.140	0.152	0.187	0.203	0.170	0.178	0.181
15	0.171	0.141	0.158	0.114	0.148	0.152	0.166	0.153	0.130	0.162	0.162	0.118	0.120	0.152	0.173	0.137	0.142	0.144
C20:1	0.055	0.053	0.062	0.048	0.055	0.055	0.055	0.057	0.043	0.064	0.070	0.048	0.045	0.055	0.055	0.045	0.045	0.049
17	0.012	0.014	0.016	0.013	0.010	0.018	0.014	0.014	0.005	0.011	0.017	0.016	0.014	0.018	0.016	0.016	0.009	0.011
18	0.011	0.011	0.017	0.005	0.009	0.025	0.012	0.014	0.011	0.009	0.008	0.008	0.007	0.021	0.010	0.010	0.009	0.011
C20:2n6	0.052	0.044	0.053	0.028	0.042	0.078	0.047	0.049	0.056	0.064	0.041	0.040	0.044	0.069	0.050	0.045	0.042	0.051
C20:3n6	0.049	0.052	0.058	0.041	0.048	0.066	0.068	0.082	0.061	0.061	0.070	0.064	0.062	0.066	0.072	0.068	0.051	0.077
C20:4n6	0.146	0.069	0.078	0.059	0.074	0.183	0.094	0.113	0.094	0.087	0.096	0.079	0.076	0.192	0.097	0.092	0.072	0.098
C20:3n3	0.021	0.013	0.021	0.022	0.010	0.016	0.013	0.011	0.016	0.014	0.017	0.017	0.017	0.025	0.019	0.018	0.018	0.016
23	0.049	0.018	0.027	0.026	0.019	0.020	0.028	0.019	0.022	0.032	0.022	0.025	0.023	0.049	0.035	0.028	0.029	0.026
C21:0	0.057	0.112	0.126	0.058	0.089	0.131	0.115	0.090	0.096	0.104	0.077	0.095	0.116	0.099	0.123	0.098	0.108	0.098
C20:5n3	0.076	0.100	0.105	0.070	0.065	0.105	0.100	0.075	0.084	0.082	0.091	0.080	0.094	0.116	0.099	0.087	0.092	0.081
C22:0	0.079	0.085	0.098	0.052	0.065	0.119	0.096	0.078	0.007	0.090	0.076	0.066	0.079	0.066	0.093	0.081	0.085	0.082
C22:1n9	0.010	0.003	0.005	0.005	0.010	0.009	0.006	0.005	0.010	0.008	0.007	0.010	0.013	0.008	0.013	0.019	0.004	0.003
28	0.017	0.006	0.006	0.003	0.032	0.013	0.006	0.008	0.023	0.017	0.003	0.003	0.015	0.020	0.003	0.017	0.023	0.016
29	0.038	0.025	0.019	0.011	0.017	0.078	0.025	0.052	0.004	0.038	0.015	0.020	0.038	0.094	0.021	0.003	0.038	0.004
C23:0	0.034	0.035	0.039	0.018	0.022	0.045	0.045	0.028	0.034	0.035	0.018	0.034	0.038	0.041	0.035	0.036	0.036	0.026
31	0.034	0.039	0.039	0.026	0.017	0.045	0.040	0.025	0.035	0.031	0.024	0.026	0.029	0.045	0.028	0.035	0.033	0.030
C22:5n3	0.083	0.089	0.090	0.069	0.061	0.116	0.106	0.073	0.033	0.074	0.086	0.086	0.095	0.130	0.104	0.098	0.106	0.022
C22:6n3	0.009	0.007	0.004	0.007	0.004	0.009	0.011	0.004	0.009	0.002	0.008	0.004	0.006	0.013	0.006	0.005	0.008	0.009
C24:0	0.025	0.023	0.025	0.014	0.025	0.031	0.032	0.022	0.003	0.028	0.019	0.025	0.019	0.028	0.024	0.015	0.025	0.006
C24:1	0.010	0.011	0.013	0.005	0.010	0.012	0.011	0.004	0.010	0.011	0.006	0.008	0.005	0.011	0.008	0.008	0.001	0.010

Table 28: Values of corrected FA peak areas (HAY Q3- Q4)

sample	Probe 91	Probe 94	Probe 95	Probe 98	Probe 99	Probe 102	Probe 103	Probe 106	Probe 107	Probe 110	Probe 111	Probe 114	Probe 115
Feeding.Quartal	Hay Q3	Hay Q3	Hay Q3	Hay Q4	Hay Q4	Hay Q4	Hay Q4	Hay Q4	Hay Q4	Hay Q4	Hay Q4	Hay Q4	Hay Q4
C18:3n3	0.729	0.983	0.936	1.017	1.129	1.038	1.078	1.033	1.197	0.745	0.779	0.905	0.845
2	0.082	0.088	0.088	0.081	0.090	0.083	0.137	0.084	0.093	0.077	0.074	0.085	0.079
3	0.150	0.170	0.152	0.144	0.163	0.133	0.262	0.164	0.169	0.131	0.129	0.152	0.143
4	0.024	0.008	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.014	0.008	0.008
ClAc9t11	1.138	1.694	1.602	0.962	1.041	0.895	1.307	0.837	0.964	0.721	0.779	0.920	0.887
6	0.009	0.009	0.010	0.006	0.010	0.005	0.010	0.005	0.005	0.014	0.005	0.007	0.005
7	0.045	0.071	0.058	0.046	0.052	0.038	0.074	0.035	0.043	0.040	0.050	0.036	0.034
8	0.026	0.045	0.034	0.024	0.032	0.025	0.041	0.027	0.031	0.024	0.027	0.026	0.022
9	0.011	0.015	0.017	0.008	0.007	0.008	0.009	0.010	0.011	0.012	0.010	0.013	0.008
10	0.067	0.085	0.079	0.060	0.067	0.065	0.098	0.059	0.065	0.058	0.072	0.067	0.062
11	0.095	0.095	0.110	0.072	0.058	0.067	0.109	0.065	0.063	0.088	0.076	0.070	0.052
C20:0	0.190	0.217	0.215	0.156	0.156	0.157	0.187	0.170	0.196	0.181	0.181	0.187	0.171
15	0.164	0.193	0.184	0.130	0.136	0.121	0.152	0.146	0.166	0.140	0.141	0.148	0.139
C20:1	0.067	0.069	0.063	0.054	0.046	0.050	0.055	0.052	0.051	0.051	0.052	0.058	0.053
17	0.023	0.020	0.007	0.021	0.014	0.005	0.019	0.003	0.002	0.007	0.022	0.020	0.005
18	0.017	0.009	0.010	0.011	0.008	0.007	0.026	0.006	0.010	0.007	0.008	0.012	0.007
C20:2n6	0.052	0.050	0.054	0.046	0.042	0.060	0.052	0.053	0.054	0.059	0.055	0.053	0.044
C20:3n6	0.085	0.075	0.082	0.082	0.053	0.075	0.066	0.036	0.056	0.075	0.066	0.059	0.061
C20:4n6	0.121	0.104	0.110	0.021	0.090	0.128	0.203	0.087	0.113	0.094	0.100	0.102	0.104
C20:3n3	0.010	0.020	0.014	0.030	0.024	0.026	0.016	0.018	0.020	0.013	0.015	0.017	0.016
23	0.018	0.022	0.020	0.023	0.019	0.016	0.038	0.020	0.025	0.013	0.023	0.024	0.020
C21:0	0.083	0.098	0.093	0.094	0.112	0.100	0.110	0.094	0.116	0.074	0.086	0.096	0.094
C20:5n3	0.069	0.080	0.076	0.074	0.104	0.085	0.116	0.096	0.116	0.070	0.075	0.087	0.086
C22:0	0.074	0.094	0.096	0.022	0.077	0.097	0.113	0.082	0.100	0.072	0.066	0.082	0.075
C22:1n9	0.016	0.014	0.025	0.011	0.010	0.010	0.019	0.017	0.006	0.020	0.015	0.008	0.006
28	0.004	0.003	0.035	0.019	0.018	0.038	0.007	0.025	0.012	0.015	0.028	0.017	0.011
29	0.035	0.021	0.038	0.025	0.055	0.028	0.052	0.040	0.049	0.019	0.038	0.029	0.029
C23:0	0.030	0.034	0.027	0.031	0.040	0.050	0.045	0.030	0.018	0.026	0.019	0.029	0.023
31	0.022	0.028	0.016	0.041	0.053	0.026	0.049	0.051	0.032	0.026	0.021	0.027	0.024
C22:5n3	0.078	0.087	0.069	0.092	0.109	0.018	0.121	0.109	0.097	0.077	0.076	0.083	0.086
C22:6n3	0.005	0.005	0.003	0.009	0.008	0.063	0.014	0.011	0.011	0.003	0.006	0.008	0.008
C24:0	0.028	0.031	0.051	0.010	0.014	0.007	0.056	0.012	0.015	0.027	0.040	0.026	0.027
C24:1	0.010	0.010	0.010	0.008	0.009	0.010	0.010	0.023	0.010	0.010	0.010	0.010	0.006

Table 29: Outlier- Test CLA (HAY)

Descriptive Statistics

Mean: 1.13407
SD: 0.42921
# of values: 45
Outlier detected? No
Significance level: 0.05 (two-sided)
Critical value of Z: 3.08542339826

Your data

Row	Value	Z	Significant Outlier?
1	0.712	0.98336	
2	0.012	2.61426	Furthest from the rest, but not a significant outlier (P > 0.05).
3	0.700	1.01131	
4	0.759	0.87385	
5	0.758	0.87618	
6	0.807	0.76202	
7	0.812	0.75037	
8	0.800	0.77833	
9	0.802	0.77367	
10	0.913	0.51505	
11	0.857	0.64553	
12	0.815	0.74338	
13	0.862	0.63388	
14	0.773	0.84123	
15	1.037	0.22615	
16	1.399	0.61726	
17	1.464	0.76870	
18	1.362	0.53105	
19	1.568	1.01100	
20	1.131	0.00714	

Table 30: Total fatty acid profile of conventional milk samples (39 total)

FA	Minimum	Maximum	Mean	Std. deviation
C18:3n3	0.518	1.244	0.720	0.178
FA 2	0.058	0.096	0.078	0.009
FA 3	0.078	0.177	0.143	0.023
FA 4	0.005	0.040	0.026	0.009
CLAc9t11	0.008	1.784	0.872	0.330
FA 6	0.005	0.051	0.014	0.012
FA 7	0.012	0.117	0.059	0.025
FA 8	0.009	0.058	0.028	0.011
FA 9	0.002	0.027	0.012	0.005
FA 10	0.030	0.096	0.060	0.013
FA 11	0.046	0.128	0.090	0.018
C20:0	0.149	0.221	0.187	0.020
FA 15	0.105	0.177	0.145	0.017
C20:1	0.019	0.071	0.057	0.009
FA 17	0.003	0.026	0.012	0.005
FA 18	0.004	0.021	0.013	0.003
C20:2	0.035	0.060	0.045	0.005
C20:3n6	0.028	0.096	0.068	0.015
C20:4n6	0.062	0.136	0.095	0.014
C20:3n3	0.005	0.020	0.013	0.004
FA 23	0.006	0.029	0.016	0.005
C21:0	0.049	0.111	0.082	0.014
C20:5n3	0.040	0.101	0.070	0.013
C22:0	0.042	0.090	0.074	0.010
C22:1n9	0.002	0.028	0.009	0.006
FA 28	0.000	0.016	0.006	0.003
FA 29	0.003	0.060	0.037	0.011
C23:0	0.011	0.041	0.027	0.006
FA 31	0.015	0.042	0.028	0.006
C22:5n3	0.013	0.096	0.066	0.021
C22:6n3	0.000	0.018	0.006	0.003
C24:0	0.003	0.032	0.021	0.007
C24:1	0.004	0.012	0.008	0.002

Table 31: Total fatty acid profile of HAY milk samples (45 total)

FA	Minimum	Maximum	Mean	Std. deviation
C18:3n3	0.729	1.293	0.979	0.124
FA 2	0.038	0.137	0.089	0.018
FA 3	0.117	0.269	0.169	0.036
FA 4	0.005	0.036	0.016	0.005
CLAc9t11	0.012	2.124	1.137	0.429
FA 6	0.000	0.024	0.010	0.005
FA 7	0.014	0.109	0.052	0.022
FA 8	0.018	0.051	0.031	0.010
FA 9	0.007	0.024	0.013	0.004
FA 10	0.027	0.109	0.063	0.019
FA 11	0.043	0.192	0.098	0.033
C20:0	0.140	0.246	0.187	0.021
FA 15	0.114	0.193	0.152	0.018
C20:1	0.043	0.070	0.055	0.007
FA 17	0.002	0.023	0.012	0.006
FA 18	0.005	0.026	0.011	0.005
C20:2	0.028	0.078	0.052	0.010
C20:3n6	0.036	0.105	0.065	0.013
C20:4n6	0.014	0.203	0.099	0.034
C20:3n3	0.010	0.030	0.017	0.005
FA 23	0.009	0.049	0.023	0.008
C21:0	0.057	0.131	0.100	0.016
C20:5n3	0.065	0.130	0.091	0.015
C22:0	0.007	0.123	0.080	0.024
C22:1n9	0.003	0.025	0.010	0.005
FA 28	0.003	0.050	0.017	0.011
FA 29	0.003	0.094	0.038	0.021
C23:0	0.016	0.051	0.034	0.009
FA 31	0.013	0.057	0.034	0.011
C22:5n3	0.006	0.130	0.083	0.026
C22:6n3	0.002	0.063	0.009	0.009
C24:0	0.003	0.056	0.025	0.012
C24:1	0.001	0.023	0.010	0.003

Table 32: Overview of fatty acid profile; means by quarter (HAY and CON)

Feeding	CON				HAY			
Quarter	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
C18:3n3	0.684	0.782	0.782	0.625	0.978	0.945	1.017	0.977
FA 2	0.079	0.082	0.079	0.071	0.088	0.092	0.086	0.088
FA 3	0.150	0.155	0.150	0.116	0.182	0.173	0.155	0.159
FA 4	0.026	0.028	0.025	0.023	0.016	0.018	0.016	0.014
CLAc9t11	0.744	1.027	0.959	0.747	0.765	1.396	1.641	0.931
FA 6	0.015	0.011	0.012	0.018	0.011	0.010	0.012	0.007
FA 7	0.055	0.059	0.063	0.059	0.034	0.061	0.078	0.045
FA 8	0.024	0.028	0.032	0.030	0.026	0.033	0.041	0.028
FA 9	0.011	0.012	0.013	0.011	0.013	0.014	0.015	0.010
FA 10	0.056	0.060	0.065	0.058	0.044	0.072	0.080	0.067
FA 11	0.096	0.089	0.090	0.082	0.097	0.104	0.120	0.072
C20:0	0.194	0.194	0.181	0.178	0.192	0.194	0.183	0.174
FA 15	0.147	0.149	0.153	0.130	0.160	0.149	0.153	0.142
C20:1	0.057	0.063	0.055	0.054	0.057	0.056	0.054	0.052
FA 17	0.010	0.014	0.014	0.011	0.010	0.013	0.015	0.012
FA 18	0.014	0.011	0.013	0.012	0.010	0.012	0.011	0.010
C20:2	0.047	0.042	0.047	0.046	0.054	0.050	0.050	0.052
C20:3n6	0.065	0.067	0.077	0.061	0.067	0.061	0.070	0.063
C20:4n6	0.093	0.092	0.099	0.096	0.094	0.095	0.104	0.104
C20:3n3	0.013	0.014	0.012	0.012	0.018	0.015	0.017	0.019
FA 23	0.013	0.017	0.016	0.018	0.021	0.023	0.028	0.022
C21:0	0.081	0.088	0.083	0.073	0.101	0.100	0.101	0.098
C20:5n3	0.067	0.077	0.070	0.065	0.095	0.087	0.087	0.091
C22:0	0.078	0.078	0.074	0.063	0.081	0.077	0.082	0.079
C22:1n9	0.006	0.005	0.012	0.012	0.009	0.007	0.012	0.012
FA 28	0.007	0.007	0.006	0.005	0.023	0.012	0.014	0.019
FA 29	0.045	0.042	0.031	0.029	0.051	0.028	0.031	0.036
C23:0	0.028	0.029	0.027	0.025	0.039	0.032	0.034	0.031
FA 31	0.030	0.029	0.028	0.024	0.039	0.032	0.029	0.035
C22:5n3	0.067	0.075	0.058	0.063	0.079	0.080	0.087	0.087
C22:6n3	0.006	0.006	0.006	0.006	0.009	0.006	0.006	0.014
C24:0	0.022	0.023	0.019	0.020	0.027	0.022	0.025	0.023
C24:1	0.009	0.008	0.008	0.008	0.011	0.009	0.008	0.011

Table 33: Variation of fatty acid profile during winter (HAY and CON)

Variable	Minimum	Maximum	Mean	Std. deviation
C18:3n3 | CON	0.518	0.895	0.656	0.139
C18:3n3 | HAY.	0.734	1.293	0.977	0.134
FA 2 | CON	0.066	0.092	0.075	0.009
FA 2 | HAY.	0.038	0.137	0.088	0.016
FA 3 | CON	0.078	0.177	0.134	0.027
FA 3 | HAY.	0.129	0.262	0.173	0.031
FA 4 | CON	0.008	0.040	0.025	0.009
FA 4 | HAY.	0.005	0.028	0.015	0.004
CLAc9t11 | CON	0.258	1.048	0.745	0.176
CLAc9t11 | HAY.	0.012	1.307	0.831	0.214
FA 6 | CON	0.005	0.051	0.016	0.016
FA 6 | HAY.	0.005	0.019	0.010	0.004
FA 7 | CON	0.012	0.117	0.057	0.028
FA 7 | HAY.	0.014	0.074	0.038	0.012
FA 8 | CON	0.009	0.047	0.027	0.011
FA 8 | HAY.	0.018	0.049	0.027	0.007
FA 9 | CON	0.002	0.027	0.011	0.006
FA 9 | HAY.	0.007	0.018	0.012	0.003
FA 10 | CON	0.044	0.085	0.057	0.011
FA 10 | HAY.	0.027	0.098	0.053	0.015
FA 11 | CON	0.046	0.126	0.089	0.020
FA 11 | HAY.	0.052	0.192	0.087	0.034
C20:0 | CON	0.157	0.220	0.186	0.020
C20:0 | HAY.	0.156	0.246	0.185	0.020
FA 15 | CON	0.105	0.176	0.139	0.018
FA 15 | HAY.	0.121	0.187	0.152	0.015
C20:1 | CON	0.049	0.070	0.056	0.006
C20:1 | HAY.	0.046	0.070	0.055	0.005
FA 17 | CON	0.003	0.017	0.011	0.004
FA 17 | HAY.	0.002	0.022	0.011	0.006
FA 18 | CON	0.006	0.021	0.013	0.004
FA 18 | HAY.	0.005	0.026	0.010	0.004
C20:2 | CON	0.039	0.060	0.046	0.005
C20:2 | HAY.	0.042	0.071	0.053	0.008
C20:3n6 | CON	0.028	0.083	0.063	0.016
C20:3n6 | HAY.	0.036	0.105	0.065	0.014
C20:4n6 | CON	0.071	0.136	0.094	0.015
C20:4n6 | HAY.	0.014	0.203	0.098	0.035
C20:3n3 | CON	0.005	0.020	0.012	0.004
C20:3n3 | HAY.	0.011	0.030	0.018	0.005
FA 23 | CON	0.006	0.025	0.016	0.006
FA 23 | HAY.	0.009	0.049	0.022	0.009
C21:0 | CON	0.049	0.108	0.077	0.015
C21:0 | HAY.	0.057	0.126	0.100	0.016
C20:5n3 | CON	0.040	0.093	0.066	0.014
C20:5n3 | HAY.	0.070	0.130	0.094	0.015
C22:0 | CON	0.042	0.090	0.071	0.013
C22:0 | HAY.	0.007	0.123	0.080	0.026
C22:1n9 | CON	0.002	0.028	0.009	0.006
C22:1n9 | HAY.	0.006	0.020	0.010	0.005

FA 28 | CON	0.000	0.016	0.006	0.004
FA 28 | HAY.	0.007	0.050	0.021	0.010
FA 29 | CON	0.014	0.060	0.037	0.010
FA 29 | HAY.	0.018	0.085	0.045	0.017
C23:0 | CON	0.011	0.034	0.026	0.007
C23:0 | HAY.	0.016	0.051	0.036	0.010
FA 31 | CON	0.015	0.042	0.027	0.007
FA 31 | HAY.	0.013	0.057	0.037	0.012
C22:5n3 | CON	0.016	0.093	0.065	0.020
C22:5n3 | HAY.	0.006	0.121	0.082	0.026
C22:6n3 | CON	0.002	0.012	0.006	0.003
C22:6n3 | HAY.	0.003	0.063	0.011	0.012
C24:0 | CON	0.005	0.032	0.021	0.007
C24:0 | HAY.	0.007	0.056	0.026	0.013
C24:1 | CON	0.004	0.011	0.008	0.002
C24:1 | HAY.	0.006	0.023	0.011	0.003

Table 34: Variation of fatty acid profile during summer (HAY and CON)

Variable	Minimum	Maximum	Mean	Std. deviation
C18:3n3 | CON	0.593	1.244	0.782	0.192
C18:3n3 | HAY.	0.729	1.162	0.981	0.114
FA 2 | CON	0.058	0.096	0.080	0.009
FA 2 | HAY.	0.046	0.133	0.089	0.020
FA 3 | CON	0.123	0.169	0.153	0.012
FA 3 | HAY.	0.117	0.269	0.164	0.041
FA 4 | CON	0.005	0.038	0.026	0.008
FA 4 | HAY.	0.008	0.036	0.017	0.005
CLAc9t11 | CON	0.008	1.784	0.993	0.396
CLAc9t11 | HAY.	0.940	2.124	1.519	0.303
FA 6 | CON	0.005	0.036	0.011	0.007
FA 6 | HAY.	0.000	0.024	0.011	0.005
FA 7 | CON	0.028	0.115	0.061	0.022
FA 7 | HAY.	0.039	0.109	0.070	0.018
FA 8 | CON	0.017	0.058	0.030	0.010
FA 8 | HAY.	0.021	0.051	0.037	0.009
FA 9 | CON	0.007	0.022	0.012	0.004
FA 9 | HAY.	0.007	0.024	0.015	0.004
FA 10 | CON	0.030	0.096	0.062	0.014
FA 10 | HAY.	0.057	0.109	0.076	0.014
FA 11 | CON	0.071	0.128	0.090	0.016
FA 11 | HAY.	0.043	0.165	0.112	0.027
C20:0 | CON	0.149	0.221	0.187	0.020
C20:0 | HAY.	0.140	0.217	0.189	0.023
FA 15 | CON	0.113	0.177	0.151	0.013
FA 15 | HAY.	0.114	0.193	0.151	0.021
C20:1 | CON	0.019	0.071	0.059	0.011
C20:1 | HAY.	0.043	0.070	0.055	0.008
FA 17 | CON	0.006	0.026	0.014	0.005
FA 17 | HAY.	0.005	0.023	0.014	0.004
FA 18 | CON	0.004	0.019	0.012	0.003
FA 18 | HAY.	0.005	0.025	0.012	0.005
C20:2 | CON	0.035	0.059	0.045	0.005
C20:2 | HAY.	0.028	0.078	0.050	0.011
C20:3n6 | CON	0.043	0.096	0.072	0.013
C20:3n6 | HAY.	0.041	0.085	0.065	0.012
C20:4n6 | CON	0.062	0.120	0.095	0.013
C20:4n6 | HAY.	0.059	0.192	0.099	0.034
C20:3n3 | CON	0.010	0.019	0.013	0.003
C20:3n3 | HAY.	0.010	0.025	0.016	0.004
FA 23 | CON	0.007	0.029	0.017	0.005
FA 23 | HAY.	0.018	0.049	0.025	0.007
C21:0 | CON	0.067	0.111	0.086	0.011
C21:0 | HAY.	0.058	0.131	0.100	0.018
C20:5n3 | CON	0.054	0.101	0.073	0.012
C20:5n3 | HAY.	0.065	0.116	0.087	0.014
C22:0 | CON	0.063	0.088	0.076	0.007
C22:0 | HAY.	0.007	0.119	0.079	0.023
C22:1n9 | CON	0.003	0.024	0.009	0.006
C22:1n9 | HAY.	0.003	0.025	0.010	0.006

FA 28 | CON	0.001	0.014	0.006	0.003
FA 28 | HAY.	0.003	0.035	0.013	0.010
FA 29 | CON	0.003	0.053	0.036	0.013
FA 29 | HAY.	0.003	0.094	0.030	0.024
C23:0 | CON	0.021	0.041	0.028	0.005
C23:0 | HAY.	0.018	0.045	0.033	0.008
FA 31 | CON	0.017	0.037	0.028	0.004
FA 31 | HAY.	0.016	0.045	0.031	0.008
C22:5n3 | CON	0.013	0.096	0.066	0.022
C22:5n3 | HAY.	0.022	0.130	0.084	0.026
C22:6n3 | CON	0.000	0.018	0.006	0.004
C22:6n3 | HAY.	0.002	0.013	0.006	0.003
C24:0 | CON	0.003	0.031	0.021	0.007
C24:0 | HAY.	0.003	0.051	0.024	0.010
C24:1 | CON	0.006	0.012	0.008	0.001
C24:1 | HAY.	0.001	0.013	0.009	0.003

Table 35: T-test by management

Variable\Test	p-value HAY-CON	Significance
C18:3n3	**< 0.0001**	***
FA 2	**0.001**	**
FA 3	**0.000**	***
FA 4	**< 0.0001**	***
CLAc9t11	**0.002**	**
FA 6	0.082	
FA 7	0.188	
FA 8	0.180	
FA 9	0.179	
FA 10	0.283	
FA 11	0.147	
C20:0	0.999	
FA 15	0.081	
C20:1	0.195	
FA 17	0.881	
FA 18	**0.035**	*
C20:2	**0.000**	***
C20:3n6	0.482	
C20:4n6	0.518	
C20:3n3	**< 0.0001**	***
FA 23	**< 0.0001**	***
C21:0	**< 0.0001**	***
C20:5n3	**< 0.0001**	***
C22:0	0.151	
C22:1n9	0.251	
FA 28	**< 0.0001**	***
FA 29	0.711	
C23:0	**< 0.0001**	***
FA 31	**0.001**	**
C22:5n3	**0.001**	**
C22:6n3	0.061	
C24:0	0.118	
C24:1	**0.003**	**

*Signif. codes: '***' 0.001 '**' 0.01 '*' 0.01*

Table 36: T-test by season

Variable\Test	p- value summer	significance	p-value winter	significance
C18:3n3	**0.000**	***	**< 0.0001**	***
FA 2	0.079		**0.003**	**
FA 3	0.224		**< 0.0001**	***
FA 4	**< 0.0001**	***	**< 0.0001**	***
CLAc9t11	**< 0.0001**	***	0.162	
FA 6	0.861		0.052	
FA 7	0.174		**0.005**	**
FA 8	**0.023**	**	1.000	
FA 9	0.078		0.630	
FA 10	**0.004**	**	0.384	
FA 11	**0.003**	**	0.801	
C20:0	0.807		0.849	
FA 15	0.994		**0.009**	**
C20:1	0.257		0.635	
FA 17	0.984		0.961	
FA 18	0.591		**0.022**	*
C20:2	0.056		**0.003**	**
C20:3n6	0.103		0.589	
C20:4n6	0.634		0.657	
C20:3n3	**0.003**	**	**< 0.0001**	***
FA 23	**< 0.0001**	***	**0.015**	*
C21:0	**0.003**	**	**< 0.0001**	***
C20:5n3	**0.001**	**	**< 0.0001**	***
C22:0	0.564		0.170	
C22:1n9	0.570		0.317	
FA 28	**0.009**	**	**< 0.0001**	***
FA 29	0.285		0.096	
C23:0	**0.033**	*	**0.001**	**
FA 31	0.230		**0.002**	**
C22:5n3	**0.028**	*	**0.025**	*
C22:6n3	0.740		0.076	
C24:0	0.390		0.212	
C24:1	0.508		**0.001**	**

*Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1*

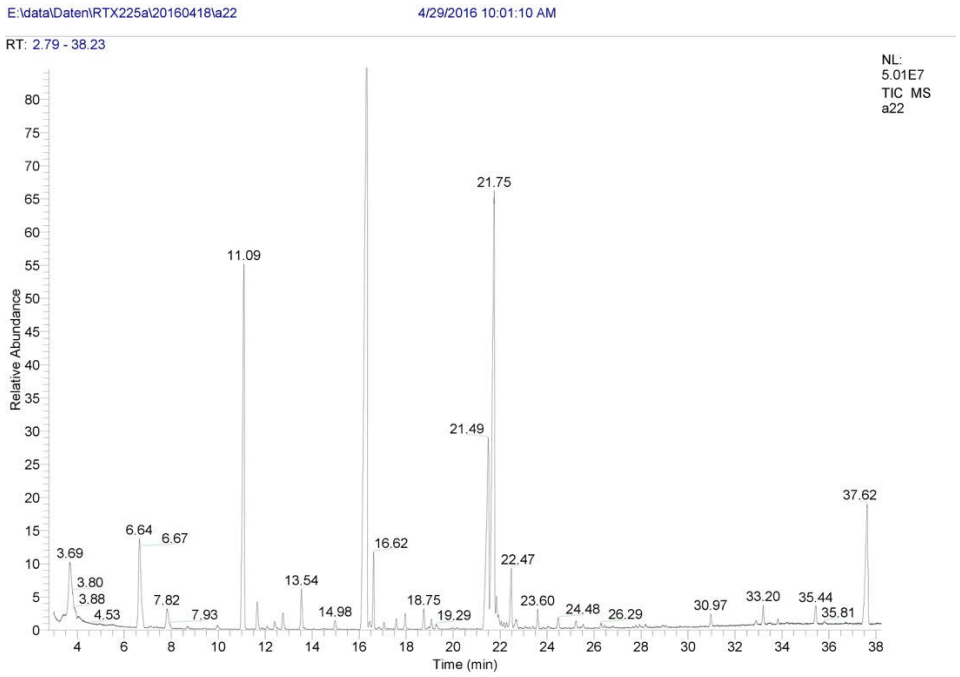

Figure 53: Splitless injection

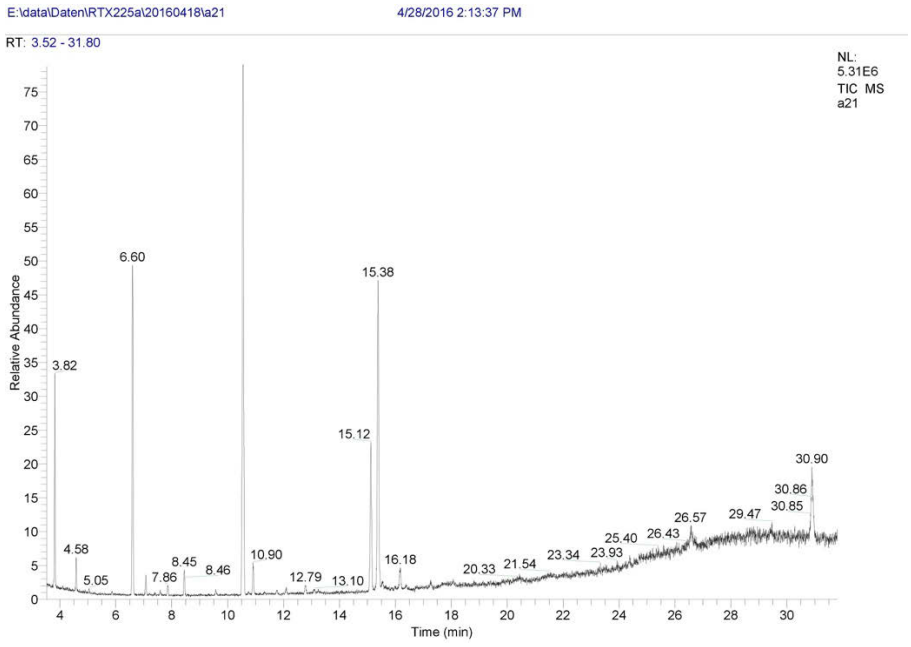

Figure 54: Split injection

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2 Theoretical background

2.1 Milk

2.1.1 Definition and composition | OI

Milk is, per definition, the “milking of the cow or many cows” and is commonly known as cow’s milk. Milk from other mammals is termed with the name of the origin, such as “mare milk; goat milk”.

Milk is a white to yellowish oil-in-water emulsion. Milk is composed of water, proteins and carbohydrates, as well as of vitamins, minerals and trace elements, as demonstrated in Figure 1. The milk fat is secreted as globules, which have a diameter of around 3 µm. The core of these globules consists mainly of triglycerides (TG), the membrane of monoglycerides, sphingolipids and stearins. Together with proteins they operate as emulgators (Bösze, 2008).

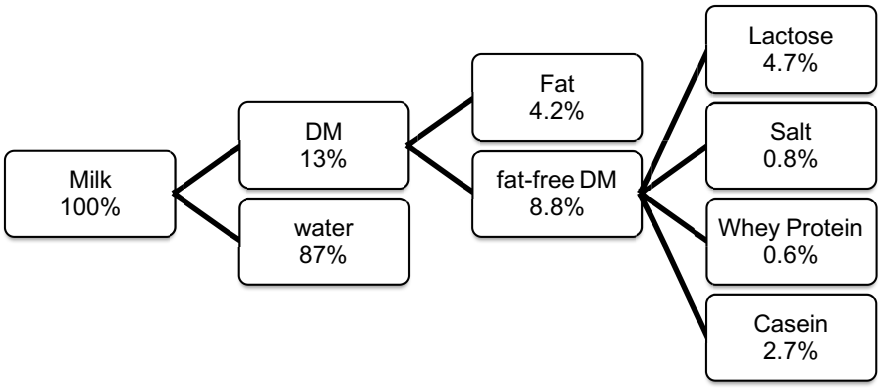

Figure 1: Average composition of cow milk (data from Eckard Schlimme, 1995)

Humans consume milk and milk products of many species, e.g. sheep and goat, but cow milk is the most important one from an economic perspective. Usually, milk is supposed to be the first and only nourishment for a new-born. This „first milk “, also called the colostrum, is essential for the newborn, because it provides all important nutrients and antibodies.

The composition of milk depends on health status and lactation stage, as well as on the composition of feed and genetics. In terms of nutrients, the protein and FA contents are of

2.1.3 Milk production in Austria | OI

Milk production in Austria contributes to a large extent to the income of Austrian farmers. In 2015, more than 3 million tons of milk has been produced, (see Figure 2; Figure 3), and the trend is still on the rise. Comparing the milk- production of May 2015 and May 2016, the results shows almost 20.000 tons more milk.

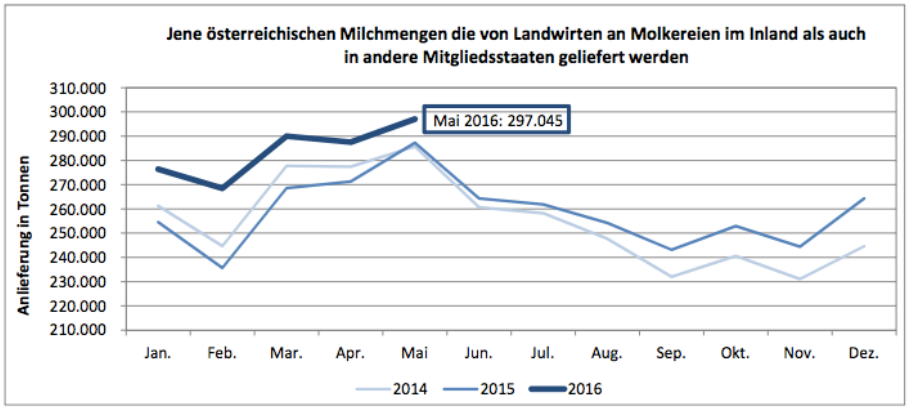

Figure 2: Milk supply in Austria (https://www.ama.at/getattachment/7265c403-a0f8-4cc2-acfb-138e20ec95e0/05_Marktbericht_Milch_Milchprodukte_05_2016.pdf)

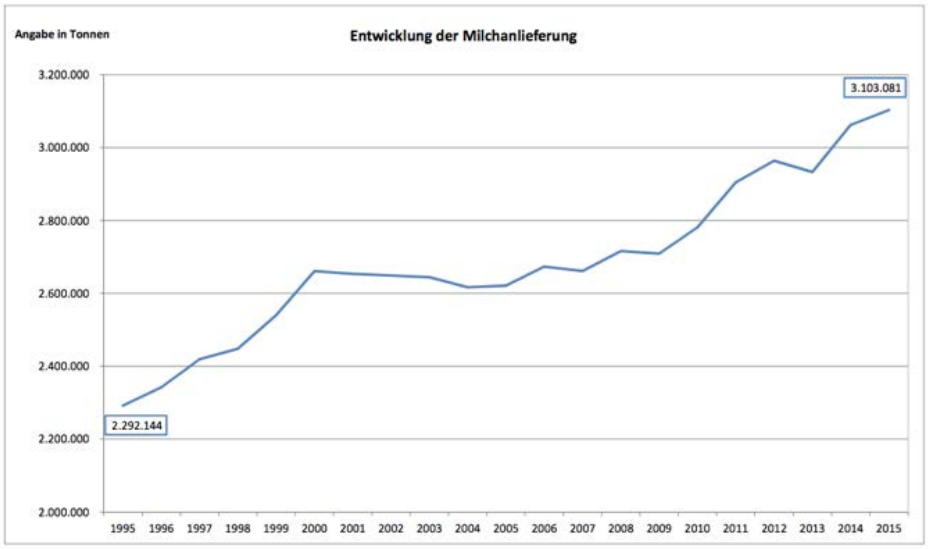

Figure 3: Development of milk supply in Austria 1995-2015 (https://www.ama.at/getattachment/3265d00f-c705-4a3f-9add-a7e84be5854a/170_Entwicklung-der-Milchanlieferung_1995-2015.pdf)

Comparing the single provinces of Austria, most of the milk is produced in Upper- (33 %) and Lower Austria (including Vienna, 18 %). Furthermore, the biggest amount of milk- cows is kept in those areas.

Over the course of the past years, the sector of milk and milk products has expanded. The consumer is not only able to select between whole milk and low- fat milk; they are further able to choose, which type of farming system they intent to support with their products. Nowadays, the diary sector in Austria undergoes a real boom in terms of demand in hay milk and the production is still rising. By this time, hay milk represents 11 % of the total milk production in Austria. Nearly every supermarket offers hay milk products (ARGE, 2016).

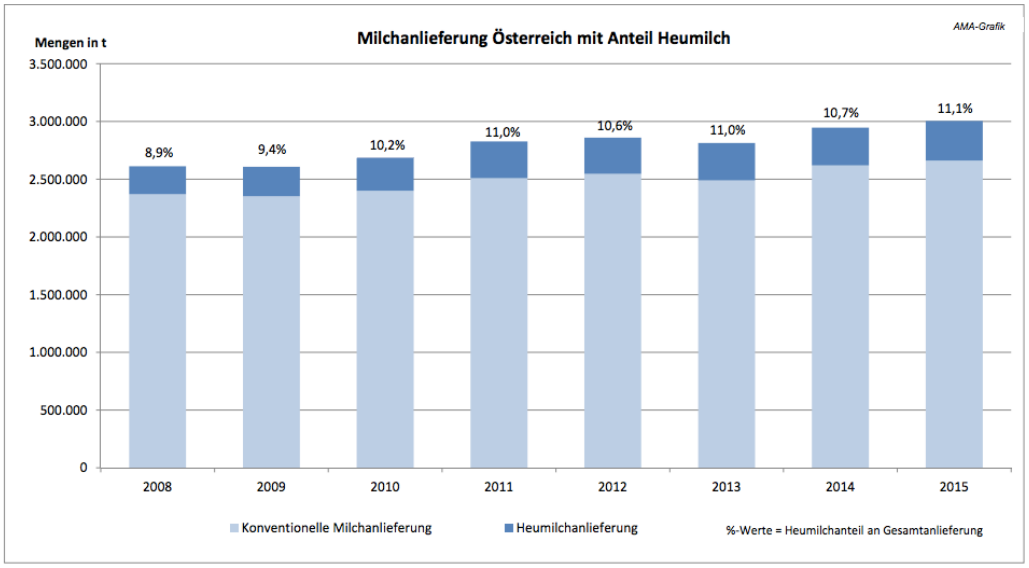

Figure 4: Milk supply in Austria, April 2016 (https://www.ama.at/getattachment/0ba4bb78-ae99-4728-9238-db7a7b38a6d1/181_Bio_Heumilchanlieferung-mit-zuschlag_1998-2015.pdf)

synthesis pathway. Acetyl-CoA carboxylase and FA- synthetase are the two key enzymes that are necessary for the synthesis.

Firstly, acetate gets converted into acetyl-CoA in the cytosol via acetyl-CoA synthetase.

Secondly, an elongation takes place by the malonyl-CoA pathway. Acetyl-CoA gets converted into malonyl-CoA by acetyl-CoA carboxylase. For further elongation malonlyl-CoA and also acetyl-CoA are used. Each cycle in this pathway results in two carbons being added to the FA chain and the FA- synthetase complex is responsible for the chain elongation. The required reducing agent is $NADPH_2$ (Harstad and Steinshamn, 2010). Finally, C16:0 is generated by the separation under enzymatic action (thioesterase I) (Smith, 1980). Thioesterase I can form FAs of various chain length, dependent of synthesis stage during release of FAs.

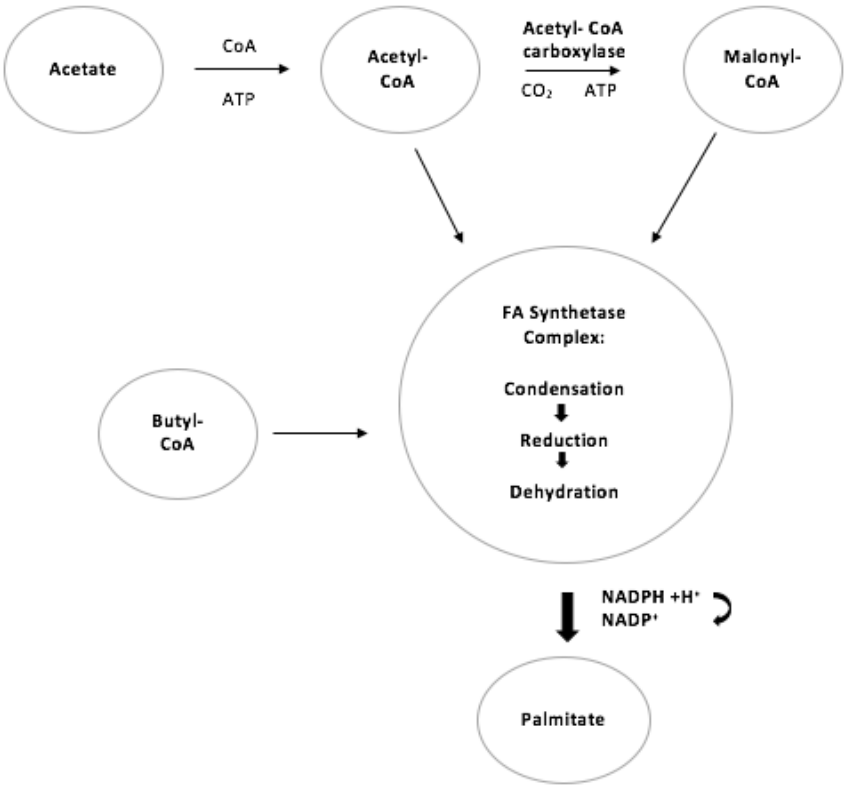

Figure 6: Overview de novo synthesis of Palmitate (data after Harstad and Steinshamn, 2010)

The main substrates for biohydrogenation are PUFAs, like C18:2n6 and C18:3n3. These long-chain FAs, originating from the diet, get hydrolysed by lipases and undergo a second transformation called biohydrogenation (Elgersma *et al.,* 2006). At this stage two different bacteria act. One group hydrogenate the C18:2n6 and C18:3n3 and the other group,

In the ruminants' gastro intestine C18:2n6 gets converted in its isoforms. The result of this partial biohydrogenation is a single bond between one or both of the double bonds, that means *cis*-9, *trans*-11 or *trans*-10, *cis*-12 (Viviani, 1970; Kennedy *et al.,* 2010). Parodi described this conjugated FAs as CLAs including its isomers. The configuration of CLAs can be *cis* or *trans*. The most frequent in ruminants is *cis*-9, *trans*-11 CLA, also called "rumenic acid" (Parodi, 1977).

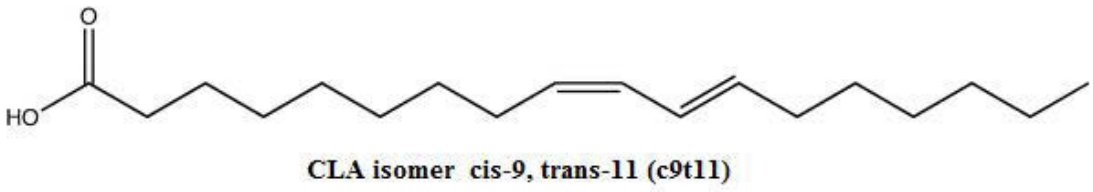

Figure 11: Chemical structure of the most common CLA isomer: cis-9, trans-11 (rumenic acid)

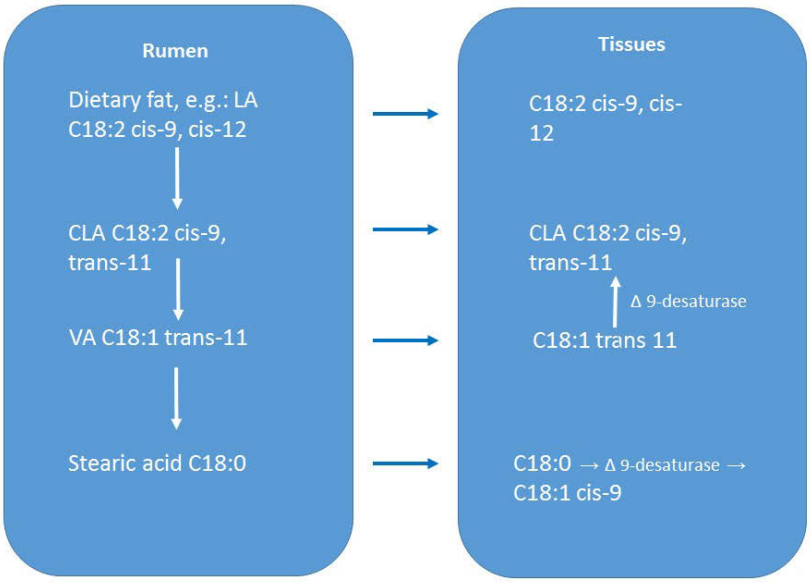

Figure 12: Role of rumen biohydrogenation and tissue ∆9--desaturase in the production of cis-9, trans-11 conjugated linoleic acid in ruminant fat, modified after: Bauman1999,: https://examine.com/supplements/Conjugated-linoleic-acid/ (accessed on 09.08.2016)

As milk and its products are a main supplier for CLAs in food (Chin *et al.,* 1992), the content of CLAs in milk shows a big variation, which depends on the CLA concentration of the used raw milk (Whigham *et al.,* 2000). Several studies confirmed the influence on CLA content in milk caused by production system and feed. There is a clear correlation between the CLA content of pastures and its concentration in milk (Dhiman *et al.,*, 1999; Jahreis *et al.,* 1999; Jahreis *et al.,* 1997a). Especially the main isomer in cow's milk, cis-9, *trans*-11 CLA, can be

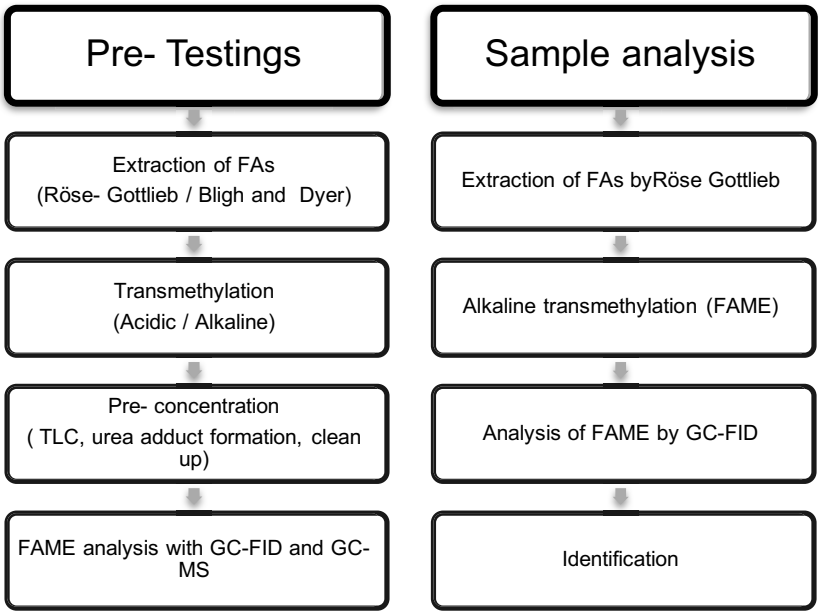

Figure 15: Scheme of pre-testing and final sample analysis methods

4.4.2 Isolation and extraction of milk fat

In order to have the milk fat present, it must get separated from the rest of the milk compounds. The isolation of the total fat was done in two different ways. One way was the isolation by an adapted Röse- Gottlieb method, the other by Bligh and Dyer (Bligh and Dyer, 1959). These extraction methods were chosen, because FAMEs were needed mainly for the preparative analysis and because no quantitative evaluation was needed.

4.4.2.1 **Bligh and Dyer method |** KJ

Four mL of methanol and 2 mL of Chloroform $CHCl_3$ were added to 2 mL of milk sample in a pyrex tube. The pyrex containing the mixture was shaken for 1 minute. Afterwards 2 mL of $CHCl_3$ and 2 mL of dH_20 were added, followed by a centrifugation step for 5 min at 1100 rpm. A pasteur pipette was cut with a knife to fit into the pyrex. Another pasteur pipette was used to reach the lower phase of the two-phase system in the pyrex tube. The lower phase was taken up, put in a clean pyrex tube and vaporized with the use of N_2 to remove the $CHCl_3$. The remaining part which contained the extracted fat was then acidly trans methylated (Bligh and Dyer, 1959).

5.2.1 Silver- ion thin layer chromatography | OI

After the GC-FID analysis, there were many not yet identified FAs. These should be identified TLC coupled with a GC-MS.

For the segregation of FAs according to the number of double bounds and their geometrical configuration, silver ion chromatography is a favourable method for it. Highly unsaturated fatty acids retain at the starting line, SFAs migrate towards the solvent front.

Figure 16 shows the 6 fluorescein bands, which could be separated by TLC.

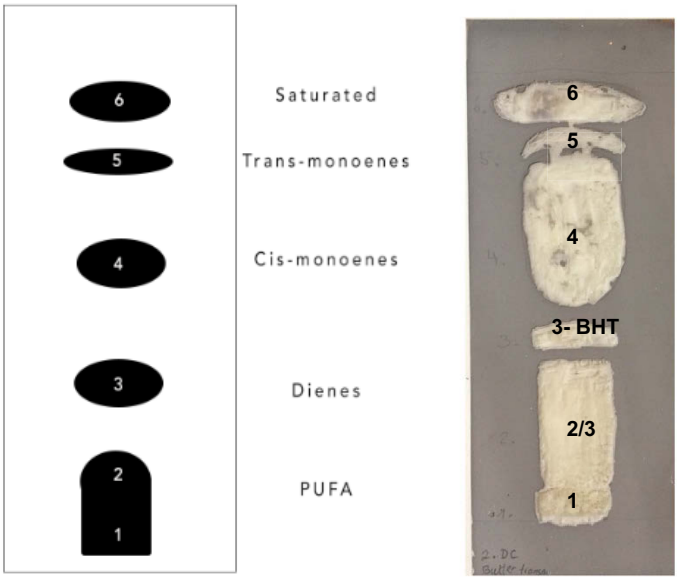

Figure 16: Schematic separation of FAME by Ag+-TLC

For qualitative evaluation of the Ag^{+}-TLC the retention factor (R_f) was used. The R_f value is defined as follows:

$$R_f = \frac{distance\ starting\ line - middle\ of\ spot}{distance\ starting\ line - solvent\ front} = \frac{a}{b}$$

The obtained R_f values for the fractions 1-6 are: 0.08; 0.29; 0.40; 0.73; 0.88; 0.94

After the extraction from the silica gel, it was possible to detect and determine the fractions by GC-MS. Further, it was possible to identify branched- isomers.

After the extraction from the silica gel, it was possible to detect and determine the fractions by GC-MS. Further, it was possible to identify branched- isomers.

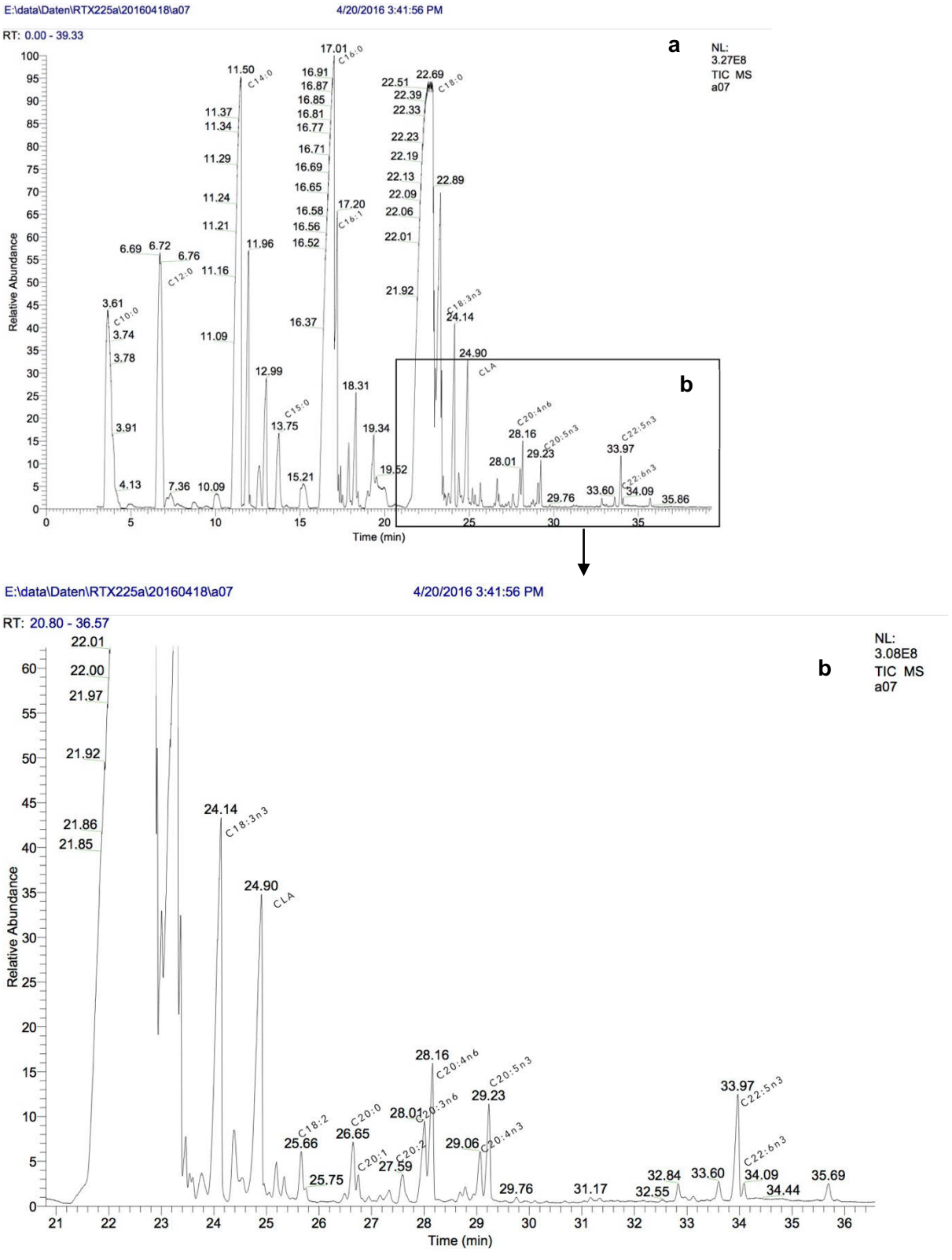

Figure 23: Chromatogram of the 1st attempt of urea adduct formation; overnight method; diluted once

The chromatogram above (Figure 23) shows the urea adduct formation from the first attempt. The solution got one time diluted with hexane. It is obvious, that the separation

improved by one further dilution step, but the chromatogram is still too overloaded. With the dilution step, the assumed SFAs could even be set.

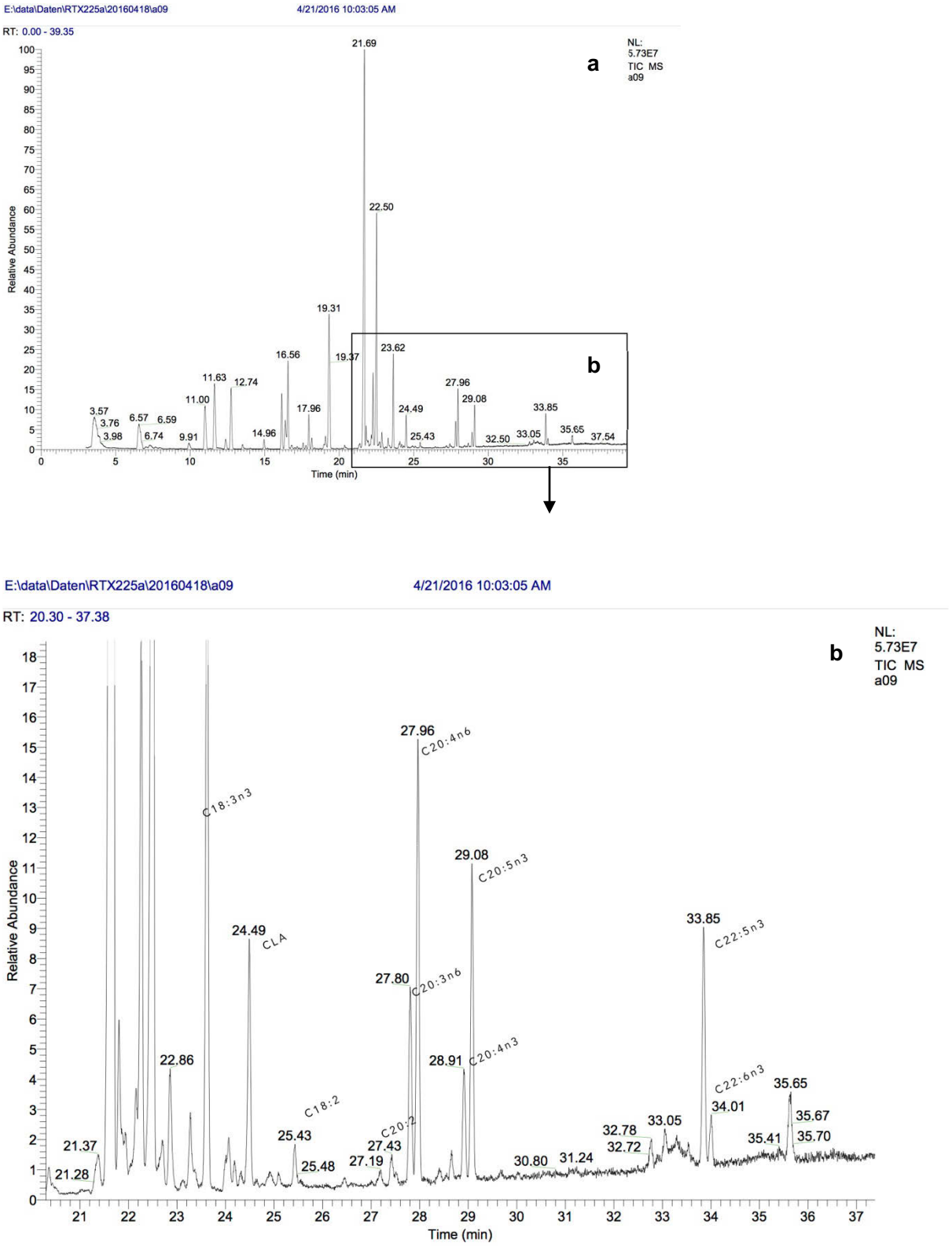

Figure 24: Chromatogram of the 2nd attempt of urea adduct formation; overnight method, diluted once

Figure 24 shows the chromatogram of the second attempt of the urea adduct formation with the overnight method. The chromatogram shows no SFAs which confirms that the complexation worked properly.

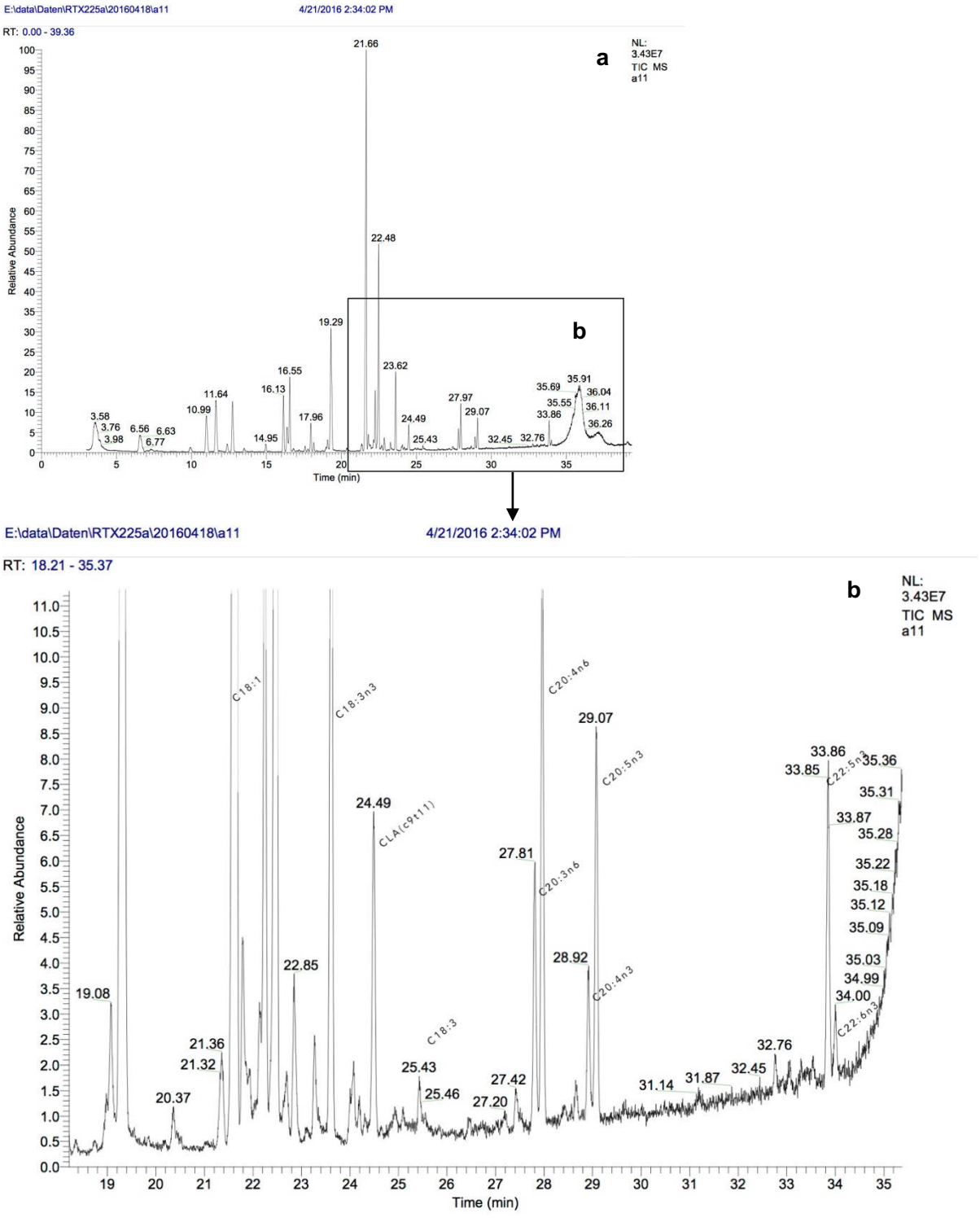

Figure 25: Chromatogram of the 2nd attempt of urea adduct formation; overnight method, 2 times diluted

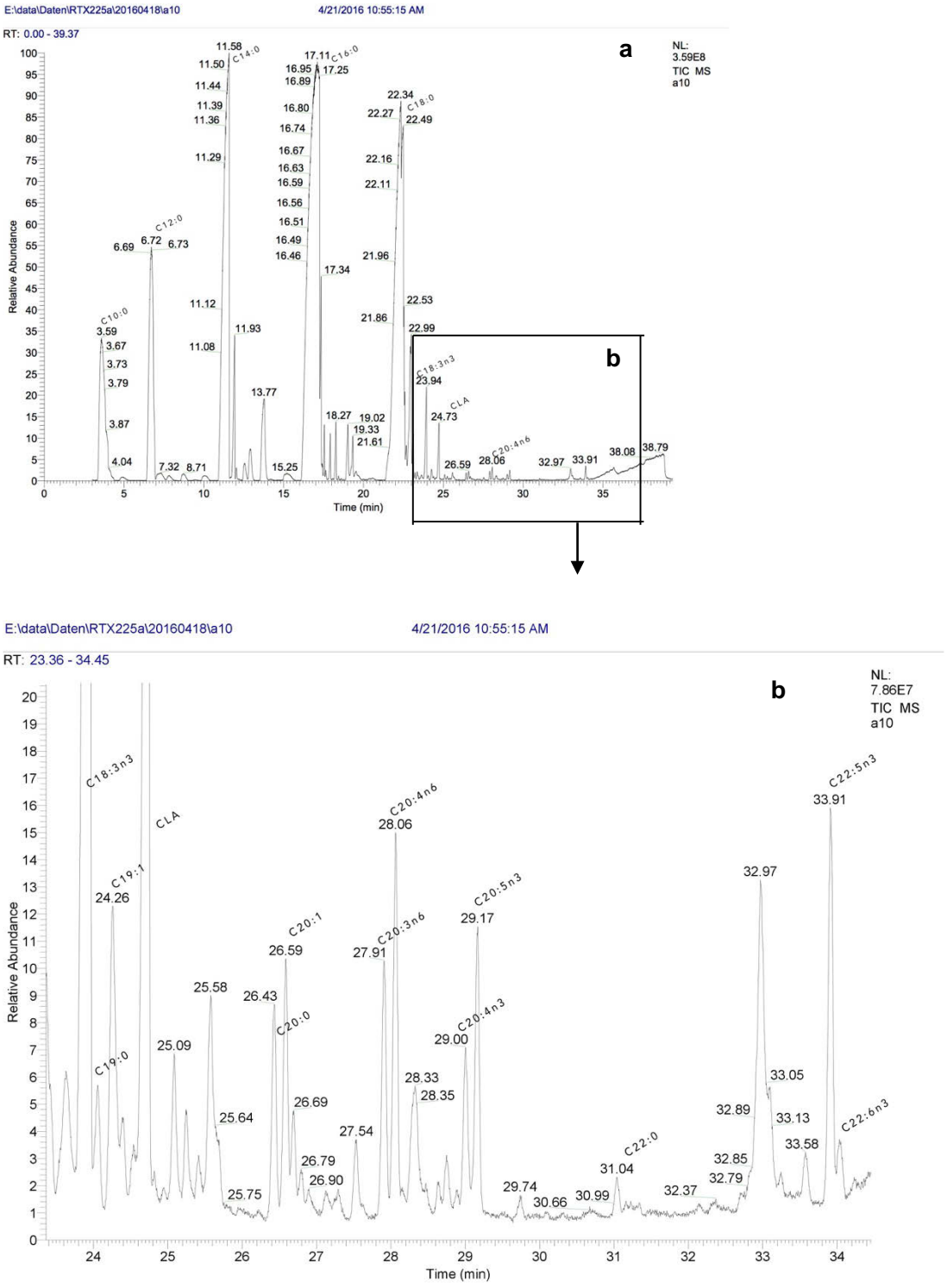

Figure 26: Chromatogram of urea adduct formation, 4h-method; diluted once

5.3.2 Comparison of different GC-MS chromatograms | OI

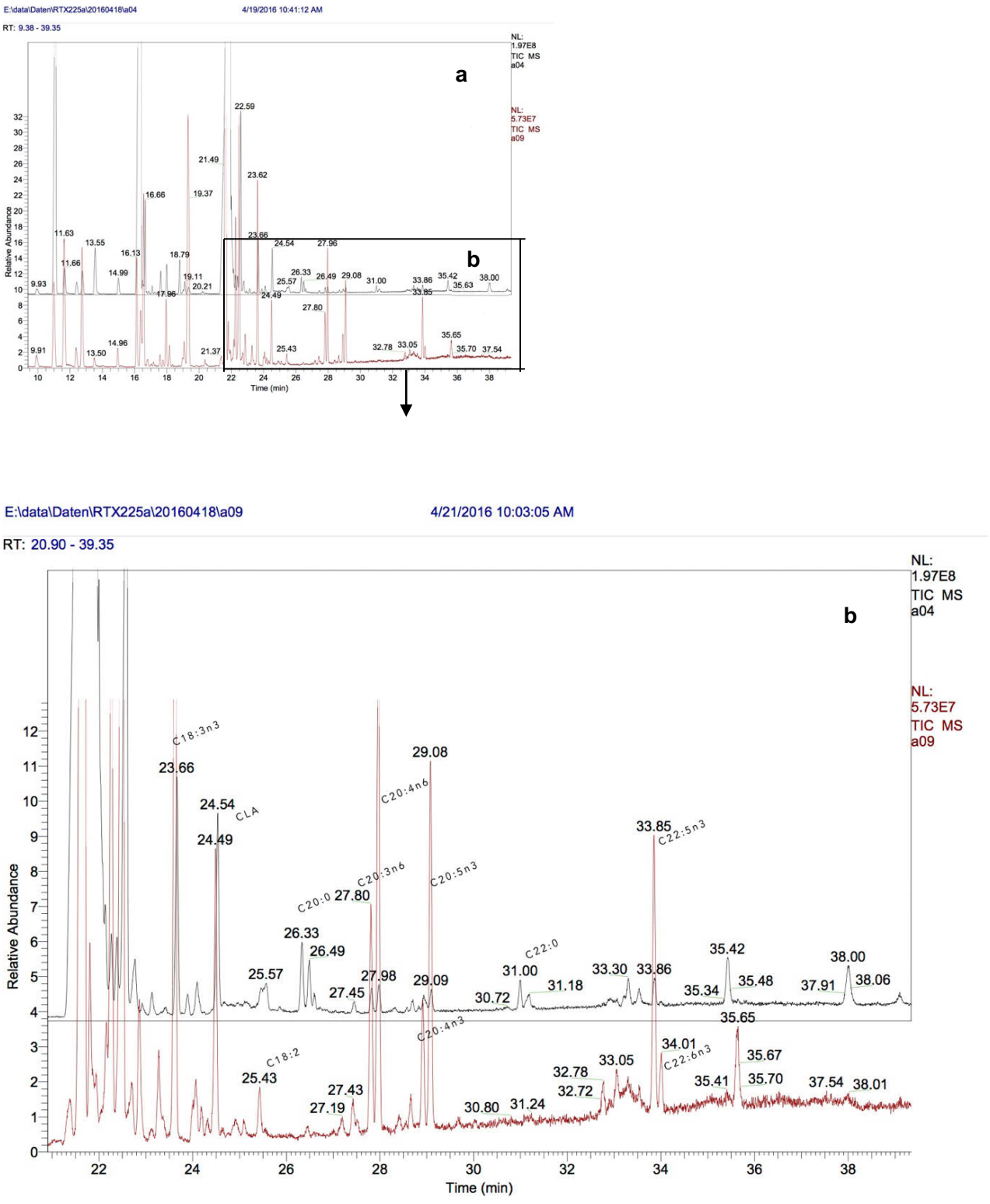

Figure 28: Chromatogram of conventional raw milk vs. urea adduct formation

A raw milk sample without urea adduct formation (a04, see black line) is compared to the urea complexion sample (a09, see red line) in Figure 28. Although C20:0 and C22:0 seem to

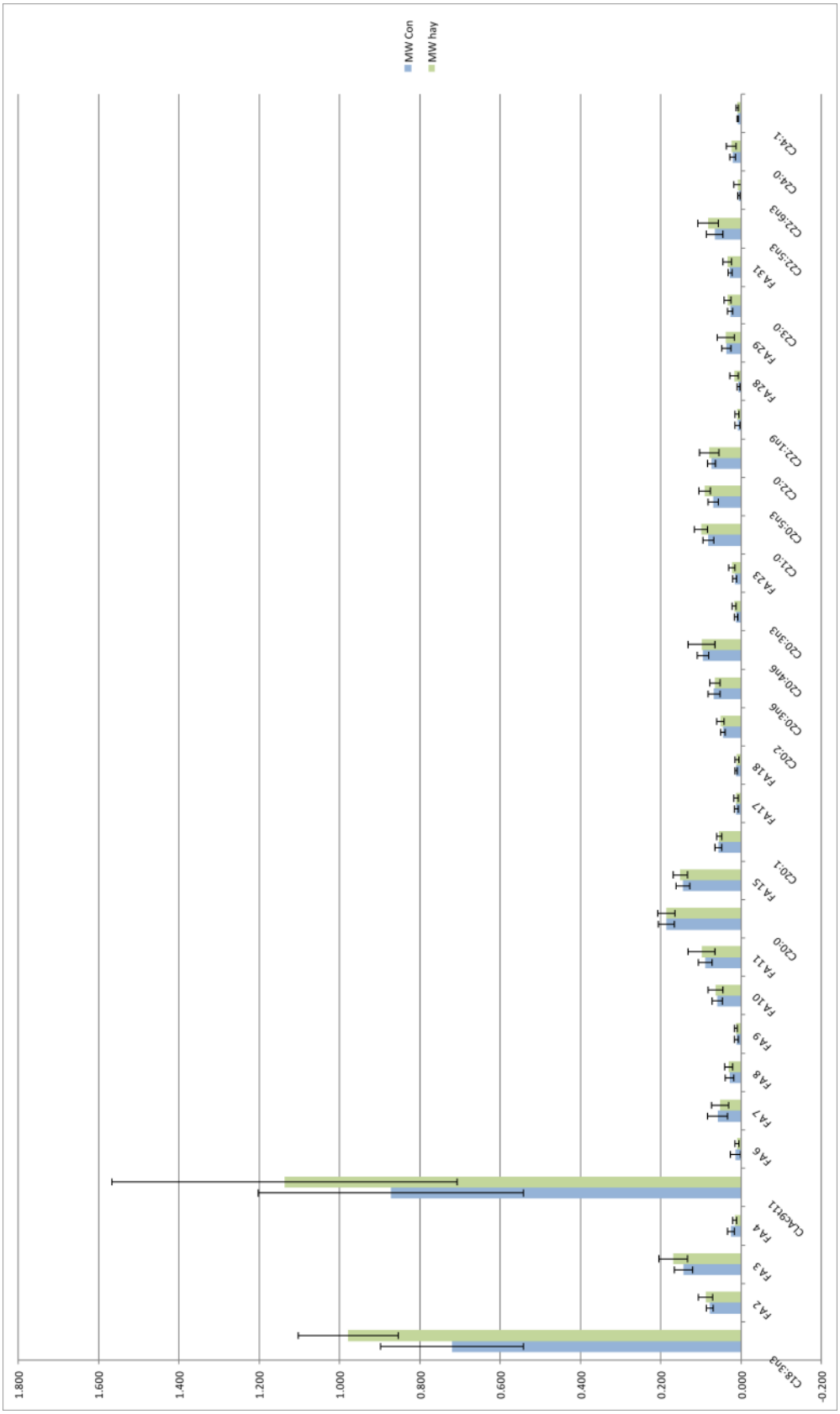

Figure 31: Overview of the whole FA composition (seasonal variation of the mean values and STD); starting at C18:3n3

Appendix

Table 24: Values of corrected FA peak areas (CON Q1- Q2)

sample	Probe 19	Probe 30	Probe 31	Probe 32	Probe 33	Probe 34	Probe 35	Probe 36	Probe 38	Probe 39	Probe 56	Probe 57	Probe 60	Probe 61	Probe 64	Probe 65	Probe 68	Probe 69
Feeding.Quartal	Con. Q1	Con. Q1	Con. Q1	Con. Q1	Con. Q1	Con. Q1	Con. Q1	Con. Q1	Con. Q1	Con. Q1	Con. Q2	Con. Q2	Con. Q2	Con. Q2	Con. Q2	Con. Q2	Con. Q2	Con. Q2
C18:3n3	0.543	0.869	0.893	0.702	0.704	0.795	0.583	0.573	0.597	0.580	0.660	0.691	0.593	0.708	0.723	0.683	1.141	1.155
2	0.085	0.084	0.085	0.091	0.092	0.076	0.067	0.066	0.066	0.079	0.065	0.082	0.078	0.085	0.084	0.079	0.089	0.081
3	0.152	0.166	0.163	0.171	0.177	0.140	0.136	0.114	0.129	0.151	0.123	0.148	0.140	0.164	0.162	0.156	0.160	0.166
4	0.039	0.014	0.017	0.033	0.035	0.009	0.026	0.017	0.027	0.040	0.005	0.030	0.027	0.038	0.038	0.034	0.026	0.026
CLAc9t11	0.258	0.868	0.936	0.859	0.868	0.980	0.618	0.589	0.712	0.747	0.898	0.678	0.838	0.912	0.820	0.768	1.634	1.779
6	0.049	0.006	0.009	0.009	0.022	0.009	0.012	0.021	0.005	0.006	0.006	0.036	0.007	0.005	0.015	0.006	0.009	0.014
7	0.012	0.088	0.095	0.043	0.052	0.101	0.030	0.025	0.050	0.049	0.040	0.028	0.070	0.072	0.055	0.048	0.115	0.063
8	0.011	0.037	0.038	0.022	0.028	0.040	0.011	0.009	0.021	0.022	0.022	0.017	0.026	0.035	0.026	0.027	0.054	0.023
9	0.027	0.010	0.013	0.009	0.012	0.014	0.006	0.003	0.007	0.008	0.011	0.017	0.007	0.013	0.013	0.008	0.020	0.012
10	0.060	0.060	0.065	0.054	0.056	0.070	0.048	0.044	0.048	0.053	0.049	0.030	0.060	0.061	0.061	0.054	0.091	0.076
11	0.090	0.087	0.103	0.093	0.102	0.126	0.104	0.102	0.078	0.075	0.074	0.128	0.083	0.101	0.077	0.071	0.104	0.090
C20:0	0.218	0.194	0.198	0.220	0.219	0.193	0.167	0.168	0.171	0.188	0.149	0.221	0.187	0.191	0.193	0.185	0.212	0.207
15	0.162	0.146	0.147	0.176	0.175	0.142	0.118	0.128	0.132	0.146	0.113	0.160	0.146	0.149	0.149	0.145	0.156	0.155
C20:1	0.063	0.051	0.053	0.070	0.068	0.052	0.049	0.053	0.053	0.057	0.057	0.067	0.067	0.061	0.057	0.054	0.065	0.071
17	0.013	0.009	0.013	0.008	0.005	0.016	0.010	0.009	0.007	0.014	0.007	0.008	0.021	0.013	0.014	0.013	0.020	0.018
18	0.013	0.012	0.011	0.016	0.014	0.006	0.020	0.021	0.012	0.016	0.004	0.013	0.014	0.014	0.014	0.011	0.012	0.009
C20:2n6	0.050	0.039	0.041	0.048	0.050	0.040	0.051	0.060	0.043	0.045	0.035	0.045	0.042	0.043	0.042	0.044	0.042	0.038
C20:3n6	0.081	0.049	0.049	0.068	0.076	0.048	0.062	0.074	0.063	0.083	0.043	0.074	0.079	0.075	0.072	0.075	0.048	0.056
C20:4n6	0.136	0.075	0.074	0.096	0.107	0.071	0.085	0.103	0.090	0.096	0.062	0.112	0.094	0.096	0.100	0.094	0.071	0.081
C20:3n3	0.011	0.020	0.020	0.014	0.010	0.015	0.012	0.005	0.011	0.012	0.012	0.014	0.011	0.012	0.014	0.011	0.019	0.018
23	0.006	0.021	0.013	0.007	0.016	0.014	0.017	0.018	0.008	0.013	0.012	0.016	0.014	0.016	0.017	0.015	0.020	0.029
C21:0	0.082	0.108	0.099	0.072	0.093	0.095	0.076	0.049	0.059	0.084	0.077	0.083	0.082	0.087	0.094	0.086	0.111	0.083
C20:5n3	0.055	0.093	0.087	0.058	0.072	0.081	0.062	0.040	0.052	0.067	0.065	0.063	0.067	0.073	0.080	0.072	0.096	0.101
C22:0	0.082	0.085	0.086	0.089	0.090	0.083	0.067	0.062	0.066	0.074	0.066	0.086	0.069	0.076	0.076	0.074	0.088	0.080
C22:1n9	0.006	0.007	0.005	0.006	0.005	0.005	0.004	0.013	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.006	0.007
28	0.005	0.007	0.012	0.006	0.005	0.009	0.006	0.007	0.008	0.004	0.002	0.006	0.009	0.005	0.011	0.009	0.007	0.008
29	0.046	0.044	0.060	0.049	0.042	0.048	0.037	0.037	0.045	0.039	0.013	0.047	0.045	0.042	0.053	0.047	0.032	0.039
C23:0	0.020	0.034	0.034	0.030	0.032	0.032	0.022	0.027	0.022	0.028	0.027	0.031	0.027	0.028	0.025	0.028	0.038	0.032
31	0.031	0.035	0.042	0.029	0.033	0.031	0.026	0.019	0.026	0.029	0.026	0.017	0.031	0.031	0.028	0.029	0.037	0.034
C22:5n3	0.063	0.086	0.093	0.080	0.077	0.076	0.055	0.016	0.060	0.068	0.069	0.050	0.068	0.073	0.079	0.074	0.092	0.096
C22:6n3	0.010	0.007	0.008	0.006	0.003	0.003	0.004	0.006	0.004	0.005	0.005	0.008	0.005	0.006	0.008	0.005	0.006	0.009
C24:0	0.019	0.028	0.024	0.032	0.027	0.022	0.025	0.005	0.019	0.022	0.022	0.019	0.022	0.026	0.025	0.025	0.028	0.020
C24:1	0.009	0.009	0.009	0.011	0.011	0.009	0.009	0.008	0.006	0.008	0.009	0.010	0.008	0.007	0.008	0.008	0.008	0.007

Table 25: Values of corrected FA peak areas (CON Q2- Q4)

sample	Probe 72	Probe 73	Probe 76	Probe 77	Probe 80	Probe 81	Probe 84	Probe 85	Probe 88	Probe 89	Probe 92	Probe 93	Probe 96	Probe 97	Probe 100	Probe 101	Probe 104
Feeding.Quartal	Con. Q2	Con. Q2	Con. Q3	Con. Q3	Con. Q3	Con. Q3	Con. Q3	Con. Q3	Con. Q3	Con. Q3	Con. Q3	Con. Q3	Con. Q4	Con. Q4	Con. Q4	Con. Q4	Con. Q4
C18:3n3	0.747	0.722	0.644	0.681	0.688	0.637	0.672	0.715	1.027	1.244	0.775	0.733	0.530	0.518	0.554	0.537	0.547
2	0.088	0.084	0.073	0.081	0.075	0.074	0.078	0.058	0.082	0.096	0.087	0.085	0.071	0.072	0.068	0.066	0.078
3	0.169	0.160	0.145	0.158	0.150	0.151	0.156	0.126	0.145	0.149	0.167	0.156	0.132	0.141	0.131	0.115	0.079
4	0.029	0.026	0.031	0.032	0.025	0.030	0.025	0.019	0.010	0.026	0.023	0.031	0.027	0.035	0.027	0.016	0.008
CLAc9t11	1.002	0.940	0.928	0.833	1.036	0.894	0.924	0.843	1.784	0.008	1.232	1.105	0.619	0.647	0.734	0.728	0.609
6	0.008	0.008	0.008	0.008	0.008	0.020	0.009	0.023	0.010	0.014	0.008	0.008	0.007	0.005	0.006	0.024	0.051
7	0.052	0.048	0.052	0.037	0.076	0.065	0.051	0.047	0.115	0.069	0.066	0.049	0.035	0.031	0.056	0.042	0.069
8	0.028	0.027	0.031	0.022	0.036	0.032	0.027	0.024	0.058	0.021	0.038	0.029	0.020	0.014	0.028	0.043	0.025
9	0.009	0.011	0.012	0.009	0.010	0.010	0.011	0.022	0.020	0.012	0.012	0.012	0.008	0.002	0.007	0.012	0.016
10	0.058	0.056	0.054	0.053	0.067	0.056	0.059	0.061	0.096	0.061	0.073	0.068	0.045	0.045	0.053	0.074	0.050
11	0.077	0.086	0.079	0.078	0.092	0.081	0.087	0.099	0.128	0.092	0.085	0.082	0.068	0.046	0.062	0.089	0.101
C20:0	0.203	0.191	0.176	0.200	0.171	0.163	0.184	0.168	0.179	0.153	0.206	0.205	0.172	0.209	0.157	0.186	0.186
15	0.163	0.157	0.148	0.165	0.142	0.142	0.155	0.140	0.141	0.148	0.177	0.166	0.133	0.143	0.127	0.105	0.145
C20:1	0.068	0.059	0.066	0.063	0.057	0.055	0.060	0.053	0.055	0.019	0.057	0.060	0.052	0.061	0.055	0.060	0.057
17	0.018	0.006	0.020	0.015	0.011	0.012	0.013	0.010	0.012	0.009	0.026	0.013	0.011	0.013	0.017	0.012	0.005
18	0.012	0.012	0.013	0.019	0.012	0.012	0.012	0.013	0.009	0.013	0.014	0.015	0.012	0.013	0.012	0.013	0.013
C20:2n6	0.046	0.045	0.042	0.052	0.041	0.045	0.048	0.050	0.040	0.046	0.048	0.059	0.049	0.042	0.047	0.045	0.045
C20:3n6	0.072	0.076	0.081	0.079	0.074	0.072	0.074	0.065	0.065	0.095	0.070	0.096	0.071	0.080	0.080	0.060	0.028
C20:4n6	0.105	0.103	0.101	0.111	0.093	0.093	0.097	0.094	0.084	0.097	0.104	0.120	0.096	0.104	0.092	0.111	0.095
C20:3n3	0.012	0.017	0.012	0.012	0.010	0.010	0.010	0.010	0.018	0.013	0.013	0.010	0.008	0.009	0.010	0.013	0.011
23	0.021	0.007	0.014	0.011	0.019	0.013	0.014	0.016	0.021	0.016	0.018	0.021	0.023	0.011	0.016	0.016	0.011
C21:0	0.096	0.084	0.083	0.086	0.080	0.072	0.078	0.077	0.099	0.104	0.089	0.067	0.061	0.067	0.062	0.081	0.081
C20:5n3	0.077	0.071	0.063	0.068	0.069	0.061	0.065	0.072	0.083	0.089	0.075	0.054	0.053	0.058	0.058	0.069	0.056
C22:0	0.081	0.079	0.073	0.079	0.068	0.064	0.072	0.063	0.081	0.075	0.084	0.083	0.064	0.074	0.055	0.073	0.042
C22:1n9	0.005	0.004	0.006	0.010	0.009	0.013	0.020	0.024	0.003	0.003	0.007	0.023	0.028	0.006	0.017	0.009	0.011
28	0.006	0.010	0.006	0.005	0.007	0.004	0.003	0.014	0.004	0.006	0.001	0.007	0.006	0.003	0.002	0.006	0.005
29	0.046	0.053	0.036	0.031	0.033	0.029	0.029	0.037	0.027	0.046	0.003	0.037	0.037	0.029	0.027	0.037	0.030
C23:0	0.026	0.025	0.025	0.027	0.023	0.021	0.022	0.022	0.032	0.041	0.035	0.025	0.022	0.022	0.017	0.027	0.011
31	0.027	0.027	0.027	0.028	0.029	0.023	0.028	0.028	0.030	0.028	0.031	0.025	0.023	0.026	0.015	0.028	0.015
C22:5n3	0.075	0.072	0.071	0.068	0.071	0.068	0.069	0.024	0.095	0.013	0.031	0.069	0.056	0.066	0.053	0.066	0.058
C22:6n3	0.005	0.005	0.007	0.002	0.006	0.004	0.005	0.018	0.008	0.000	0.006	0.004	0.005	0.004	0.002	0.006	0.005
C24:0	0.026	0.021	0.019	0.024	0.020	0.022	0.031	0.007	0.021	0.031	0.003	0.016	0.022	0.023	0.030	0.021	0.013
C24:1	0.006	0.007	0.008	0.010	0.008	0.009	0.012	0.008	0.007	0.008	0.008	0.006	0.010	0.007	0.008	0.008	0.004

Table 26: Values of corrected FA peak areas (CON Q4- HAY Q1)

sample	Probe 108	Probe 109	Probe 112	Probe 113	Probe 20	Probe 21	Probe 22	Probe 23	Probe 24	Probe 25	Probe 26	Probe 27	Probe 28	Probe 29	Probe 3797	probe 3750	Probe 0956	Probe 2760
Feeding.Quartal	Con. Q4	Con. Q4	Con. Q4	Con. Q4	Hay Q1	Hay Q1	Hay Q1	Hay Q1	Hay Q1	Hay Q1	Hay Q1	Hay Q1	Hay Q1	Hay Q1	Hay Q1	Hay Q1	Hay Q1	Hay Q1
C18:3n3	0.895	0.873	0.573	0.594	0.950	0.833	0.811	1.006	0.734	1.053	1.010	1.040	1.046	1.293	0.928	1.009	0.989	0.970
2	0.078	0.073	0.067	0.069	0.091	0.081	0.081	0.038	0.086	0.101	0.104	0.087	0.087	0.098	0.091	0.089	0.102	0.088
3	0.078	0.116	0.123	0.127	0.205	0.171	0.170	0.153	0.153	0.211	0.192	0.173	0.181	0.209	0.177	0.159	0.208	0.164
4	0.026	0.026	0.022	0.023	0.016	0.016	0.016	0.021	0.028	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.010	0.005
CLAc9t11	0.838	1.048	0.771	0.732	0.713	0.012	0.700	0.804	0.758	0.807	0.812	0.800	0.802	0.915	0.858	0.815	0.862	0.773
6	0.050	0.008	0.005	0.008	0.019	0.017	0.010	0.009	0.009	0.008	0.009	0.009	0.009	0.009	0.019	0.005	0.015	0.013
7	0.088	0.117	0.053	0.045	0.031	0.014	0.020	0.053	0.037	0.036	0.044	0.032	0.030	0.031	0.033	0.033	0.049	0.030
8	0.034	0.047	0.029	0.025	0.024	0.018	0.018	0.049	0.020	0.022	0.028	0.023	0.020	0.024	0.030	0.027	0.033	0.029
9	0.012	0.018	0.008	0.019	0.013	0.013	0.018	0.012	0.016	0.010	0.016	0.014	0.014	0.013	0.017	0.010	0.009	0.011
10	0.065	0.085	0.055	0.052	0.034	0.064	0.041	0.027	0.047	0.044	0.040	0.039	0.038	0.053	0.046	0.045	0.044	0.042
11	0.090	0.118	0.064	0.100	0.178	0.192	0.094	0.068	0.103	0.086	0.095	0.088	0.079	0.087	0.084	0.059	0.058	0.063
C20:0	0.189	0.178	0.157	0.168	0.195	0.164	0.187	0.192	0.208	0.185	0.188	0.198	0.196	0.246	0.177	0.177	0.181	0.169
15	0.131	0.130	0.125	0.134	0.165	0.153	0.159	0.168	0.155	0.153	0.155	0.168	0.172	0.187	0.150	0.148	0.149	0.141
C20:1	0.052	0.051	0.049	0.053	0.060	0.055	0.058	0.064	0.054	0.055	0.055	0.052	0.056	0.050	0.070	0.051	0.060	0.056
17	0.015	0.003	0.005	0.015	0.007	0.010	0.015	0.008	0.014	0.016	0.006	0.016	0.012	0.007	0.003	0.004	0.007	0.011
18	0.008	0.016	0.009	0.012	0.008	0.011	0.008	0.011	0.011	0.009	0.009	0.007	0.019	0.011	0.011	0.005	0.010	0.008
C20:2n6	0.050	0.047	0.046	0.040	0.049	0.070	0.047	0.053	0.054	0.044	0.043	0.048	0.046	0.063	0.071	0.053	0.048	0.069
C20:3n6	0.042	0.043	0.077	0.064	0.064	0.105	0.074	0.064	0.077	0.058	0.046	0.065	0.064	0.080	0.062	0.067	0.054	0.078
C20:4n6	0.083	0.082	0.096	0.103	0.101	0.014	0.103	0.089	0.100	0.085	0.076	0.096	0.094	0.124	0.099	0.094	0.088	0.104
C20:3n3	0.020	0.019	0.008	0.010	0.018	0.011	0.011	0.012	0.014	0.019	0.021	0.021	0.021	0.026	0.016	0.019	0.017	0.021
23	0.023	0.024	0.025	0.015	0.013	0.023	0.023	0.015	0.009	0.016	0.015	0.016	0.016	0.022	0.039	0.018	0.018	0.023
C21:0	0.084	0.084	0.067	0.070	0.113	0.083	0.100	0.093	0.095	0.126	0.121	0.119	0.123	0.100	0.095	0.101	0.106	0.088
C20:5n3	0.083	0.084	0.059	0.066	0.097	0.088	0.080	0.100	0.076	0.110	0.103	0.101	0.101	0.130	0.090	0.095	0.100	0.086
C22:0	0.073	0.069	0.059	0.061	0.090	0.007	0.086	0.037	0.087	0.094	0.094	0.097	0.100	0.123	0.083	0.082	0.085	0.075
C22:1n9	0.016	0.015	0.003	0.002	0.007	0.018	0.007	0.011	0.006	0.007	0.006	0.008	0.009	0.006	0.018	0.011	0.007	0.009
28	0.003	0.016	0.006	0.000	0.019	0.050	0.017	0.014	0.012	0.020	0.021	0.016	0.018	0.027	0.033	0.010	0.034	0.030
29	0.023	0.032	0.037	0.014	0.057	0.037	0.051	0.018	0.056	0.060	0.065	0.055	0.056	0.077	0.038	0.032	0.085	0.035
C23:0	0.034	0.033	0.021	0.034	0.039	0.037	0.034	0.016	0.031	0.044	0.041	0.045	0.044	0.048	0.051	0.036	0.041	0.037
31	0.035	0.031	0.025	0.022	0.044	0.035	0.032	0.013	0.030	0.057	0.051	0.042	0.037	0.051	0.034	0.027	0.056	0.038
C22:5n3	0.089	0.086	0.067	0.022	0.087	0.006	0.077	0.085	0.078	0.097	0.086	0.092	0.091	0.113	0.042	0.075	0.095	0.075
C22:6n3	0.012	0.010	0.006	0.006	0.007	0.028	0.004	0.009	0.005	0.009	0.007	0.007	0.004	0.005	0.009	0.006	0.013	0.011
C24:0	0.024	0.021	0.021	0.007	0.025	0.009	0.029	0.025	0.029	0.028	0.028	0.032	0.034	0.040	0.012	0.056	0.016	0.017
C24:1	0.008	0.008	0.008	0.008	0.014	0.010	0.011	0.010	0.009	0.012	0.013	0.011	0.012	0.017	0.010	0.010	0.011	0.010

Table 27: Values of correctedFA peak areas (HAY Q1- Q3)

sample	Probe 1111	Probe 58	Probe 59	Probe 62	Probe 63	Probe 66	Probe 67	Probe 70	Probe 71	Probe 74	Probe 75	Probe 78	Probe 79	Probe 82	Probe 83	Probe 86	Probe 87	Probe 90
Feeding.Quartal	Hay Q1	Hay Q2	Hay Q2	Hay Q2	Hay Q2	Hay Q2	Hay Q2	Hay Q2	Hay Q2	Hay Q2	Hay Q2	Hay Q3	Hay Q3	Hay Q3	Hay Q3	Hay Q3	Hay Q3	Hay Q3
C18:3n3	0.993	0.957	1.004	0.846	1.045	1.029	0.993	0.766	0.902	0.934	0.971	1.052	1.113	1.110	1.162	1.069	1.106	0.910
2	0.104	0.101	0.110	0.046	0.102	0.133	0.095	0.088	0.089	0.088	0.061	0.069	0.079	0.126	0.092	0.076	0.081	0.084
3	0.210	0.203	0.216	0.127	0.170	0.253	0.161	0.153	0.151	0.150	0.147	0.117	0.133	0.269	0.156	0.130	0.137	0.141
4	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.036	0.015	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.017
CLAc9t11	1.039	1.399	1.463	1.363	1.578	1.145	1.368	0.940	1.164	1.654	1.889	1.635	1.862	1.357	2.124	1.782	1.868	1.350
6	0.010	0.009	0.014	0.000	0.010	0.011	0.008	0.010	0.022	0.010	0.010	0.020	0.011	0.005	0.024	0.010	0.010	0.009
7	0.029	0.071	0.067	0.066	0.073	0.052	0.060	0.039	0.054	0.057	0.073	0.088	0.109	0.072	0.100	0.083	0.088	0.067
8	0.019	0.038	0.024	0.030	0.036	0.031	0.034	0.021	0.032	0.035	0.051	0.045	0.051	0.044	0.031	0.049	0.051	0.039
9	0.013	0.018	0.012	0.007	0.013	0.014	0.013	0.011	0.016	0.017	0.024	0.012	0.018	0.013	0.013	0.020	0.014	0.015
10	0.059	0.065	0.068	0.063	0.065	0.073	0.078	0.057	0.065	0.074	0.109	0.084	0.087	0.101	0.096	0.063	0.063	0.078
11	0.122	0.111	0.165	0.043	0.098	0.117	0.096	0.093	0.119	0.123	0.077	0.111	0.148	0.110	0.134	0.148	0.134	0.115
C20:0	0.220	0.184	0.210	0.148	0.205	0.187	0.217	0.200	0.188	0.202	0.201	0.140	0.152	0.187	0.203	0.170	0.178	0.181
15	0.171	0.141	0.158	0.114	0.148	0.152	0.166	0.153	0.130	0.162	0.162	0.118	0.120	0.152	0.173	0.137	0.142	0.144
C20:1	0.055	0.053	0.062	0.048	0.055	0.055	0.055	0.057	0.043	0.064	0.070	0.048	0.045	0.055	0.055	0.045	0.045	0.049
17	0.012	0.014	0.016	0.013	0.010	0.018	0.014	0.014	0.005	0.011	0.017	0.016	0.014	0.018	0.016	0.016	0.009	0.011
18	0.011	0.011	0.017	0.005	0.009	0.025	0.012	0.014	0.011	0.009	0.008	0.008	0.007	0.021	0.010	0.010	0.009	0.011
C20:2n6	0.052	0.044	0.053	0.028	0.042	0.078	0.047	0.049	0.056	0.064	0.041	0.040	0.044	0.069	0.050	0.045	0.042	0.051
C20:3n6	0.049	0.052	0.058	0.041	0.048	0.066	0.068	0.082	0.061	0.061	0.070	0.064	0.062	0.066	0.072	0.068	0.051	0.077
C20:4n6	0.146	0.069	0.078	0.059	0.074	0.183	0.094	0.113	0.094	0.087	0.096	0.079	0.076	0.192	0.097	0.092	0.072	0.098
C20:3n3	0.021	0.013	0.021	0.022	0.010	0.016	0.013	0.011	0.016	0.014	0.017	0.017	0.017	0.025	0.019	0.018	0.018	0.016
23	0.049	0.018	0.027	0.026	0.019	0.020	0.028	0.019	0.022	0.032	0.022	0.025	0.023	0.049	0.035	0.028	0.029	0.026
C21:0	0.057	0.112	0.126	0.058	0.089	0.131	0.115	0.090	0.096	0.104	0.077	0.095	0.116	0.099	0.123	0.098	0.108	0.098
C20:5n3	0.076	0.100	0.105	0.070	0.065	0.105	0.100	0.075	0.084	0.082	0.091	0.080	0.094	0.116	0.099	0.087	0.092	0.081
C22:0	0.079	0.085	0.098	0.052	0.065	0.119	0.096	0.078	0.007	0.090	0.076	0.066	0.079	0.066	0.093	0.081	0.085	0.082
C22:1n9	0.010	0.003	0.005	0.005	0.010	0.009	0.006	0.005	0.010	0.008	0.007	0.010	0.013	0.008	0.013	0.019	0.004	0.003
28	0.017	0.006	0.006	0.003	0.032	0.013	0.006	0.008	0.023	0.017	0.003	0.003	0.015	0.020	0.003	0.017	0.023	0.016
29	0.038	0.025	0.019	0.011	0.017	0.078	0.025	0.052	0.004	0.038	0.015	0.020	0.038	0.094	0.021	0.003	0.038	0.004
C23:0	0.034	0.035	0.039	0.018	0.022	0.045	0.045	0.028	0.034	0.035	0.018	0.034	0.038	0.041	0.035	0.036	0.036	0.026
31	0.034	0.039	0.039	0.026	0.017	0.045	0.040	0.025	0.035	0.031	0.024	0.026	0.029	0.045	0.028	0.035	0.033	0.030
C22:5n3	0.083	0.089	0.090	0.069	0.061	0.116	0.106	0.073	0.033	0.074	0.086	0.086	0.095	0.130	0.104	0.098	0.106	0.022
C22:6n3	0.009	0.007	0.004	0.007	0.004	0.009	0.011	0.004	0.009	0.002	0.008	0.004	0.006	0.013	0.006	0.005	0.008	0.009
C24:0	0.025	0.023	0.025	0.014	0.025	0.031	0.032	0.022	0.003	0.028	0.019	0.025	0.019	0.028	0.024	0.015	0.025	0.006
C24:1	0.010	0.011	0.013	0.005	0.010	0.012	0.011	0.004	0.010	0.011	0.006	0.008	0.005	0.011	0.008	0.008	0.001	0.010

Table 28: Values of corrected FA peak areas (HAY Q3- Q4)

sample	Probe 91	Probe 94	Probe 95	Probe 98	Probe 99	Probe 102	Probe 103	Probe 106	Probe 107	Probe 110	Probe 111	Probe 114	Probe 115
Feeding.Quartal	Hay Q3	Hay Q3	Hay Q3	Hay Q4	Hay Q4	Hay Q4	Hay Q4	Hay Q4	Hay Q4	Hay Q4	Hay Q4	Hay Q4	Hay Q4
C18:3n3	0.729	0.983	0.936	1.017	1.129	1.038	1.078	1.033	1.197	0.745	0.779	0.905	0.845
2	0.082	0.088	0.088	0.081	0.090	0.083	0.137	0.084	0.093	0.077	0.074	0.085	0.079
3	0.150	0.170	0.152	0.144	0.163	0.133	0.262	0.164	0.169	0.131	0.129	0.152	0.143
4	0.024	0.008	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.014	0.008	0.008
CLAc9t11	1.138	1.694	1.602	0.962	1.041	0.895	1.307	0.837	0.964	0.721	0.779	0.920	0.887
6	0.009	0.009	0.010	0.006	0.010	0.005	0.010	0.005	0.005	0.014	0.005	0.007	0.005
7	0.045	0.071	0.058	0.046	0.052	0.038	0.074	0.035	0.043	0.040	0.050	0.036	0.034
8	0.026	0.045	0.034	0.024	0.032	0.025	0.041	0.027	0.031	0.024	0.027	0.026	0.022
9	0.011	0.015	0.017	0.008	0.007	0.008	0.009	0.010	0.011	0.012	0.010	0.013	0.008
10	0.067	0.085	0.079	0.060	0.067	0.065	0.098	0.059	0.065	0.058	0.072	0.067	0.062
11	0.095	0.095	0.110	0.072	0.058	0.067	0.109	0.065	0.063	0.088	0.076	0.070	0.052
C20:0	0.190	0.217	0.215	0.156	0.156	0.157	0.187	0.170	0.196	0.181	0.181	0.187	0.171
15	0.164	0.193	0.184	0.130	0.136	0.121	0.152	0.146	0.166	0.140	0.141	0.148	0.139
C20:1	0.067	0.069	0.063	0.054	0.046	0.050	0.055	0.052	0.051	0.051	0.052	0.058	0.053
17	0.023	0.020	0.007	0.021	0.014	0.005	0.019	0.003	0.002	0.007	0.022	0.020	0.005
18	0.017	0.009	0.010	0.011	0.008	0.007	0.026	0.006	0.010	0.007	0.008	0.012	0.007
C20:2n6	0.052	0.050	0.054	0.046	0.042	0.060	0.052	0.053	0.054	0.059	0.055	0.053	0.044
C20:3n6	0.085	0.075	0.082	0.082	0.053	0.075	0.066	0.036	0.056	0.075	0.066	0.059	0.061
C20:4n6	0.121	0.104	0.110	0.021	0.090	0.128	0.203	0.087	0.113	0.094	0.100	0.102	0.104
C20:3n3	0.010	0.020	0.014	0.030	0.024	0.026	0.016	0.018	0.020	0.013	0.015	0.017	0.016
23	0.018	0.022	0.020	0.023	0.019	0.016	0.038	0.020	0.025	0.013	0.023	0.024	0.020
C21:0	0.083	0.098	0.093	0.094	0.112	0.100	0.110	0.094	0.116	0.074	0.086	0.096	0.094
C20:5n3	0.069	0.080	0.076	0.074	0.104	0.085	0.116	0.096	0.116	0.070	0.075	0.087	0.086
C22:0	0.074	0.094	0.096	0.022	0.077	0.097	0.113	0.082	0.100	0.072	0.066	0.082	0.075
C22:1n9	0.016	0.014	0.025	0.011	0.010	0.010	0.019	0.017	0.006	0.020	0.015	0.008	0.006
28	0.004	0.003	0.035	0.019	0.018	0.038	0.007	0.025	0.012	0.015	0.028	0.017	0.011
29	0.035	0.021	0.038	0.025	0.055	0.028	0.052	0.040	0.049	0.019	0.038	0.029	0.029
C23:0	0.030	0.034	0.027	0.031	0.040	0.050	0.045	0.030	0.018	0.026	0.019	0.029	0.023
31	0.022	0.028	0.016	0.041	0.053	0.026	0.049	0.051	0.032	0.026	0.021	0.027	0.024
C22:5n3	0.078	0.087	0.069	0.092	0.109	0.018	0.121	0.109	0.097	0.077	0.076	0.083	0.086
C22:6n3	0.005	0.005	0.003	0.009	0.008	0.063	0.014	0.011	0.011	0.003	0.006	0.008	0.008
C24:0	0.028	0.031	0.051	0.010	0.014	0.007	0.056	0.012	0.015	0.027	0.040	0.026	0.027
C24:1	0.010	0.010	0.010	0.008	0.009	0.010	0.010	0.023	0.010	0.010	0.010	0.010	0.006

Table 29: Outlier- Test CLA (HAY)

Descriptive Statistics

Mean: 1.13407
SD: 0.42921
# of values: 45
Outlier detected? No
Significance level: 0.05 (two-sided)
Critical value of Z: 3.08542339826

Your data

Row	Value	Z	Significant Outlier?
1	0.712	0.98336	
2	0.012	2.61426	Furthest from the rest, but not a significant outlier (P > 0.05).
3	0.700	1.01131	
4	0.759	0.87385	
5	0.758	0.87618	
6	0.807	0.76202	
7	0.812	0.75037	
8	0.800	0.77833	
9	0.802	0.77367	
10	0.913	0.51505	
11	0.857	0.64553	
12	0.815	0.74338	
13	0.862	0.63388	
14	0.773	0.84123	
15	1.037	0.22615	
16	1.399	0.61726	
17	1.464	0.76870	
18	1.362	0.53105	
19	1.568	1.01100	
20	1.131	0.00714	