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Influence of dairy farming method and other production factors on lipid metabolism and quality parameters of cow's milk

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Abstract

Organic milk has been popularly developed in western dairy industry. This milk was believed to bring better health benefits than conventional milk and thus, receiving high attention from consumers in recent years. The chemical composition of organic milk versus conventional milk remains an attractive research direction in order to improve knowledge using advanced techniques such as metabolic profiling. On the other hand, milk constituents could vary with other production factors as well as processing parameters. The purpose of this study was to investigate the effects of production types together with other factors, such as season, geographical regions and fat content, on cow's milk composition, nutritional and potential sensory quality of the milk. The study included two phases of experiment. In Phase 1, organic and conventional semi-skimmed milk (1.2 – 1.8% fat) was collected during 5 periods from December 2012 to October 2013 from Norway, Germany, Sweden and Denmark. In Phase 2, organic and conventional whole milk (3.5 – 4% fat) and low fat milk (1.2 – 1.5% fat) were collected from June to October 2014 in Middle Norway. Lipid compounds and multi-elements were investigated in Phase 1 using ultra-performance liquid chromatography coupled with time-of-flight/mass spectrometry (UPLC-TOF-MS) and inductively coupled plasma-mass spectrometry (ICP-MS), respectively. In Phase 2, antioxidant activity and metabolites were detected and measured based on ferric reducing antioxidant power (FRAP assay) and gas chromatography-mass spectrometry (GC-MS), respectively.

The results indicated that production types had no significant influence on lipid compounds distribution, major metabolites, minerals as well as antioxidant activity. Concentration of a few metabolites (xylose, tryptophan, gluconic acid, capric acid and lauric acid) and copper (Cu) were found significantly different between organic and conventional milk. Besides, season had strong effect on distribution of lipid compounds as shown by principal component analysis (PCA). Level of major elements, except Selenium (Se), were significantly changed within a year with a remarkable drop in August. In addition, selenium level in milk was strongly affected by geographical regions. Whole milk had significantly higher antioxidant activity compared to low fat milk. Concentration of capric acid, lauric acid, palmitic acid, stearic acid, oleic acid, elaidic acid and cholesterol were significantly different between the whole and the low fat milk. Overall, the findings of this study provide a better understanding about potential factors which significantly affect milk composition and nutritional quality.

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Abbreviations

ANOVA	=	Analysis of Variance
CLA	=	Conjugated linoleic acid
CON	=	Conventional milk
ECO	=	Ecological/organic milk
F	=	Full fat
L	=	Low fat
FA	=	Fatty acid
FRAP	=	Ferric reducing antioxidant power
GC-MS	=	Gas chromatography-mass spectrometry
ICP-MS	=	Inductively coupled plasma-mass spectrometry
m/z	=	Mass–to–charge ratio
MeV	=	MultiExperiment Viewer software
MS	=	Mass spectrometry
MUFAs	=	Monounsaturated fatty acids
PC1 & PC2	=	Principal component 1 & 2
PCA	=	Principal component analysis
PUFAs	=	Polyunsaturated fatty acids
UHT milk	=	Ultra-high temperature processing milk
UPLC-TOF-MS	=	Ultra-performance liquid chromatography coupled with time-of-flight-mass spectrometry

1. Introduction

1.1 Why is milk an important food?

Milk is a complicated mixture of water, lipids, carbohydrates, proteins, vitamins and minerals which provides sufficient nutrients for newly born mammals (Damodaran and others, 2007). Milk is a valuable food source which is suitable for all age group (Schönfeldt and others, 2012). It brings us a high nutrition and tasty food. Milk can be derived from various mammal species such as cow, goat, ewe and mare (Jahreis and others, 1999). (Damodaran and others, 2007) stated that cow's milk has been used as the most popular source of milk from husbandry and become the main source of milk for human consumption, especially in the Western world . Besides, dairy products such as cheese, yogurt, butter, casein, ice cream require a large amount of milk in the production. Thus, milk is the most important raw material in dairy production and needs to be studied in-depth in order to provide essential information for the food industry.

The biosynthesis of milk constituents occurs in the secretory epithelial cells of the mammary gland. Metabolites from blood enter the cells and are used to produce the basic components of milk such as proteins, lipids and carbohydrates. Then, these milk ingredients are translocated to a storage place named lumen, which is a round chamber surrounded by epithelial cells. However, some milk ingredients, e.g. serum albumin and immunoglobulin, originate from blood and pass through the cells to arrive lumen, without being synthesized in the cells. When receiving a hormonal signal, the whole system of cells and lumen contracts to excrete the milk (Damodaran and others, 2007).

Milk is an important food which contains high level of nutrients, with various amount of fatty acids, essential amino acids, carbohydrates, vitamins and minerals (Laben, 1963). According to (Parodi and others, 2003), (Butler and others, 2007) reported that monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) found in milk have brought health benefits which decrease the risk of cancer and cardiovascular disease. These important unsaturated fatty acids include α-linolenic acid (α-LA; an omega-3 fatty acid), conjugated linoleic acid (CLA; an omega-6 fatty acid) and vaccenic acid. CLA has been reported due to its potential in preventing atherogenesis and diabetes (Dhiman and others, 2005). Besides, researches have shown that milk fat consumption is negatively correlated to asthma and allergies in very young children (Kusche and others, 2015; Wijga and others, 2003). Cow's

milk contain higher total content of minerals (approximately 7.3 g/L) than human milk (2g/L) Fat-soluble antioxidants found mostly in milk are α-tocopherol (vitamin E) and β-carotene (precursor of vitamin A). The other vitamins in milk include D, riboflavin, pantothenic acid, biotin and nicotinic acid which are stable during pasteurization or ultra-high temperature processing (UHT milk) (Damodaran and others, 2007). Thus, milk is composed of various types of vitamins and antioxidants, providing consumers with a food rich in health-beneficial components.

Milk is a source of protein which offers specific nutritive values. It supplies 30-36 g total protein/L and comprises essential amino acids which cannot be naturally synthesized by human body but have to be absorbed from diet. Two major types of bovine milk protein are casein (80% of milk protein) and whey protein. The casein exists in milk as a spherical complex in combination with calcium phosphate, known as casein micelle, and is the major protein component in cheese. The other important protein fraction found in milk is whey protein (Damodaran et al., 2007). Whey protein contains serum albumin and immunoglobulin originated from blood and being transferred to milk, making milk a good food for babies. In addition, whey protein concentrates is utilized as dietary supplement which has approved functionality and nutritional values. It is documented that whey protein promotes muscle protein synthesis by providing essential amino acid, e.g. leucine, which are necessary for protein metabolism. Therefore, whey protein is popularly used by those who attend physical training and sport activities (Ha and Zemel, 2003). Consequently, milk supplies a qualified source of proteins and essential amino acids.

Another major component of milk is lipid (milk fat). In bovine milk fat, triacylglycerols (triglycerides) comprise 95.8% of the total fat weight in whole milk (3.2% fat content). The other fat components include diacylglycerols (diglycerides), monoacylglycerols (monoglycerides), free fatty acids, phospholipids and cholesterol (Damodaran and others, 2007; Jensen and others, 1991). Based on Wisconsin Milk Marketing Board in 1988 (Grummer, 1991), a ratio of 8% saturated fatty acids, 10% PUFAs, and 82% MUFAs is reported to provide a perfect nutritive value for milk. The fatty acid molecular structure is indicated by the ratio of carbon chain length : number of double bond (e.g. C16:0, C18:1). Milk lipid shows a complex composition because of the presence of fatty acids having an odd number of carbons in a straight chain which does not usually happen in other natural food source. Besides, dairy fats

have been found to contain short-chain fatty acids (<14 carbons), which display a diversity of fat components in milk (Damodaran and others, 2007).

Lactose is the principle carbohydrate in human and cow's milk (Fusch and others, 2011). The lactose content ranges from 4.6 to 4.8 g/100 g milk based on the cow breed (Schroeder, 2012) and reaches 5.26 g/100 g in whole bovine milk produced in USA (Schönfeldt and others, 2012). Lactose, together with chlorides, accounted for approximately 77% of the freezing point depression of cow's milk which contribute to the thermal sensitivity of the milk (Dharamarajan and others, 1950; Shipe, 1959). Because milk is the only natural source of lactose, lactose intolerance is related to consumption of milk and dairy products. Lactase (β-galactosidase) is responsible for the hydrolysis of lactose into its monosaccharides, i.e. glucose and galactose (Scrimshaw and Murray, 1988). Lactose intolerance happens due to deficiency of the intestinal enzyme lactase which results in milk intolerance in many people. The case is common in Asia and Africa, but not in European countries (Damodaran and others, 2007).

Beside nutritive values, the other quality aspects, i.e. sensory quality and safety, have also been related to the chemical composition. It is reported that some amino acids were responsible for the taste of food. L-arginine, L-histidine, L-isoleucine, L-phenylalanine and L-tryptophan cause a bitter taste, while other amino acids such as L-alanine, L-glutamic acid, glycine and L-serine contribute to sweetness of food (JoMarLaboratories, 2010). In addition, volatile compounds function in the odor of food, and the molecules such as carotenoids, anthocyanins and chlorophylls are responsible for food color (Coultate, 2009). The level of toxic residues and other undesirable components reflects the safety of food. Many studies showed that harmful heavy metals such as cadmium (Cd), chromium (Cr), lead (Pb) (Enb and others, 2009) and arsenic (As) (Licata and others, 2004), were detected at different concentration in cow's milk collected from various dairy farms in Egypt and Italy, respectively. Moreover, the level of pesticide and hormone residues are important criteria in the authentication of organic milk (Packer and Dalmia, 2013).

1.2 Production type and environment affect milk composition

Several factors which may responsible for the variation of milk ingredients have been reported in many reports and publications. The internal factors were genotype (breed), age of dairy cow, stage of lactation and the external factors included feeding regime, seasonal and regional

effects, milking frequency and milking systems (Dangour and others, 2009; Lindmark-Månsson and others, 2003). This section will provide an introduction to the factors which are mostly discussed in recent researches.

1.2.1 Ecological versus conventional farming methods

Dairy manufacturer are trying to modify milk properties to adapt to large-scale customer demands. By adjustment of the milk composition, many types of milk such as “fat free”, “low fat”, “vitamin D added”, “lactose tolerant” are being produced, beside the conventional milk. The organic (or ecological) milk is one of these products.

The term organic milk refers to milk which is produced by organic agriculture. Production of ecological milk has become a focus area in dairy industry which highlights environmental issues during the production activity and follows current environmental friendly trends worldwide. During the period from January to October, 2011, there was a noticeable increase in consumption of organic whole milk by 17% and organic low fat milk by 15% in US (Packer and Dalmia, 2013). In European countries, specifically France, Germany and Austria, the ecological milk got significantly higher price than did the conventional milk from 2004-2009, and also faster in the rate of increasing price, according to European Commission-Farm Accounting Data Network (FADN)(EuropeanCommission, 2013). The literature showed that because the consumers were willing to pay more on ecological product, the organic milk farms gained higher margin per unit of production. The ecological milk is also popularly produced in Norway at the current period. A large number of Norwegian milk farmers started the green production from late 1980s, and a remarkable increase in volume of organic milk was recorded in 1999 (Larssæther, 2011; Stø and others, 2005). The manufacture of organic milk in Norway has been developed with financial support by the government and the dairy company Tine (Flø, 2003; Larssæther, 2011) and various milk products were generated, from the first green packaged low fat milk to ecological cheese and others. The consumer demand continuously promotes the development of organic milk because organic products are believed to have higher nutritional value, be safer and more wholesome (Bergamo and others, 2003).

Ecological production of milk requires farmers to follow specific conditions, and these are slightly different between countries. In USA, the compulsory grazing time is not less than 120 days per year and pasture grass encounters at least 30% of animal feed. Besides, the organic milk production has to follow strictly rules about the use of antibiotics, bovine growth hormone

and pesticide. Antibiotics are used only at a limited scale and once the treatment is applied in a continuous time, the dairy production is postponed until receiving an antibiotic-free certificate. The other rules are the inhibition of bovine growth hormone used for dairy cow and insecticide on the ecological farm (Packer and Dalmia, 2013). In Brazil, the principles for agri-ecological dairy production were applied, including optimizing endogenous resources (pasture), intensive use of solar energy, environmental protection, economical sustainability, respecting farmers' culture and animal welfare. Therefore, organic farming is prohibited to use insecticides, antibiotics, anti-parasitic agents, chemical inputs or genetically modified organisms (GMO) (Kuhnen and others, 2014).

However, several research questions are related to production of ecological milk. Is there any difference in nutrition and flavors between the organic and conventional milk? Does the organic milk bring additive value to the consumer in terms of quality?

Farming methods involved in the factors affecting milk quality as reported in several studies. The amount of total phenolic compound in milk from ecological production was lower than those from conventional system, particularly in summer and autumn (Kuhnen and others, 2014). Besides, the fatty acid concentration and milk yield were different between organic and conventional dairy farm. Bulk tank organic milk contained higher saturated fatty acid content which was considered as negative impact on health (Adler and others, 2013). However, it also contained a significantly higher level of healthy compounds such as mono- and poly-unsaturated fatty acids as well as fat soluble antioxidants, compared to the conventional milk (Butler and others, 2007). In addition, the level of hippuric acid in organic milk were found to be higher than conventional cow's and goat's milk (Boudonck and others, 2009; Carpio and others, 2010; Packer and Dalmia, 2013). The findings of these studies suggested that composition of milk may vary by different farming methods. Thus, further research on effect of farming methods on milk composition, particularly in relation to health benefits, should be performed with the goal of improving its nutritional quality.

1.2.2 Seasonal variation

Regarding the influence of production time on milk quality, research has shown differences in milk ingredients between seasons (Schönfeldt and others, 2012). A seasonal effect was found on 90 compounds in Swedish dairy milk in a study performed from November 1995 until November 1996. Firstly, there was a significant difference in content of proteins and amino

acids around the year. The concentration of total protein and particularly, the amount of casein, whey proteins and β-lactoglobulin A (g/100 g milk), were significantly different among the periods of production. For the amino acid concentration, the variation was also displayed in most of the amino acids composition, except for proline and tyrosine. Secondly, nitrogen compounds in Swedish dairy milk were effected by seasonal factor, and this was clearly shown for non-protein nitrogen and carnitine ($P < 0.001$). In addition, the research also indicated high variation with regard to lipids composition such as free fatty acids, cholesterols, phospholipids and sphingomyelin. The total content of monounsaturated fatty acids (cis) and that of polyunsaturated fatty acids (cis) were found to be significantly different within the year. Besides, the amount of essential fat-soluble vitamins in milk such as vitamin D, α-tocopherol, vitamin K and vitamin A (retinol and β-carotene) clearly showed seasonal differences. The investigation on the water-soluble vitamins composition also proved significant differences regarding vitamin content, excluded vitamin C. Moreover, the effect was clearly displayed in other mineral and trace elements such as zinc (Zn), copper (Cu), iron (Fe), phosphorus (P), potassium (K), sodium (Na) and calcium (Ca) (Lindmark-Månsson and others, 2003).

The influence of production time on variation of chemical compounds in milk was also reported in several dairy studies, with particular focus on summer (outdoor period) and winter (indoor period). The dairy cow received different types of diets according to seasons. In the summer, they were pastured or fed fresh-cut grass when they were in the cowhouse. In contrast, hay was used instead during the winter period. Silages of grass/clover and maize as well as supplemented concentrates were optionally given in both periods (Kusche and others, 2015). The majority of fatty acids were affected by seasonal factor in Spain, especially the unsaturated fatty acids (except C18:1). Regarding the chain length of fatty acids, the concentration of both short (C4:0-C10:1) and medium chain (C12:0-C17:0) fatty acids in milk produced in summer was higher than that in winter (Alonso and others, 2004).

Because a large number of milk components might change their concentration over time, a study on seasonal variation in milk from Norway and neighbor countries would provide necessary information to assess the milk quality in recent years.

1.2.3 Geographical variation

Apart from feeding type and season, the milk ingredients alteration is also related to geographical parameter. The fatty acids C4, C14, C16, C17, iC17, C18:0, C18:2, C18:3 and

conjugated linoleic acid were significantly affected regarding geographical areas ($p<0.01$) in cow's milk in Spain (Alonso and others, 2004). (Collomb and others, 2002) stated that there was a significant difference in concentration of fatty acids in milk produced at lowlands, mountains and highlands of Switzerland. The amount of C18:2, conjugated linoleic acid, C18:1 *(trans)* were found significantly different at three vegetation sites. Milk fat in highland contained more monounsaturated fatty acids (oleic, elaidic fatty acids) and less saturated short- and medium- chain fatty acids, 4-16C atom, than that in lowland.

A study on concentration of fatty acid compositions of retail milk in USA indicated statistical differences in not only the fatty acids mentioned above, but also saturated fatty acids (i.e. C6:0, C8:0, C10:0, C12:0, C15:0, C20:0) and several unsaturated ones (i.e. C14:1, C16:1, C18:2 (cis-9, trans-11)) (O'Donnell-Megaro and others, 2011). In addition, the composition of milk also varies from country to country as reported by (Schönfeldt and others, 2012). In the report, there was a large difference in the content of lactose between Denmark and the United States of America (USA) whereas the difference was narrow among the three countries, i.e. United Kingdom (UK), South Africa (SA) and Australia-New Zealand (AUS-NZ), (4.70 - 4.80g lactose/100g whole bovine milk). Regarding the health benefits, conjugated linoleic acid (omega-6 fatty acid) (C18:2, cis-9, cis-12), an important fatty acid, varied in concentration under the effect of geographical difference. Hence, the literature has proved that the quality of milk is possibly altered due to regional variation.

1.2.4 Breeds of dairy cow

Many previous studies suggested that genetic difference is one of the factors causing the variation in milk composition from cow to cow. Breed types were known to affect milk fat concentration, especially the CLA and antioxidant content (Butler and others, 2008; Lawless and others, 1999), as well as omega-3 FA content (Ellis and others, 2006). The level of difference could be up to 15-20% of content between breeds of dairy cow (Butler and others, 2008; Dhiman and others, 2005; Jensen and others, 1999). Genotype was also associated with the changes in the amount of minerals in milk (Lindmark-Månsson and others, 2003). The selection of dairy cow herd is therefore important for specific purpose, e.g. CLA-high producing breeds. Current dairy cow breeds in Sweden are Swedish Red, White, and Swedish Friesian, while the most common dairy breeds in Norway are the Norwegian Red (94%) and the crossbreeds with Norwegian Red (4%) (Østerås and others, 2007).

1.3 Methods overview

Detection of chemical compounds in milk or animal feed can be carried out by several analytical methods. The most commonly used technique for authentication of ecological milk is isotopic ratio mass spectrometry (IRMS). However, literature also reported that the other methods which are chromatography-based such as liquid chromatography-mass spectrometry (LC-MS) or high-performance liquid chromatography (HPLC) are possibly applied (Packer and Dalmia, 2013).

The four major methods used in this study are ultra-performance liquid chromatography coupled with time-of-flight-mass spectrometry (UPLC-TOF-MS), inductively coupled plasma-mass spectrometry (ICP-MS), gas chromatography-mass spectrometry (GC-MS) and ferric reducing antioxidant power (FRAP). The UPLC-TOF-MS was applied to investigate lipid profile, ICP-MS for multi-elemental distribution pattern, FRAP assay for assessment of antioxidant activity and GC-MS for metabolic profiling. In this section, short introduction of these analytical methods and their application in milk-related studies so far is given.

1.3.1 UPLC-TOF-MS

Ultra-Performance Liquid Chromatography coupled with Time-of-Flight-Mass Spectrometry (UPLC-TOF-MS) is a technique for chemical analysis based on chromatographic performance. The UPLC is a recently developed analytical method with similar principles as high performance liquid chromatography (HPLC) but with higher speed, sensitivity and resolution (Swartz, 2005). The principle of how UPLC technique works is described according to (Waters, 2015). A UPLC machine includes major parts such as solvent (mobile phase) manager, sample injector, column chamber, detector and a computer to display the chromatogram. The sample after being injected is carried by the mobile phase. The mixture passes the chromatographic column and is separated to individually analyzed bands which are later detected and present in the chromatogram. The reason for bands separation during the flow through the column is that the components in mixture have different affinity to a stationary phase located inside the chromatographic column which make them move along the column with different speed. Those which are highly attracted to the stationary phase will move slower than the others and therefore, come out of the column later. This order provides a scale of retention time with different peaks in chromatogram. The height of each peak shows the concentration of a specific compound.

UPLC has been applied in some researches on quantification of milk ingredients. Fusch and others (2011) applied the UPLC-tandem mass spectrometry (UPLC-MS/MS) at the first time for measurement of lactose content in cow's and human milk. These milk samples were diluted without subsequent removal of proteins and fats. The samples were operated in negative mode and the detection of lactose was finished in 5 min. The method has advantages such as fast, sensitive and accurate in determination of the concentration of lactose in milk. In previous study, UPLC-TOF-MS was applied for the detection and quantification of veterinary drugs in milk (Stolker and others, 2008). The analytical method was appropriate for screening the veterinary drugs present in milk in the form of residuals. According to the criteria on maximum residual limit (MRLs), the method satisfied and validated in terms of repeatability, reproducibility and accuracy. LC-MS technique has been applied on analysis of biochemical composition in milk because of its capacity to detect a large number of metabolites (Boudonck and others, 2009). However, the application targeted only on lactose, veterinary drugs or specific constituents. Milk is a complex mixture of nutritional compositions as mentioned in section 1.1, particularly the lipid compounds. (Zhao and others, 2014) suggested that UPLC-MS could be sensitive and powerful technique to investigate lipid profile in disease, drug, food and other fields. Lipidomics in milk using UPLC-TOF-MS is therefore a potential method providing high efficiency and is applied in this study.

1.3.2 ICP-MS

The screening and quantification of multi-elements is important. According to (Ataro and others, 2008), the amount of an element transferred to and accumulated in food determines its level of toxic or health benefit. For examples, Cr and Mn are normally necessary but turn to toxic at a higher amount whereas Pb and Cd are naturally poisons even at low levels (Ataro and others, 2008; Martino and others, 2000; Onianwa and others, 1999; Underwood, 1977). The investigation on multi-elements in milk is therefore a key point to evaluate milk quality, both regarding nutritive value and safety aspects.

Inductively coupled plasma-mass spectrometry (ICP-MS) is a recently developed technique for elemental analysis of biological samples. Ions formed from elements in ICP plasma, are detected and quantified by mass spectrometry. The ICP-MS has been used in milk research at certain scale. (Martino and others, 2001) applied double-focusing ICP-MS for investigation of multi-elemental distribution patterns in human and cow's milk with different milk types (i.e.

whole milk, skimmed milk) and milk whey. Both essential and toxic elements which are important in milk such as Na, Ca, Mg, Al, Cr, Mn, Fe, Ni, Cu, Zn, Se, Sr, Cd, Hg and Pb were studied. This method was proved a useful analytical method which removed many polyatomic interferences by conducting the measurement at a suitable medium resolution. In another research, trace elements, particularly heavy metals (i.e. V, Cr, Mn, Sr, Cd and Pb) in raw cow's milk were quantified using ICP-MS technique. The obtained results showed agreement with the references used to evaluate the accuracy of the method (Ataro, McCrindle, Botha, McCrindle, & Ndibewu, 2008). Based on the applications of the technique in milk mentioned above, ICP-MS is used for screening and quantification of multi-elements in milk as part of this thesis.

1.3.3 FRAP assay

The antioxidant capacity of milk is important in terms of maintaining quality of the product. Casein, the major protein type in whole milk, is mainly responsible for the antioxidant activity of the milk, while vitamin C, uric acid and other hydrophilic antioxidants are the contributors to the antioxidant capacity (Zulueta and others, 2009).

Ferric reducing antioxidant power assay (FRAP) is a method developed by (Benzie and Strain, 1996). The purpose of the analytical method was to measure the antioxidant capacity at the first time in human plasma. After that, the method was modified and widely utilized in other research fields such as tea, vegetables and fruit (Chen and others, 2003). Ferric (Fe^{3+}) is reduced to ferrous (Fe2+) at low pH generating a blue color of which absorbance can be measured at 593 nm. The ability of a sample to reduce ferric to ferrous demonstrates the total antioxidant capacity. The amount of generated ferrous shows the antioxidant activity of the samples which is calculated based on the standard curve of the known concentration of ferrous ions. The FRAP assay has advantages being a fast, simple and highly reproducible method (Benzie and Strain, 1996), being widely applied to study the total antioxidant activity of over 3,100 foods, beverages, spices, herbs and supplements used worldwide (Carlsen and others, 2010).

FRAP has been applied to detect antioxidant capacity in milk and dairy products. The sensitivity of this method was recognized in pasteurized milk (Smet and others, 2008). (Chen and others, 2003) applied this method in studying antioxidant activity of bovine milk. Besides,

oxidative stability of UHT milk under impact of fatty acid composition and packaging conditions was researched using FRAP method (Smet and others, 2009).

1.3.4 GC-MS

Metabolomics is an important field of study with the purpose of assessing the quality of milk. The complicated nutrient ingredients in cow's milk can be detected and quantified by a method called gas chromatography/mass spectrometry (GC-MS). According to (Kataria, 2011), gas chromatography linked to mass spectrometry (GC-MS) is an analytical method for detection of individual compound in a testing sample. The gas chromatography functions to separate volatile compounds and mass spectrometry functions to detect them. Different molecules in the sample have different characteristics which is the basic principle for the separation, which occurs when they pass through a column in gas chromatography. The traveling time called the retention time is notified by the mass spectrometry. The ionized fragments which have the same retention time in gas chromatography are easily identified using their mass to charge ratio (m/z) in the mass spectrometry.

GC-MS was applied to determine pharmacologically active substances residues in milk with different fat content such as whole, half-skimmed, skimmed milk, which were originated from cow, goat and human. These substances mainly consisted of antibacterials, anti-inflammatories, antiepileptic and hormones (Azzouz and others, 2011). In addition, GC-MS was suggested in previous study as a powerful method to investigate metabolite profile in complex mixture, particularly in milk (Boudonck and others, 2009).

1.4 Aim of study

The individual effect of different production factors on milk composition has been described in many previous studies. However, these reports did not combine the three parameters, i.e. farming method (organic and conventional production systems), seasonal and geographical variation at once within one study. In addition, several papers considered two of these three factors, but only focusing on fatty acid composition. The present study was carried out with the purpose of investigating milk quality under impact of these production conditions, not only with respect to milk fat composition but also multi-elements, metabolite profile and antioxidant activity of milk.

The following main questions were established for this study:

Organic milk has been used widely and receives a higher price compared to conventional milk. However, can potential quality differences be detected by advanced analytical profiling methods?

How does nutrient composition change according to different production season, region, as well as processing by skimming?

The results of this project contribute novel and useful information about milk components and quality in Norway and neighbor countries in recent years. Besides, it potentially provides the dairy industry with a knowledge base on the effect of farming methods, region and season. The thesis was carried out in the framework of NFR project "Eco-values as product quality attributes in manufacturing of agricultural food ingredients"(NFR no. 207761), in cooperation with TINE Norwegian dairy company.

2. Materials and methods

The project included 2 different batches of cow's milk samples which were collected from December 2012 to October 2013 (Phase 1), and from May to October 2014 (Phase 2). In Phase 1 experiment, Ultra-Performance Liquid Chromatography coupled with Time-of-Flight-Mass Spectrometry (UPLC-TOF-MS) was used to investigate lipid profile and Inductively-Coupled Plasma Mass Spectrometry (ICP-MS) was applied to quantify multi-elements in the milk samples. In Phase 2 of the study, Ferric Reducing Antioxidant Power (FRAP) assay and Gas Chromatography coupled to Mass Spectrometry (GC-MS) were utilized to measure antioxidant capacity and investigate metabolite profiles of the milk samples, respectively. All of the chemical analyses were performed at Norwegian University of Science and Technology (NTNU), Trondheim, Norway.

2.1 Phase 1

2.1.1 Sample collection

In Phase 1, fresh semi-skimmed milk (1.2-1.8% fat content) samples were collected with regard to production types (ecological and conventional milk), production periods (5 periods) and production places (11 locations). Sampling of milk samples was carried out at five time points from December 2012 to October 2013 (**Table 2.1**). Production types and locations of sampling were illustrated in **Figure 2.1**. The samples were collected at eight different production locations in Norway and also consisted of samples from Germany, Sweden and Denmark. The Norwegian dairy plants are located in Ålesund, Sandnessjøen, Harstad, Sem, Sola, Trondheim, Oslo and Bergen, which were regionally grouped as North, Middle, East and West Norway, as indicated colors in **Figure 2.1**. A full description of milk samples in Phase 1 is presented in **Appendix 1**. In Phase 1 experiment, total 90 samples were represented by: Place of Production – Sampling Period – Type of Production (e.g, ÅLE-1-C, ÅLE-1-E, GE-2-C). Norwegian milk samples were labeled by the first three letters of location names (i.e. ÅLE, SAN, HAR, SEM, SOL, TRO, OSL, BER). Milk samples from Germany, Sweden and Denmark were labeled by two letters which are abbreviation of country names, i.e. GE, SW and DK, respectively. After one week of storage at 4°C, 15 mL of each milk sample, pooled from two milk cartons, was fresh-frozen and stored in a -80°C freezer (Dep. Biology, NTNU) and thawed at 4°C prior to chemical analysis.

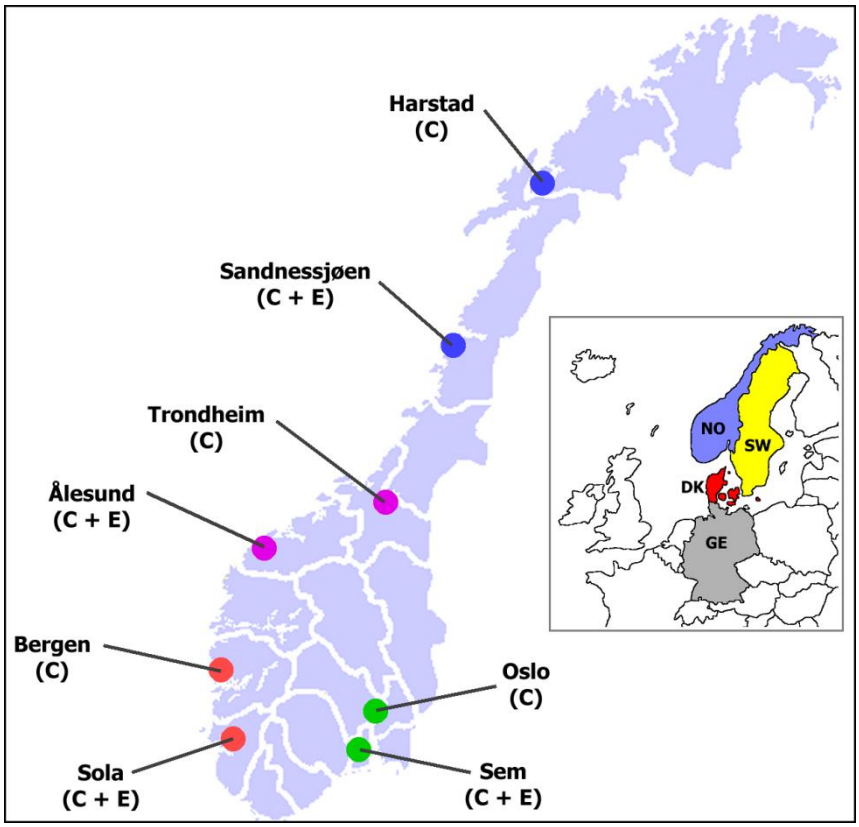

Figure 2.1: Locations and production types of milk collected in Phase 1 in Norway and other countries. The colored dots represent production regions in Norway (North, Middle, West and East Norway). Conventional and ecological milk are denoted by C and E, respectively.

Table 2.1: Milk sampling periods in Phase 1

Sampling period	1	2	3	4	5
Month	December	March	June	August	October
Date	03.12.2012	11.03.2013	10.06.2013	19.08.2013	14.10.2013

2.1.2 Lipid profiling

Ultra Performance Liquid Chromatography coupled with Time of Flight-Mass Spectrometry (UPLC-TOF-MS) was applied in the study in order to investigate the lipid profile of milk samples. The analysis was based on instrumental method for UPLC-TOF-MS instrument located at the joint MS Lab facility, NTNU, Norway. The chemicals CH_3OH and $CHCl_3$ were purchased from Merck Company, Germany. For extraction, 20µL sample was dissolved in 1mL ice-cold H2O: CH_3OH: $CHCl_3$ with volume ratio 1:2.5:1. The mixture was vortexed for 1 min

at maximum speed. After that, the mixture was left at room temperature for 10 min to let the protein precipitate. A centrifuge (Heraeus Biofuge Fresco, UK) was set up at 13,000 rpm at 4°C in 10 min. 750 µL aliquot of each sample was transferred to 1.5 mL glass vial and was kept in the fridge at -18°C prior to UPLC-TOF-MS analysis. Methanol was used as a control in this experiment.

A Waters Acquity UPLC I-class system (Waters, Milford, MA) coupled to a SYNAPT G2-Si HDMS QTOF-MS was used for untargeted lipid profiling. A CSH C18 column (2.1 mm × 100 mm, 1.7 µm particle size) (Waters, Milford, MA) was used. The mobile phase consisted of A: acetonitrile:H_2O (60:40), 10mM AA pH9; and B: acetonitrile:isopropanol (10:90), 10mM NH_3 0.1% FA, delivered at 300 µL min−1. A multi-step gradient was applied (10 min analysis time) to deconvolute lipid species: 0.5 min A:B 60:40, 2 min 30:70, 6.8 min 0:100, and 10 min 60:40. The QTOF-MS was operated in positive ion electrospray mode (+ESI) because only few compounds were being detected at negative mode in trial test.

Data processing was conducted using software named TransOmics™ Informatics for Metabolomics and Lipidomics (Nonlinear Dynamics/Water, Milford, USA) in order to detect, quantify and compare lipid levels in milk samples. The analysis included alignment, multivariate analysis and tentative identification of compounds based on Lipid Maps LMSD database, an available mass library of the software (LIPIDMAPS, 2015). In here, minimum peak width was chosen to be 0.2 min for peak picking limit.

2.1.3 Quantification of multi-elements

The purpose of using ICP-MS in this project was to investigate the multi-elemental distribution pattern in different types of milk. The elemental composition in milk was determined following a method by (Overjordet and others, 2015) at Dep. Chemistry, NTNU, with some modifications: Samples (2 mL) were pipetted into PTFE-vials and 3 mL concentrated nitric acid, HNO_3 (Scanpure, equal to ultrapure grade, Chem Scan, Elverum, Norway) was added. Digestion was carried out using a high-pressure microwave emitter (Milestone Ultra Clave, EMLS, Leutkirch, Germany) through a gradual temperature increasing from room temperature up to 250°C within 1 h. The digested samples were diluted with ultrapure water in acid washed polypropylene vials (BD Falcon 50 mL conical, BD Biosciences, Bradford, MA, US) to a final volume of 60 mL. The elemental composition (61 elements) was determined by high-resolution inductively coupled plasma mass spectrometry (HR-ICP-MS, Thermo Finnigan model Element

2, Bremen, Germany). Instrument settings are described in detail by (Sørmo and others, 2011). Four blank samples containing ultrapure water and HNO_3 were prepared in the same way as the samples.

2.2 Phase 2

2.2.1 Sample collection

In Phase 2 of the project, milk samples were collected with regard to production types (ecological and conventional milk), production periods (8 periods) and fat content (full and low fat). The milk samples were Tine Lettmelk (1.2 - 1.5% fat, conventional product), Tine Lettmelk (1.2% fat, ecological product), Tine Helmelk (3.5% fat, conventional product) and Rørosmeieriet Helmelk (4% fat, ecological product) **(Figure 2.2).**

These fresh milk samples were collected at local supermarkets in Trondheim, Norway every 3 weeks, starting from May to October, 2014 **(Table 2.2).** The milk was bulk milk and was manufactured by production plant in Middle Norway. Phase 2 experiment excluded consideration of geographical regions.

Figure 2.2: Four types of milk which were collected in Phase 2. From left to right, low fat – conventional, low fat – ecological, full fat – conventional and full fat – ecological milk. The cover of bottles of the low fat and full fat – conventional milk were changed from the date of sample collection, but keeping the same type of milk.

After purchase, milk was kept in a cooler with refrigerant ice gel pack. The samples were fresh-frozen at -80°C and stored at Dep. Biology, NTNU. Prior to each of biochemical assays, samples were thawed at 4°C. Total 31 samples as being presented in **Appendix 2** were labeled by Fat Content (Full fat (F) or Low fat (L)) – Sampling Period (1 - 8) – Type of Production (Conventional (C) or Ecological (E)); for example, F-1-C, F-2-E and L-2-C.

Table 2.2: Sampling period of milk in Phase 2

Sampling period	1	2	3	4	5	6	7	8
Date (in 2014)	15.05.	05.06.	26.06.	17.07.	07.08.	28.08.	18.09.	09.10.

2.2.2 Antioxidant activity

FRAP assay was applied to estimate antioxidant activity of milk in this study. The antioxidant capacity was determined by method by (Benzie and Strain, 1996) with slight modifications. The FRAP reagent was prepared from Acetate buffer pH 3.6 (3.1 g $C_2H_3NaO_2.3H_2O$/L, 16 ml $C_2H_4O_2$ /L, pH adjusted with acetic acid), 10 mmol/L 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) in 40 mmol/L HCl (stored cold), and 20 mmol/L $FeCl_3$. 6 H_2O (stored cold) in a volume ratio 10:1:1, respectively. Because the FRAP reagent quickly degrades, a mixture of three FRAP chemicals had to be made freshly and discarded after 2 hours. Modifications started from the following step. 5 µL of sample was mixed with 150 µL of FRAP reagent and was incubated at 37°C in 10 min in incubator (Termaks, Norway). 155 µL blanks were inserted to the first three wells of a 96-well plate and the mixtures of 5 µL of sample and 150 µL of FRAP reagent were added to the other wells. The absorbance was measured at 595 nm by microplate reader (Thermo Scientific / Labsystems Multiskan MS), starting with vigorous shaking for 10 sec, and using FRAP reagent as blanks. Results were recorded using Ascent Software, version 2.6 (Labsystems Multiskan MS, Helsinki, Finland).

Calibration curve was made from different concentrations of $FeSO_4 \cdot 7H_2O$, ranging from 1000 to 10,000 µmol/L. At low pH, the antioxidant capacity of milk was determined by Fe^{2+} generated from reduction of Fe^{3+} to Fe^{2+} in the presence of antioxidants in milk (Smet and others, 2009). Antioxidant activity is represented by equivalent concentration of generated Fe^{2+}

in milk, which is calculated from the standard curve based on Equation 2.1 and displayed in mmol/L. The standard curve and absorbance values of all samples are presented in **Appendix 3A** and **3C.**

Concentration of generated Fe^{2+} (mmol/L) = (Absorbance value – 0.0644)/0.0003/1000	**(2.1)**

Where absorbance values was the measured absorbance of samples. Division of 1000 in the equation was due to synchronize units (µmol/L to mmol/L).

2.2.3 Metabolite profiling

Gas chromatography coupled to mass spectrometry detector (GC-MS) is an analytical method used in metabolomics studies. The method helps to quantify and qualify the entire metabolome of specific biological samples (Gullberg and others, 2004). In this study, GC-MS was applied to assess the milk composition in order to identify freely-extractable metabolites in milk.

The method followed the technique of metabolite analysis described by (Rohloff and others, 2012) and (Sanchez and others, 2008). The chemicals were obtained from Sigma-Aldrich, USA. 250 µL milk was transferred into round-bottomed 1.5 mL Eppendorf tube. 875 µL ice-cold mixture of CH_3OH:$CHCl_3$ (with volume ratio 2.5:1) and ribitol (100 µg/mL) was added along with a vigorous shake. Ribitol was used as internal standard in this experiment. The sample was incubated at 60°C for 60 min with a release of internal pressure after the first 10 min and continuous incubation in the rest 50 min. Then, the sample was transferred directly to cooled centrifuge. The mixture was centrifuged at 13,000 rpm at 3°C in 10 min. The pellet at bottom of centrifuge tube was precipitated protein in milk. 700 µL aliquots from the clear supernatant (the polar phase) was taken to be dried in the Savant SpeedVac Plus (ThermoQuest, USA) for at least 16 hours without applying heat. Samples were stored at -80°C prior to derivatization.

To derivatize samples, 80 µL of cold 20 mg/mL methoxyamine hydrochloride in pyridine was added to each sample. The sample was incubated at 30°C in 90 min. After that, samples were added with 80 µL N-Methyl–N–(trimethylsilyl) trifluoroacetamide (MSTFA) and were incubated at 37°C in 30 min. The samples were transferred to 1.5 ml autosampler vials with glass inserts, and stored at 4°C prior to GC-MS analysis.

GC-MS quadrupole (Agilent Technologies, USA) was programmed for MSTFA with standard duration 60 min. The GC-MS syringe was cleaned with hexane and absolute ethanol prior to the running of samples. Separations were performed on an Agilent 6890/5975 GC-MS (Agilent Technologies, Palo Alto, CA) equipped with a HP-5MS capillary column (30 m × 0.25 mm i.d., film thickness 0.25 µm) (Agilent Technologies). Sample volumes of 3 µL were injected with a split ratio of 15:1. Injection and interface temperature were set to 230°C and 250°C, respectively. The GC temperature program was held isothermically at 70°C for 5 min, ramped from 70 to 310°C at 5°C/min, and finally held at 310°C for 7 min (run time: 60 min). The MS source was adjusted to 230°C and a mass range of m/z 70–600 was recorded.

Further data alignment and processing was achieved using MetAlign software (Rikilt, Wageningen, NL). Compound identification was carried out using available MS libraries. They were NIST05 spectral library (National Institute of Standards and Technology, Gaithersburgh, MD) in combination with the Golm Metabolome Database containing MS spectra of derivatized metabolites (Hummel and others, 2010). Automated Mass Spectral Deconvolution and Identification System (AMDIS) software was used to interpret GC-MS data. The result chromatograms show detected peaks at a relative retention time and mass to charge ratio (m/z). The peak which had the highest intensity and was typical for a compound was selected during peak picking. Besides, MS library search suggested several compounds at a given retention time in a GC-MS chromatogram. The selection of these compounds was based on the quality of matching between the data of the library and that of the samples, which was above 80% of matching.

2.3 Statistical analyses

Data profiles from UPLC-TOF-MS, ICP-MS and GC-MS were subjected to statistical analyses using MultiExperiment Viewer software (MeV). Student's t-test was applied to test for significant difference between two treatments (i.e. ecological and conventional production systems) as well as between whole milk and low fat milk. One-way Analysis of Variance (ANOVA) was used for statistical analysis regarding production periods and production regions. In addition, two-way ANOVA was applied to study the interaction effect potentially made by each pair of the three factors including production method, production time and production regions (Phase 1 experiment) or fat content (Phase 2 experiment). Besides, their mean and standard deviation were estimated. The same statistical analyses were performed in

Minitab® Statistical software (version 17) for data from antioxidant assay (FRAP results). In Phase 1 experiment analysis of samples was carried out once (n=1) due to large sample size (90 samples). However, chemical analysis was conducted in triplicates (n=3) in Phase 2 (31 original samples).

Typical composition in milk derived from mass spectrometry data profiles was selected for further discussion. Thus, Tukey's test for pairwise comparison using Minitab® was applied to point out differences between treatments ($P < 0.05$).

Principal components analysis (PCA), a multivariate analysis, was used to investigate the variance of lipid compounds in UPLC-TOF-MS dataset. The Minitab® software was employed in this statistical test.

Critical P-values were 0.05, 0.01 and 0.001 in order to show the level of statistical significance. When the calculated P-values were above or equal to 0.05, the results were considered as not significant.

3. Results

In this section, the main outcomes of the analytical methods will be presented in relation to the two experimental phases. In Phase 1 experiment, 90 milk samples were collected in Norway, Germany, Sweden and Denmark from December 2012 to October 2013. The UPLC-TOF-MS and ICP-MS technical analyses were used to investigate the lipid profile and multi-elements distribution pattern, respectively. In Phase 2 experiment, 31 milk samples were collected in Norway from May to October 2014. Two analytical methods used in Phase 2 were FRAP assay and GC-MS, which were applied to estimate the antioxidant activity and investigate metabolite profiles, respectively.

3.1 Phase 1

3.1.1 UPLC-TOF-MS

Based on UPLC-TOF-MS analysis, 205 mass peaks with specific retention time and mass-to-charge ratio (m/z) were detected. The data includes a total of 46 lipid compounds being identified, while other metabolites remain unidentified. The full data was statistically treated with Excel to obtain mean values and standard deviations, according to production type, production time and production location. Besides, the variation in concentration of the 46 lipid compounds between samples was investigated using principle component analysis (PCA) in which the most significant contribution to the variation derived from the first principal component (PC1), followed by the second PC (PC2). The result shows the loading plot of lipid and other compounds **(Figure 3.1).** Besides, results of samples are also displayed based on production type **(Figure 3.2),** period (**Figure 3.3**), countries **(Figure 3.4)** and domestic geographical regions within Norway **(Figure 3.5)**. One sample from Norway (BER-1-C) was excluded from these figures due to its outlier performance. The pattern, grouping and main trend of sample distribution based on lipid composition can be obtained from the following plots. The full list of lipid compositions in milk and their m/z ratio (lipids profiling) as well as chemical description is presented in **Appendix 4**.

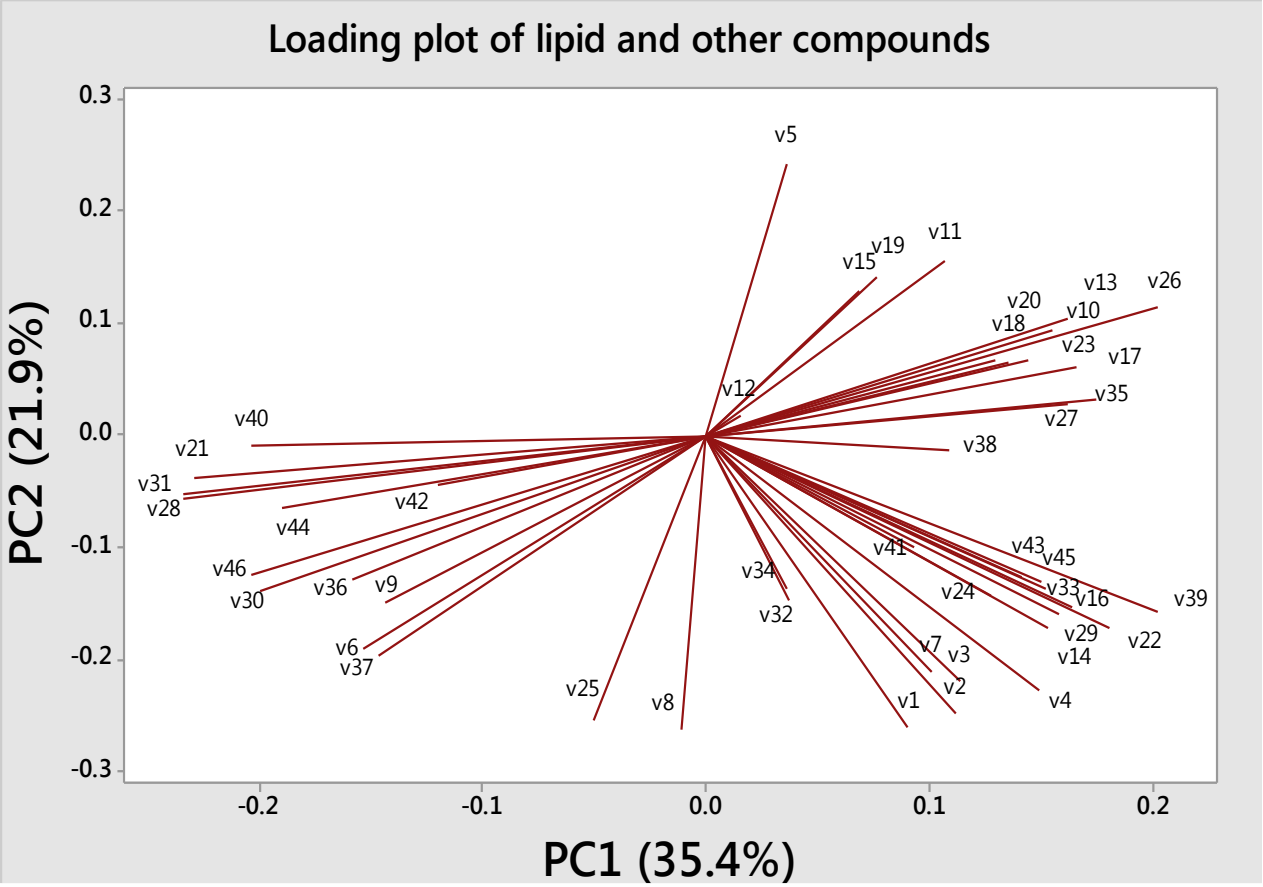

Figure 3.1: Loading plot from PCA of 46 lipid compounds in all milk samples. The name of lipids was replaced by v1-v46 due to the length of compound names. Details are presented in Table 3.1. The replaced names have been slightly moved for better visibility, keeping the same order.

The loading plot in **Figure 3.1** shows that PC1 contributes to 35.4% of the variation in the dataset, whereas PC2 contributes to 21.9% of the variation. The lipid compounds were assembled mostly on the negative side of the second component axis. In the opposite, they were distributed on both positive and negative sides along the first component which formed two groups on the plot. The lipids v12 (20:3 Cholesteryl ester) and v34 (1-O-alpha-D-glucopyranosyl-(2-hexadecanoyloxy)-eicosan-1-ol) were located closely to the center point which showed less effect on the variation between samples. Meanwhile, the lipids v26 (Coenzyme Q10), v39 (TG (14:0/16:1(9Z)/14:0) (d5)), v31 (13, 14-Dihydroxy-docosanoic acid) and v28 (18:1 Cholesteryl ester (d5)) had very long distances to the center point indicating their large contribution to variance of lipids among samples.

Table 3.1: List of lipid and other compounds found in milk, as being illustrated in Figure 3.1

Compound ID	Compound name
v1	Sulfoglycolithocholate
v2	LacCer(d18:1/12:0)
v3	PG(12:0/0:0)
v4	1-(6-[3]-Ladderane-hexanoyl)-2-(8-[3]-ladderane-octanyl)-sn-glycerophosphocholine
v5	Jurubine
v6	22:6 Cholesteryl ester
v7	PG(O-16:0/12:0)
v8	MG(20:0/0:0/0:0)[rac]
v9	Oceanalin A
v10	Anhydrorhodovibrin
v11	Bacteriorubixanthinal
v12	20:3 Cholesteryl ester
v13	34:6(16Z,19Z,22Z,25Z,28Z,31Z)
v14	Termitomycesphin A
v15	(-)-11-Hydroxy-9,10-dihydrojasmonic acid 11-beta-D-glucoside
v16	Ketospirilloxanthin
v17	2-Bromopalmitaldehyde
v18	CerP(d18:1/24:1(15Z))
v19	Depdecin
v20	C19 Sphingosine-1-phosphate
v21	DG(O-16:0/18:1(9Z))
v22	1-(2E,6E-phytadienyl)-2-(2E,6E-phytadienyl)-sn-glycero-3-phosphocholine
v23	TG(22:5(7Z,10Z,13Z,16Z,19Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))[iso3]
v24	Diketospirilloxanthin
v25	2-Arachidonoyl glycerol-d5
v26	Coenzyme Q10

v27	LacCer(d18:0/22:0)
v28	18:1 Cholesteryl ester (d5)
v29	Bacteriohopane-,32,33,34-triol-35-cyclitolguanine
v30	MG(18:0/0:0/0:0)[rac]
v31	13,14-Dihydroxy-docosanoic acid
v32	PA(O-16:0/O-16:0)
v33	N-ornithinyl-35-aminobacteriohopane-32,33,34-triol
v34	1-O-alpha-D-glucopyranosyl-(2-hexadecanoyloxy)-eicosan-1-ol
v35	1-(6-[3]-Ladderane-hexanoyl)-2-(8-[3]-ladderane-octanyl)-sn-glycerophosphoethanolamine
v36	PC(O-20:0/22:4(7Z,10Z,13Z,16Z))
v37	DG(13:0/13:0/0:0)
v38	Oleandomycin
v39	TG(14:0/16:1(9Z)/14:0) (d5)
v40	DG(18:2(9Z,12Z)/0:0/18:2(9Z,12Z)) (d5)
v41	27-Nor-5b-cholestane-3a,7a,12a,24,25-pentol
v42	N-stearoyl histidine
v43	3Z,6Z,9Z,12Z,15Z-Pentacosapentaene
v44	DG(21:0/22:3(10Z,13Z,16Z)/0:0)[iso2]
v45	1α-hydroxy-18-[m-(1-hydroxy-1-ethylpropyl)-benzyloxy]-23,24,25,26,27-pentanorvitamin D3
v46	8E-Heptadecenedioic acid

MG = Monoacylglycerol, DG = Diacylglycerol, TG = Triacylglycerol, PG = Phosphatidylglycerol, PC = Phosphocholine, PA = Phosphatidic acid, CerP = Ceramide-1-phosphate, LacCer = Lactosylceramide,

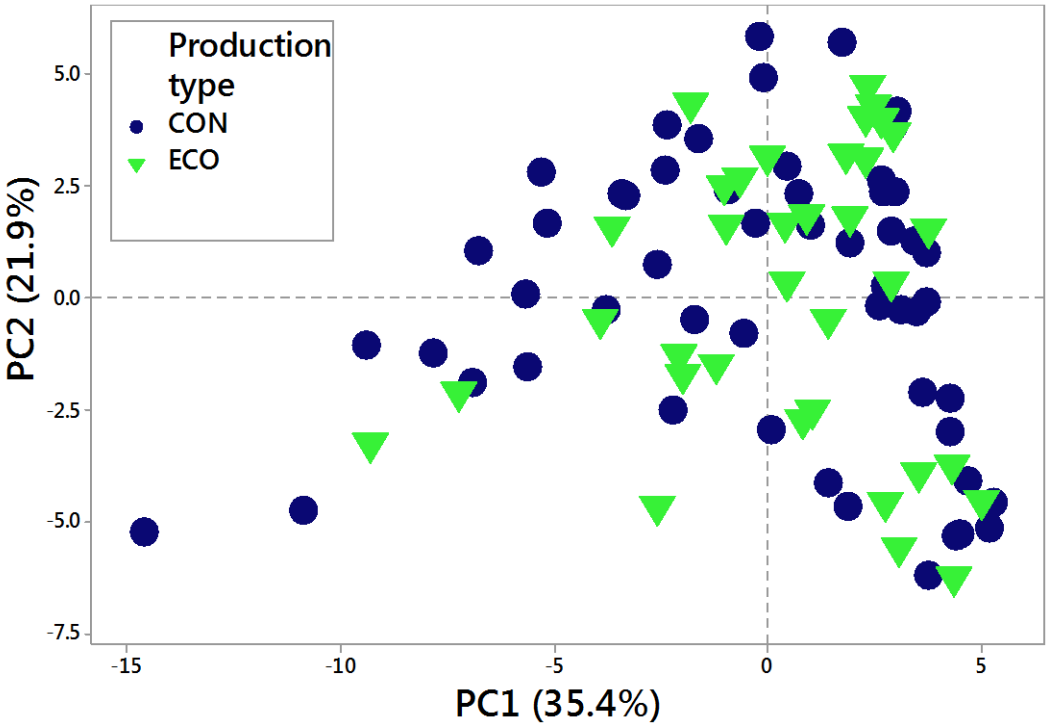

Figure 3.2: Score plot from PCA of lipid compounds in milk samples from Norway, Germany, Sweden and Denmark. The samples are colour grouped according to the production types, i.e. ecologically (ECO) and conventionally (CON) agricultural methods.

Figure 3.2 displays the variation in lipid components by a score plot of ecological and conventional samples. The samples are widely spread along both axes of PC1 and PC2. No distinct sample grouping pattern related to production type could be observed.

The score plot in **Figure 3.3** indicates that samples of period 1 and 2 (December and March) are clearly separated from those of period 4 and 5 (August and October) across the axis of PC2. Besides, there was a downward trend from period 1 to 5 as illustrated by the arrow in the figure. Samples of period 3 (June) were not clearly separated from periods 2 and 4. Superimposition of the loading plot (**Figure 3.1**) on the score plots in **Figure 3.3** revealed that group of compounds in the uppermost right corner (v5, v11, v26, and other lipid compounds) were mainly responsible for the variance of lipid compounds in samples of period 1. The variation between countries is displayed in **Figure 3.4**. The samples from Germany were clustered on the positive site of PC1 whereas samples from the other countries spread along PC1 and PC2. The group of compounds on the positive site of PC1 in the loading plot (**Figure 3.1**) may have a great influence on the variation of German samples.

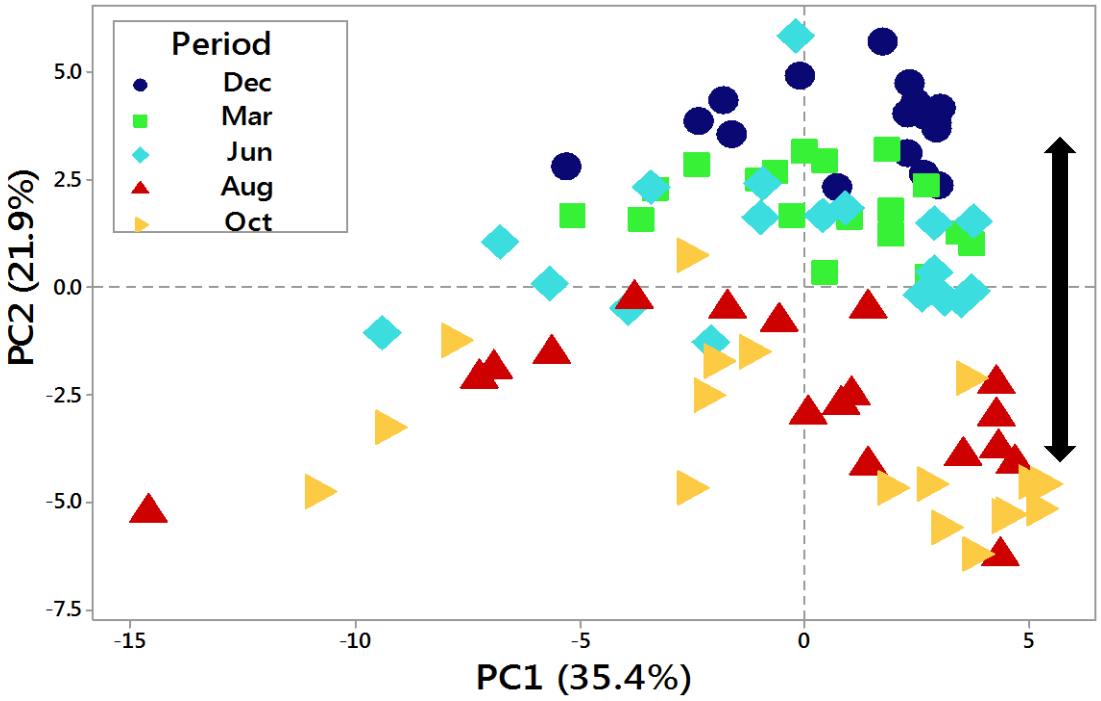

Figure 3.3: Score plot from PCA of lipid compounds in milk samples. The samples are colour grouped according to the production periods (1-5). The arrow indicates the trend of distribution of compounds.

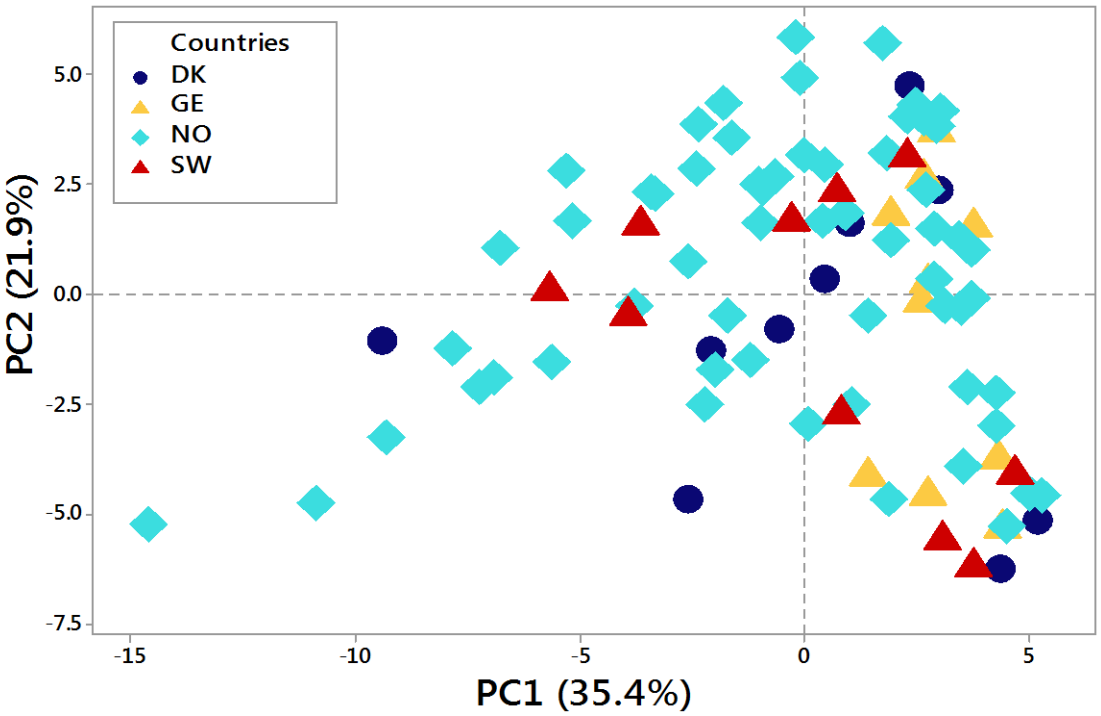

Figure 3.4: Score plot from PCA of lipid compounds in milk samples from Denmark (DK), Germany (GE), Norway (NO) and Sweden (SW).

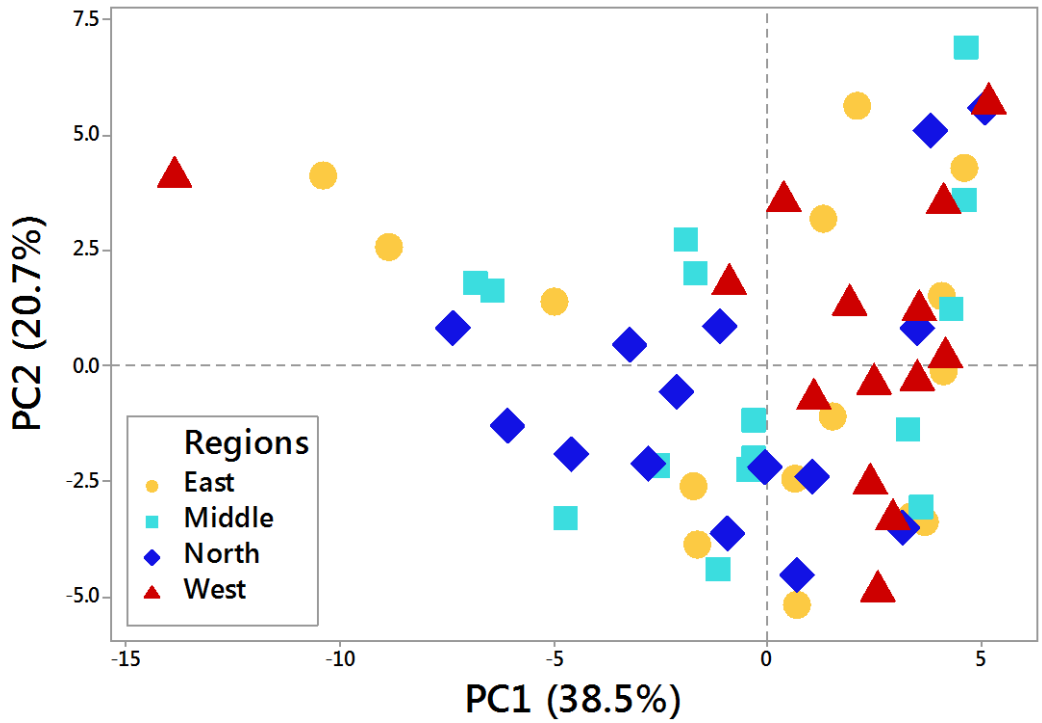

Figure 3.5: Score plot from PCA of milk samples from Norway. The samples are grouped by the production regions. The specific locations within each region are described in Materials and Methods.

The score plot in **Figure 3.5** represents only samples from Norway with regard to regional variation, showing that PC1 contributed to 38.5% of the variation in the dataset of samples whereas PC2 contributed to 20.7% of the variation. No clear grouping pattern could be found within the Norwegian samples. However, samples from North and West Norway were slightly separated from each other, forming weak clusters. Besides, samples from Middle and East Norway were evenly spread along the axes of PC1 and PC2.

3.1.2 ICP-MS

The ICP-MS analysis result includes the concentration of multi-elements in the milk samples. A total of 61 elements were detected in this study and corresponding ICP-MS profiles are presented in **Appendix 5**. Selected and nutritionally-relevant elements with regard to quantity and/or milk quality are displayed in the result section and further discussed.

The concentration of typical elements in semi-skimmed milk (1.2-1.8% fat) is shown in **Table 3.2**. The table contains statistical P-values of production types (ecological and conventional method) obtained using t-test, and P-values of production time (5 periods) and production places (four regions in Norway and countries level) by one-way ANOVA statistical test.

Regarding production type, no significant differences in the elemental concentration of Na, Mg, P, K, Ca, Zn, Se, Se and Fe between ecologically and conventionally-produced cow's milk could be observed, except for copper (Cu).

Table 3.2: Content of selected elements (mean value) in semi-skimmed milk (1.2-1.8% fat) including P-values according to the production types, period and places of milk production. All table values are based on 100 g of milk. P-values indicate significant differences: * P < 0.05; ** P < 0.01; *** P < 0.001.

			***P*-value**			
Elements	**Mean**	**Unit**	**Production type**	**Production period**	**4 Regions in Norway**	**4 Countries**
Na	34.97	mg	0.64	*	0.22	0.89
Mg	10.32	mg	0.12	***	0.14	0.46
P	91.01	mg	0.37	***	0.11	0.46
K	146.59	mg	0.99	**	0.24	0.73
Ca	104.02	mg	0.33	**	0.11	0.17
Zn	0.36	mg	0.57	***	0.06	0.29
Se	1.34	µg	0.99	0.09	*	***
Fe	13.72	µg	0.64	*	0.36	**
Cu	3.88	µg	*	***	0.09	0.81

In contrast to production type parameter, the concentration of all selected elements, excluding selenium (Se), was significantly different among five production periods, with higher level of significance (P<0.001) for elements Mg, P, Zn and Cu. The results indicate a significant effect of production time on the level of typical elements. In order to clarify differences between samples with regard to seasonal variation, Tukey's HSD test was applied and results are presented in **Table 3.3**. Major elements had the lowest concentration in August and elements Mg, P, Zn and Cu showed distinct results in this period as highlighted rows in the table. No significant differences in the level of elements (Na, Mg, P, K, Ca, Zn, Fe, Se and Cu) between the other four periods (Dec, Mar, Jun, Oct) could be found.

The four regions of milk production in Norway include North, Middle, West and East. The countries are referred to as NO, GE, SW and DK (Phase 1, sample collection). Firstly, the levels of Na, Mg, P, K, Ca, Zn and Cu in the milk were not significantly different among the domestic areas in Norway (NO) as well as among the four countries (**Table 3.2**). In contrast, element Se

was affected by geographical parameter, resulting in significant differences in the amount of Se in milk produced in different regions in Norway, as well as in the different countries. Regarding the Fe concentration, no significant differences were found within Norway, but among the countries.

Table 3.3: Amount of selected elements (mean and standard deviation) in 100 g semi-skimmed milk (1.2-1.8% fat content) according to period of production. Means within the same row which do not share the same letter(s), are significantly different.

	December	March	June	August	October	Unit
Na	34.95 ± *1.38* ^{ab}	36.41 ± *5.32* ^{a}	35.87 ± *3.07* ^{ab}	**32.84 ± *3.39*** ^{b}	34.78 ± *3.75* ^{ab}	mg
Mg	10.50 ± *0.49* ^{a}	10.85 ± *1.54* ^{a}	10.49 ± *0.76* ^{a}	**9.33 ± 1.*03*** ^{b}	10.43 ± *1.01*^{a}	mg
P	93.35 ± *4.46* ^{a}	94.82 ± *12.35* ^{a}	91.75 ± *4.82* ^{a}	**82.79 ± *8.25*** ^{b}	92.36 ± *10.81* ^{a}	mg
K	150.21 ± *6.93* ^{a}	153.92 ± *21.87* ^{a}	147.88 ± *10.47* ^{ab}	**135.95 ± *12.48*** ^{b}	145.01 ± *12.27* ^{ab}	mg
Ca	107.58 ± *4.87* ^{a}	108.5 ± *14.90* ^{a}	104.42 ± *5.90* ^{ab}	**94.62 ± *9.06*** ^{b}	104.95 ± *14.20* ^{a}	mg
Zn	0.37 ± *0.02* ^{a}	0.37 ± *0.05* ^{a}	0.37 ± *0.02* ^{a}	**0.33 ± *0.03*** ^{b}	0.37 ± *0.04* ^{a}	mg
Se	1.37 ± *0.23* ^{a}	1.44 ± *0.23* ^{a}	1.33 ± *0.22* ^{a}	**1.21 ± *0.26*** ^{a}	1.35 ± *0.27* ^{a}	µg
Fe	14.42 ± *0.91* ^{a}	13.77 ± *1.99* ^{ab}	13.61 ± *1.22* ^{ab}	**12.74 ± *1.11*** ^{b}	14.04 ± *2.08* ^{ab}	µg
Cu	3.95 ± *0.37* ^{a}	4.15 ± *0.61* ^{a}	3.88 ± *0.50* ^{a}	**3.39 ± *0.49*** ^{b}	4.04 ± *0.53* ^{a}	µg

3.2 Phase 2

3.2.1 FRAP

Antioxidant activity of milk (mmol/L) measured by FRAP method is shown in **Figure 3.5** according to production type (ecological and conventional production), production period (1-8) and the content of fat in milk (full fat and low fat). The antioxidant values of all samples including standard curve of FRAP assay are presented in **Appendix 3A** and **Appendix 3C**. The critical P-value was set at 0.05.

Regarding production type, no significant difference in antioxidant activity between the organic and the conventional milk could be observed.

The antioxidant activity of milk was slightly fluctuating throughout the season from May to October (Phase 2, sample collection). The milk antioxidant capacity continuously increased in the first three periods, reaching a top at period 3 (end of June) and then gradually decreased. Despite the fluctuation, no significant effect of production time on the antioxidant activity was found.

Regarding the fat content on the other hand, antioxidant measurements revealed that whole milk (red column) had significantly higher total antioxidant capacity than low fat milk (orange column).

The possibility of interaction effects was analyzed using two-way ANOVA statistical analysis, and the calculated P-values are shown in **Table 3.4**. The combination of the level of fat and the type of production resulted in a significant interaction effect ($P < 0.01$). In other words, significant differences in antioxidant capacity between the four investigated milk types, i.e. full fat – organic, full fat – conventional, low fat – organic and low fat – conventional milk were found. Referring to **Appendix 3B**, organic whole milk contains the highest antioxidant level, followed by conventional whole milk.

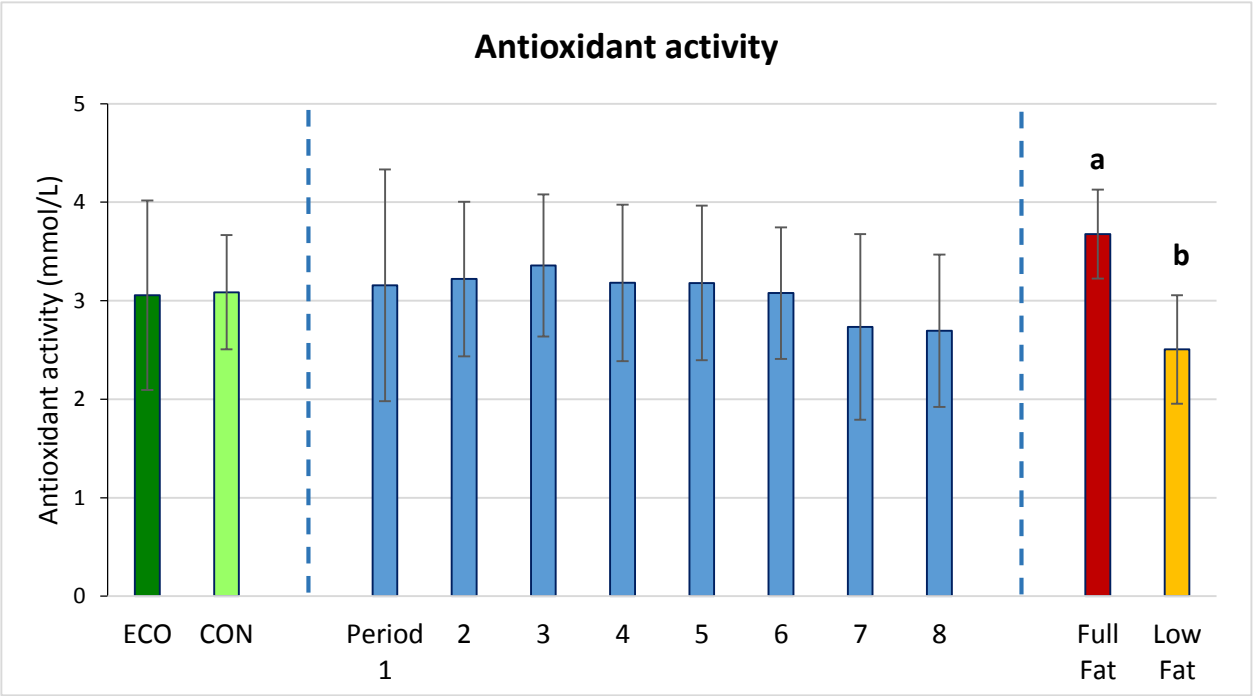

Figure 3.5: Antioxidant activity of milk (Mean ± standard deviation). Samples are grouped by color according to three individual effects, i.e. content of fat in milk (full and low fat), production type (ECO-ecological; CON - conventional) and production period (1 to 8). Different letters above the columns denote significant difference between factors.

Table 3.4: P-value from testing for interaction effect of pair of factors on the antioxidant activity of milk (two-way ANOVA)

Interaction effect	**Period × Production type**	**Fat content × Production Type**	**Fat content × Period**
P-value	0.936	0.001	0.882

3.2.2 GC-MS

Extractable metabolites in cow milk were identified using GC-MS analysis. A full list of metabolites and their estimated concentration in individual samples (metabolite profile) is presented in **Appendix 6**. Selected metabolites which are potentially related to milk flavor and quality taste are given in **Table 3.5**.

The medium chain fatty acids capric acid (C10:0) and lauric acid (C12:0) were found to show significant difference between the ecological and the conventional milk. In the sugars group, only xylose had significantly different values. Both tryptophan and gluconic acid performed

significantly different values. There were no metabolites with significant different values in other lipids and sugar alcohols group.

Regarding the seasonal variation, in the fatty acids group, heptanoic acid (C7:0) showed very strong significance (P<0.001). In the sugars group, all metabolites except fructose, glucose and maltose were found to be significantly different over periods of production. In the sugar alcohols group, only galactinol had significantly different value. In the amino acids group, aspartic acid, 4-hydroxyproline and pyroglutamic acid performed significant difference over production time. Many organic acids were been found to show significant differences according to period of production, including galacturonic acid, gluconic acid, glucuronic acid, 3-hydroxybutanoic acid, pyruvic acid, quinic acid and succinic acid.

Regarding fat content, most of lipid metabolites, except for heptanoic acid (C7:0), caprylic acid (C8:0) and myristic acid (C14:0), had significantly different values. In the sugars group, glucose and galactose showed significant difference between the full fat and the low fat milk. In the amino acids group, only lysine was found to be significantly different. Metabolites in both sugar alcohols and organic acids groups showed no significantly different value.

Table 3.5: Metabolites in milk (µg/mL) including mean values (n=3) and results of statistical analysis one-way and two-way ANOVA according to production type, period of production and fat content and their combination effects.

Metabolites	Mean (µg/mL)	*P*-value					
		Production type	Period	Fat content	Production Type x Period	Period x Fat	Production Type x Fat
Fatty acids							
Heptanoic acid (C7:0)	3.72	0.68	***	0.15	0.35	0.10	0.82
Caprylic acid (C8:0)	0.29	0.08	0.36	0.81	**	**	0.46
Capric acid (C10:0)	0.43	*	0.08	**	0.21	***	0.82
Lauric acid (C12:0)	0.44	*	0.34	***	0.11	*	0.67
Myristic acid (C14:0)	0.09	0.92	0.25	0.12	0.8	0.26	0.90
Palmitic acid (C16:0)	3.71	0.15	0.30	***	0.44	***	0.48
Stearic acid (C18:0)	2.24	0.78	0.20	***	0.39	0.11	0.38
Oleic acid (C18:1)	1.60	0.90	0.50	***	0.55	***	0.97
Elaidic (C18:1)	0.11	0.82	0.42	**	0.51	**	0.18

Table to be continued.

Table 3.5 continued.

Metabolites	Mean (µg/mL)	*P*-value					
		Production type	Period	Fat content	Production Type x Period	Period x Fat	Production Type x Fat
Other lipids							
Cholesterol	4.35	0.53	0.55	***	0.83	*	0.46
n.i. (sterol)	0.35	0.68	0.85	***	0.82	**	0.84
Sugars							
Arabinose	1.74	0.56	***	0.54	0.13	0.65	0.64
Erythrulose	0.18	0.31	***	0.47	*	0.73	0.27
Fructose	5.01	0.70	0.22	0.32	0.42	*	0.63
Galactose	5.23	0.52	*	**	0.16	0.09	0.73
Glucose	12.13	0.47	0.45	*	0.15	0.08	0.09
Lactose	17.07	0.63	*	0.25	0.34	0.25	0.84
Maltose	28.91	0.31	0.06	0.79	0.39	0.96	0.61
Maltotriose	1.86	0.86	***	0.84	0.38	*	0.98
6-Deoxy-mannose	0.18	0.08	**	0.74	*	0.05	0.42
Mannose	0.76	0.44	**	0.10	0.14	0.05	0.95
Ribose	0.35	0.33	*	0.17	0.16	0.75	*
Xylose	0.62	*	**	0.06	0.11	0.39	0.44
Sugar alcohols & polyols							
Galactinol	7.22	0.41	*	0.88	0.43	*	0.52
Galactitol	7.59	0.05	0.07	0.81	*	0.34	0.34
Glycerol	4.95	0.87	0.07	0.93	0.26	0.18	0.74
Myo-inositol	21.60	0.05	0.29	0.55	0.62	0.37	0.83
Scyllo-inositol	0.31	0.87	0.84	0.92	0.28	0.74	0.36
Amino acids							
Alanine	0.07	0.30	0.8	0.07	0.11	0.97	0.41
Aspartic acid	0.37	0.05	*	0.12	0.13	0.13	0.09
Glutamic acid	7.49	0.38	0.23	0.62	0.56	0.13	0.99
Glycine	1.40	0.54	0.40	**	0.40	0.38	0.98
Lysine	0.17	0.92	0.10	0.22	0.42	0.92	0.97
Phenylalanine	0.42	0.41	0.45	0.32	0.37	0.14	0.67

Table to be continued.

Table 3.5 continued.

Metabolites	Mean (µg/mL)	*P*-value					
		Production type	Period	Fat content	Production Type x Period	Period x Fat	Production Type x Fat
Proline	0.50	0.66	0.14	0.85	0.09	0.10	0.97
4-Hydroxyproline	0.43	0.58	*	0.47	0.09	*	0.46
Pyroglutamic acid	2.38	0.80	**	0.89	0.19	*	0.75
Tryptophan	2.07	**	0.40	0.63	0.70	0.72	0.57
Valine	0.10	0.70	0.07	0.48	0.10	0.09	0.64
Organic acids							
Citric acid	187.48	0.57	0.15	0.54	0.20	0.19	0.85
Fumaric acid	0.74	0.30	0.24	0.39	0.41	*	0.49
Galacturonic acid	0.44	0.10	***	0.84	0.19	0.69	0.49
Gluconic acid	0.82	*	**	0.11	***	**	0.11
Glucuronic acid	2.55	0.20	***	0.78	*	0.50	0.81
Glyceric acid	0.99	0.20	0.07	0.45	0.44	0.24	0.66
2-Hydroxyglutaric acid	1.89	0.16	0.16	0.81	0.16	0.13	0.83
3-Hydroxybutanoic acid	1.38	0.68	**	0.24	0.53	*	0.55
Itaconic acid	0.22	0.06	0.72	0.69	0.92	0.09	0.69
Lactic acid	1.26	0.06	0.19	0.61	0.06	0.20	0.47
Malic acid	8.10	0.58	0.49	0.38	0.20	**	0.18
Methylmaleic acid	0.37	0.23	0.31	0.91	0.81	0.7	0.68
Pantothenic acid	0.38	0.96	0.43	0.16	0.40	*	0.14
Pyruvic acid	0.13	0.24	***	0.42	***	**	0.05
Quinic acid	0.24	0.36	**	0.66	0.19	0.88	*
Succinic acid	1.76	0.58	*	0.88	0.24	**	0.96

n.i., Not identified

*, ** and *** correspond to levels of significant difference, $P<0.05$, $P<0.01$ and $P<0.001$, respectively.

Production type: organic and conventional farming methods

Fat content: whole milk (full fat) and low fat milk

Period: Production period (8 periods, Phase 2-sample collection)

4. Discussion

This chapter follows the same structure as Chapters 2 and 3, with section titles in the order of group of compounds analyzed by each analytical method. The discussion is finalized by a section emphasizing characteristics of milk composition and quality discussing nutritional and sensory aspects based on typical constituents of milk. A large number of metabolites and elements were detected from three analytical methods linked to mass spectrometry (UPLC-TOF-MS, ICP-MS and GC-MS). In Phase 1 experiment, the discussion focuses on three effects, i.e. production systems, season and geographical regions; in Phase 2 experiment, the discussion assesses the impact of three effects, i.e. production system, season and fat content on milk composition.

The study shows some limitations belonging to methodological points. Regarding the sampling in Phase 1 experiment, conventional milk had larger sample size (55 samples) compared to ecological milk (35 samples). In Phase 2 experiment, the number of conventional milk and ecological milk were 16 and 15 samples, respectively. Difference in sample size may slightly effect discussion on impact of production system on milk composition and quality in this study. Besides, comparison between regular milk and organic milk of the same production plant were not fulfilled because all locations produced regular milk but only half of them had ecological milk collected as in Phase 1 experiment (**Figure 2.1**, Materials and methods).

In Phase 1, the outcomes of chemical analyses may be affected by peak picking limits. Any ion that has eluted in less than "minimum peak width" (in here 0.2 minutes) is rejected. Therefore, some ions with a potential peak width less than 0.2 min may be lost in the result from UPLC-TOF-MS. Iodine, an important element related to milk quality, was not detected in elemental analysis by ICP-MS analytical method. The GC-MS analytical method which was used in this study, did not cover all of the metabolites present in milk. Therefore, some important compounds such as ω-3 and ω-6 fatty acids, several amino acids and metabolites were not considered in this study. Further investigations which are particularly focused on these milk constituents, should be carried out to fully assess the milk quality in Norway.

When seasonal effects are discussed, a suitable comparison to similar period in other countries is considered. According to the special climate in Norway, spring is from March to May, summer June – August, autumn September – November, and winter December – February.

4.1 Phase 1

4.1.1 Lipid compounds

Lipid compounds distribution was unaffected by different production types, which resulted in no distinct grouping pattern between organic milk and conventional milk **(Figure 3.2)**. However, this result was obtained by applying principal component analysis (PCA) on all periods, but not on individual production period. Further PCA was carried out to investigate any clear separation between organic and conventional milk of the same production period and results are shown in **Appendix 7**. Although the analysis was performed for individual production period, no clear separation between the two milk types could be found in this study. Samples of organic milk formed a small cluster while the conventional milk samples spread widely in period 1 and 2, but the two milk types were not separated from each other. Thus, no systematic difference was observed from distribution pattern of lipid compounds between the two milk types. The study could not find a separation between organic milk and conventional milk based on lipid compounds profile.

The score plot of PCA in **Figure 3.3** gave distinct results relating to seasonal effect. Pattern of grouping and main trend of lipids distribution were clearly observed. Samples of period 1 and 2 (December and March) are clearly separated from those of period 4 and 5 (August and October) forming two large groups of season. This showed a seasonal effect based on lipid compounds distribution pattern. Besides, samples of period 3 (June) were located at intermediate regions, forming overlaps area between the two groups due to transition state of seasons. Moreover, samples in December not only separated from the others periods but also clustered at the top of the score plot, indicating that samples of cold winter period do not share the same lipid compound distribution with the other seasons. The loading plot exhibited compounds which were important for classification of samples according to production period. The major lipid compounds contributing to the separation of samples of December were v5 (Jurubine), v11 (Bacteriorubixanthinal) and v26 (Coenzyme Q10), whereas compounds v30 (MG(18:0/0:0/0:0)), v39 (TG (14:0/16:1(9Z)/14:0) (d5)), v31 (13,14-Dihydroxy-docosanoic acid) and v28 (18:1 Cholesteryl ester (d5)) were responsible for the separation of samples of August and October from the other periods. The first two PC accounted for more than half (57.3%) of the variance of all samples, showing their large contribution to the overall dataset. The grouping pattern and main trend of sample distribution were clearly observed in the score

plot. The result from this study indicated that season had strong effect on overall lipid composition.

PCA was performed for samples with regard to four countries as presented in the score plot of lipid compounds included in the above result section **(Figure 3.4).** However, a discussion based on these data could cause a bias due to the higher number of samples collected from Norway compared with the other countries. Although the variation in lipid composition between the countries was not presented as part of this study, characteristic cluster patterns on one side of the plot were found for German samples. The loading plot indicated that glycerophospholipids, glycerolipids, prenol lipids and several fatty acyls located on right side of PC1, which had the longest lines from center point, mostly contributed to the variation in lipid composition of German samples.

Norwegian samples analyzed by PCA gave no distinct results relating to production regions. However, slight separation were observed for samples from North and West Norway. The samples from Middle and East evenly spread and overlapped samples from other production regions. PC1 and PC2 contributed largely (59.2%) to the variance of all Norwegian samples. Based on the results, milk produced from different regions in Norway had mostly identical variation in lipid composition, with slight difference between samples from North and West Norway.

4.1.2 Multi-elements

Minerals are important because of their functions in many metabolic processes and deficiency from diet causes serious symptoms. Essential minerals are classified into major elements and trace (or micro-) elements by concentration presented in animal body or required amounts from diet. Human requires trace elements in small amount which is less than 100 mg/kg diet. Farming animals obtain minerals naturally from plant feed or via supplement diet, and minerals are secreted into milk during lactation (McDonald and others, 2010). Influences of farming method, season and geographical region on level of mineral elements of cow's milk are discussed in this section.

Farming method had no impact on concentration of selected elements, except for Cu. The content of macro-mineral elements (Na, Mg, P, K and Ca) was maintained in both types of milk produced by organic and conventional methods. Besides, this group was found to be stable regarding geographical parameter, not only in Norwegian domestic regions but also among

four countries (Norway, Germany, Sweden and Denmark). Regarding the group of micro-mineral elements (Zn, Se, Fe and Cu), production systems showed effect solely on element Cu. Very few differences in term of mineral and trace elements composition was reported for organic and conventional milk (Hermansen and others, 2005). In the current study, Cu was found to be affected by production type. Element Cu could be a potential marker for discrimination of organic and conventional milk as being found in this study. Selenium (Se) concentration in this study was found to be not affected by farming methods. This finding was in disagreement with the result from previous study which reported the higher level of Se in organic milk compared with conventional milk in Norwegian farms (Adler and others, 2013). According to the study, the possible explanation for the difference was the higher Se concentration in fishmeal used on organic farm. In addition, organically bound Se had greater availability than inorganic Se in the diet and thus, was transferred to milk at higher efficiency. A large amount of trace elements (Fe, Cu and Zn) bind to casein protein fraction in cow's milk and concentration of these elements was significantly changed in the early of lactation stage (Fransson and Lönnerdal, 1983).

Season had strong impact on elements composition. Based on the result (**Table 3.2**), most of the elements, except for Se, significantly altered their concentration in milk throughout the year (from December 2012 to October 2013). This is in agreement with a study on concentration of minerals and trace elements which reported that seasonal variation resulted in significant differences of many elements, excluding Se (Sola-Larrañaga and Navarro-Blasco, 2009). The result from the current study was also similar with another study on milk from 28 dairy farms in central Norway, which stated that no significant difference in Se content was found between indoor and outdoor period (Adler and others, 2013).

Elemental concentration tends to drop in August, followed by an increase in October, as a general trend of all elements (**Table 3.3**). Level of Mg, P, Zn and Cu in August was significantly lower than the other four periods. Although no significant difference in concentration of Na, K and Ca could be found between December, March, June and October, these elements share the same trend of fluctuation around the year, with an increase from December to March, followed by continuously reduction in June until reaching the bottom in August. Element Fe showed a different trend of fluctuation which is a steady decrease from period 1 (December) to 4 (August). Se content remained stable throughout periods, proved by the fact that no significant difference among periods could be found for this element. Despite

the variation in fluctuation features, all elements were observed to have the same pattern from June to October with a drop in Aug. In this outdoor period, animals were fed fresh grass in cow-house or were allowed to graze. The remarkable fall in level of all elements in Aug may be explained by different reasons. Firstly, during pasture period, the cows, which normally adapt to indoor conditions, used extra amount of energy and nutrients for many voluntary muscular activities such as grazing, walking, climbing, etc. It was reported that the grazing animals have requirements for maintenance 25-50 % higher than housed animals (McDonald and others, 2010). Secondly, a large number of samples in Phase 1 experiment were from Norway (two thirds of sample size), and July and August are reported with higher temperature than the other months in Norway (Dannevig and Harstveit, 2013). Cattle respond to hot climate in summer by losing water and heat via sweating. In addition, they reduce amount of feed and energy intake because a large portion of body heat derived from metabolism of nutrients after meals (McDonald and others, 2010). The adaptation to warm weather possibly reduced minerals intake and thus, decreased mineral content secreted to milk via directly absorption from blood to mammary gland.

Trace element Se was not affected by farming method and season, but it was strongly influenced by geographical regions as in this study. The regional differences in Se content within Norway as well as between countries may be explained by soil characteristics of pastures and feeding regimes. Firstly, Se level in agricultural land soil was reported to affect grazing animals (NGU, 2008). This chemical element was found at different level in soil across European regions. Most parts of agricultural land in Europe have low level of Se, while coastal areas (i.e. Ireland, the south-western coast of Norway) have high level of this element due to a steady enrichment by marine aerosols. Beside, Se is directly absorbed from blood and secreted into milk by mammary gland (McDonald and others, 2010). The result of this study could be associated with geographical differences in Se content in blood samples collected from Norwegian dairy cow herds, which was reported in a previous study by (Sivertsen and others, 2005). The study also stated that blood Se level varied with the content of Se provided in feed. Regarding the intake of Se from diet, Se was supplied to dairy cow via concentrates and in form of mineral supplements. Standardized Se content added to concentrates for lactating dairy cows in Norway were 0.2 mg/kg (Sivertsen and others, 2005). However, the level given in feed could be up to 0.5 mg/kg dry matter according to EU recommendations in 1993 (Lindmark-

Månsson and others, 2003). Therefore, Se level in milk possibly varies from country to country due to different feeding regimes applied on this element.

All these findings suggested that level of major elemental compositions was strongly associated with season and they were independent on production types and production regions, except for Se.

4.2 Phase 2

4.2.1 Antioxidant activity

Antioxidant capacity expresses the ability of free radical scavenging. Milk naturally contains antioxidants in order to inhibit internal oxidation (e.g. lipid peroxidation) (Chen and others, 2003; Haug and others, 2007). The lower oxidation stability of milk is important in terms of nutritional value and sensory quality of dairy products, i.e. shortening shelf-life and inducing off-flavours. It is therefore essential to measure the antioxidant capacity of milk (Smet and others, 2009). In comparing organic milk to conventional milk, no significant difference was found between the two milk types in this study. This result indicated that antioxidant activity in Norwegian milk was not affected by farming methods. However, an interaction effect between farming methods and fat content showed significant level ($P < 0.01$) (**Table 3.4**), with distinct antioxidant activity of four types of milk, i.e. organic whole milk, conventional whole milk, organic skimmed milk and conventional skimmed milk (**Appendix 3B**) .

The antioxidant capacity was not significantly different over production period (May to October) as found in this study. Milk samples showed antioxidant capacity ranging from 2.29 - 3.85 mmol/L. (Kuhnen and others, 2014) reported a lower antioxidant level of crude milk in summer and autumn period, ranged from 0.15-0.17 mmol/L. The difference between Norwegian milk samples and that from previous study which was carried out in Brazil may be explained by different reasons. Milk from cow which was fed grass-clover silage contained higher level of polyunsaturated fatty acids than those fed with hay. This was suggested as a responsible factor that enhanced transferring efficiency of α-tocopherol from feed to milk (Havemose and others, 2006). Another study investigated effect of feed composition on antioxidant status of cow during mid to late lactation. Cow which was fed with supplemented dietary antioxidant increased antioxidant status via improving activity of glutathione peroxidase (Vazquez-Anon and others, 2008) which is an important enzyme in cellular antioxidant capacity.

Whole milk performed significantly higher antioxidant activity (3.7 mmol/L) than low fat milk (2.5 mmol/L) in this study. This result may be explained by removal of fat-soluble antioxidant components and other hydrophobic antioxidants due to separation of fat phase in processing of low fat milk. Both casein and whey protein were reported to be mainly responsible for antioxidant capacity of skimmed milk (Taylor and Richardson, 1980). The major contributors to total antioxidant activity of bovine milk were fat-soluble antioxidants such as α-tocopherol (vitamin E), β-carotene (precursor of vitamin A) (Smet and others, 2008); hydrophilic antioxidants such as phenolic compounds, vitamin C and uric acid (Kuhnen and others, 2014) ; and casein (a major protein in milk) (Zulueta and others, 2009).

In short, an interaction effect between production type and fat content was found with the higher antioxidant activity of organic whole milk. Milk was not affected by season, but it was influenced by skimming which removed several important antioxidants.

4.2.2 Metabolite profiling

The findings of this study suggested that farming methods had weak effect on metabolites. In this study five of selected metabolites showed significant differences between conventional and organic milk, including xylose, tryptophan, gluconic acid, capric acid and lauric acid. The level of xylose and tryptophan significantly decreased in organic milk. However, organic milk had higher level of gluconic acid than organic milk. The result of this study revealed that these metabolites could be potential marker classifying samples according to farming methods. Several metabolites which were reported as significantly different between organic and conventional milk in previous study did not show statistical significant value in the current study. These metabolites included mannose, ribose, proline and trans-4-hydroxyproline (Boudonck and others, 2009).

Regarding free fatty acids composition, the study found that ecological milk was characterized by significantly higher level of capric acid (C10:0) and lauric acid (C12:0) than the conventional milk. These results were in the agreement with the findings from previous study on bulk tank milk of 14 organic and 14 conventional dairy farms in central Norway in 2007 and 2008 (Adler and others, 2013). Oleic acid and elaidic acid, the cis- and trans- isomers of C18:1 (ω-9 fatty acids), were not affected by farming methods in this study. In the opposite, the previous study found that the organic milk contained lower level of oleic acid and higher level of elaidic acid, compared to conventional milk. In the current study, difference in fatty

acids composition might have been expected between the two production types because fatty acid content could be altered by different feeding regimes and composition of diet, as reported by many studies (Bergamo and others, 2003; Dangour and others, 2009; Kusche and others, 2015). However, the results of this study showed that minor number of fatty acids were found to be significantly different between organic and conventional milk produced during the outdoor period. This result could be explained by good culture conditions in Norway, where fresh grass fed to dairy cows in conventional farms was managed at as high quality as grazing in organic farm in outdoor period (May to October) (Rohloff, 2015; personal communication). Another explanation for low variation might be due to the fact, that only free fatty acids were detected by GC-MS, thus not covering glyceride-bound FAs.

Season had less effect on overall metabolites, but it showed wide effect on group of sugars. Concentration of a large number of sugar metabolites, excluding glucose, fructose and mannose, were significantly changed over production periods as found in this study. This could be explained by causes related to botanical composition in diet because the milk samples in Phase 2 experiment were produced in outdoor period when dairy cows were fed with pasture-based diet. Several studies found a seasonal variation in the quantity and quality of pasture. Immature pastures contained higher amount of soluble carbohydrate compared to mature pastures (Edwards and Parker, 1994). In a study on pasture nutrients from 1992-1994, (Parker and Edwards, 1996) reported that content of soluble carbohydrate varied within a year and had highest quantity in late autumn, winter and spring. These findings suggested a possible explanation for seasonal change in sugar content of milk in outdoor period.

Concentration of pyruvic acid was significantly changed over time ($P < 0.001$). Moreover, the result in this study showed that interaction between seasonal factor and either farming method or fat content caused significant effect on content of this metabolite. These findings indicated that pyruvic acid strongly depends on period of production. Pyruvic acid is an essential metabolite which takes part in synthesis of oxaloacetate and acetyl-CoA prior to Krebs cycle to generate energy in cell (Chien, 2000; Krebs and Eggleston, 1940).

As the result of this study, content of cholesterol, the sterol and most free fatty acids, excluding heptanoic acid, caprylic acid and myristic acid, were significantly higher in whole milk compared to low fat milk due to process of fat separation in skimming. This finding is in agreement with an earlier study of metabolite profile of bovine milk of different fat content,

which claimed that whole milk was distinguishable from low fat based on higher level of these lipid metabolites and 1,2-dipalmitoylglycerol (Boudonck and others, 2009). The result also indicated that interaction effect between period and fat level has strong influence on this compound group, except for heptanoic acid, myristic acid and stearic acid.

4.3 Characteristics of milk composition and quality

Due to the addition of a mixture of CH_3OH:$CHCl_3$ in preparation of GC-MS analysis, protein and large lipid molecules were removed from milk samples. The amino acids presented in this study were free amino acids which remained in samples. Therefore, the comparison with other studies on the amino acids content refers to free amino acids.

Density of whole milk and low fat milk collected in Phase 2 experiment were 1.03 and 1.035 g/mL, respectively (Charrondiere and others, 2012; Habberstad, 2015). In this section, it is assumed as 1 g/mL for convenience, and is not considered as a major effect during assessing the quantity of milk compositions.

Mean weight of tryptophan and valine in this study were 2.1 and 0.1 µg/mL, respectively. In Swedish dairy milk the average content of these amino acids were 0.01 and 0.04 µg/g (Lindmark-Månsson and others, 2003). The equivalent estimated concentration (w/w) of two amino acids in the current study were possibly higher than that in Swedish milk. The results indicate high nutritive level, but regarding the sensory aspect high content of tryptophan and valine might cause a bitter taste in milk (JoMarLaboratories, 2010).

Regarding sensory quality, several amino acids were reported to cause bitterness in food, especially when they were in L-form of chemical structure. Phenylalanine, tryptophan and tyrosine were bitter in taste. Other examples of bitter amino acids were leucine, isoleucine and valine (JoMarLaboratories, 2010; Lindqvist, 2011). In this study, phenylalanine, tryptophan and valine were detected at levels of 0.4 µg/mL, 2.1 µg/mL and 0.1 µg/mL, respectively. Taste threshold for these bitter amino acids were 1.1 mg/mL, 0.4 mg/mL and 0.4 mg/mL, respectively. Calculated values are based on values of detection threshold for individual amino acid (mol/L) (Schiffman and others, 1981) x molar mass (g/mol). The level of free amino acids in this study was much lower than the level of detection of bitterness, even though sensibility may vary between individuals. Therefore, it is assumed that the detected level of these amino acids did not contribute to a bitter taste of milk samples.

Element Ca has received high attention because low calcium intake is associated with risk of hip and vertebral fracture. Supplement of diet with high Ca in milk powder delayed bone loss (Lau and others, 2001). Element Ca in milk samples (1.2-1.8% fat) in this study had a concentration of 104 ± 0.01 mg/100 g milk. This value is lower than the content of Ca in semi-skimmed milk (1.2% fat) (134 mg/100 g milk) found in the Norwegian Nutrient Database (Matvaretabellen, 2014). However, the Ca level changed around year as illustrated in **Table 3.3**. This can be a reason for the difference between samples in Phase 1 which was produced in 2013 and the value in the database notified in 2014. Content of Ca in this study was also lower than that in Swedish milk which was collected from bulk tanks of dairy plants (114 mg/100 g milk) (Lindmark-Månsson and others, 2003).

Mean levels of typical macro-minerals (Na, Mg, P, K and Ca) in the present study were 35, 10, 91, 146 and 104 mg/100 g, respectively. The finding compared well with mean concentrations in cow's milk reported by (Cashman, 2006). The author reported that the levels were 53, 11, 89, 136 and 112 mg/100 ml (approximately mg/100 g) for Na, Mg, P, K and Ca, respectively.

Element Zn was found at 0.36 mg/100 g milk in this study. This level is in the same range of previous study which reported the content of Zn in raw cow's milk was 0.30-0.39 mg/100g milk (Enb and others, 2009).

Pantothenic acid (vitamin B5) was found in this study (Phase 2). The mean value of pantothenic acid in milk samples (0.38 mg/L) is equivalent to 12 % recommended dietary intake of pantothenic acid in children of 4-8 years old (3 mg/day) (Yates and others, 1998). Pantothenic acid is reported to be essential constituent in synthesis of coenzyme A, involved in fatty acid and acetate metabolism (McDonald and others, 2010). This result was found in Norwegian milk samples produced in the outdoor period.

In the UPLC-TOF-MS results, very long-chained polyunsaturated fatty acid (VLC-PUFAs, Cn>22) was found in milk, i.e. 34:6(16Z,19Z,22Z,25Z,28Z,31Z), an omega-3 fatty acid named 34:6 (n-3) (LIPIDMAPS, 2015). This lipid in milk samples (1.2-1.8% fat) was found is contrast to value of cis-poly unsaturated fatty acids in semi-skimmed milk (1.2% fat) (0g/100g milk) (Matvaretabellen, 2014), with information provided by Tine dairy industry (personal communication). Polyunsaturated fatty acids are reported to bring health benefits, but researches on VLC-PUFAs in milk had less attention at current time due to their minor abundance. This rarely found lipid and some VLC-PUFAs were reported to be associated with

retinal health and diseases, particularly age-related macular degeneration (AMD). Deficiency in VLC-PUFAs were stated to possibly participate in AMD pathology (Liu and others, 2010).

5. Conclusion

Production types had little effect on overall milk composition, but not the variation pattern of lipid compounds as shown in PCA. However, Copper (Cu) and 5 metabolites which displayed statistical difference between the two production systems could serve as potential biomarkers separating organic and conventional milk. A larger sample size in further investigation is recommended.

Based on the results from Phase 1 and Phase 2 experiments, season showed strong impact on overall milk composition, but not for antioxidant activity. Due to warm climate and change in culture conditions from indoor to pasture periods, the dairy cows possibly reduced feed and energy intake, and nutrients were lost for extra-muscular activities on grazing time which may affect their nutrient requirements for maintenance and production of milk. Therefore, the minerals secreted in milk obviously drop in August, compared to other periods. In the scale of this study it is suggested that the farmers may assist the cows by feeding them diet supplemented with minerals, except for Se, or reduce effect of heat on animal health by improving living conditions in cow-house or manage suitable time for grazing.

Geographical variation in relation to milk quality was one of the major factors investigated in Phase 1 experiment. A slight separation in lipid compound distribution pattern between Norwegian domestic regions was found. Selenium content was strongly affected by geographical changes both within regions in Norway and between countries.

Processing of fat content showed significant lower antioxidant activity in low fat milk, compared to whole milk, and strategies to enhance antioxidants level in skimmed milk are suggested. Besides, reduced concentration of most free fatty acids and cholesterol are potentially typical biochemical characteristics of skimmed milk.

The application of UPLC-TOF-MS and GC-MS analytical methods enabled detection of 46 identified lipid compounds and over 100 metabolites present in milk in this study although several ones remained unnamed chemical structures. These profiling methods have been widely applied in lipidomics and metabolomics in food research in recent years. These techniques could be useful in further investigation of milk composition and be modified in order to improve quality of analytical detection.

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APPENDIXES

Appendix 1: Description of milk samples collected in Phase 1 experiment

Total: 90 samples (frozen) of semi-skimmed milk (1.2-1.8% fat content)

Sampling period	**Period 1**	**Period 2**	**Period 3**	**Period 4**	**Period 5**
Date	03.12.2012	11.03.2013	10.06.2013	19.08.2013	14.10.2013
Sample no.	1.1,2.1,3.1,… 18.1	1.2,2.2,3.2,… 18.2	1.3,2.3,3.3,… 18.3	1.4,2.4,3.4,… 18.4	1.5,2.5,3.5,… 18.5

LOCATION	**TYPE**	**SAMPLE no***	**COUNTRY**
Ålesund	conventional	**1**	Norway
Ålesund	ecological	**2**	Norway
Sandnessjøen	conventional	**3**	Norway
Sandnessjøen	ecological	**4**	Norway
Harstad	conventional	**5**	Norway
Sem	conventional	**6**	Norway
Sem	ecological	**7**	Norway
Sola	conventional	**8**	Norway
Sola	ecological	**9**	Norway
Trondheim	conventional	**10**	Norway
Oslo	conventional	**11**	Norway
Bergen	conventional	**12**	Norway
Germany	conventional	**13**	Germany
Germany	ecological	**14**	Germany
Sweden	conventional	**15**	Sweden
Sweden	ecological	**16**	Sweden
Denmark	conventional	**17**	Denmark
Denmark	ecological	**18**	Denmark

* Sample number corresponds to table above

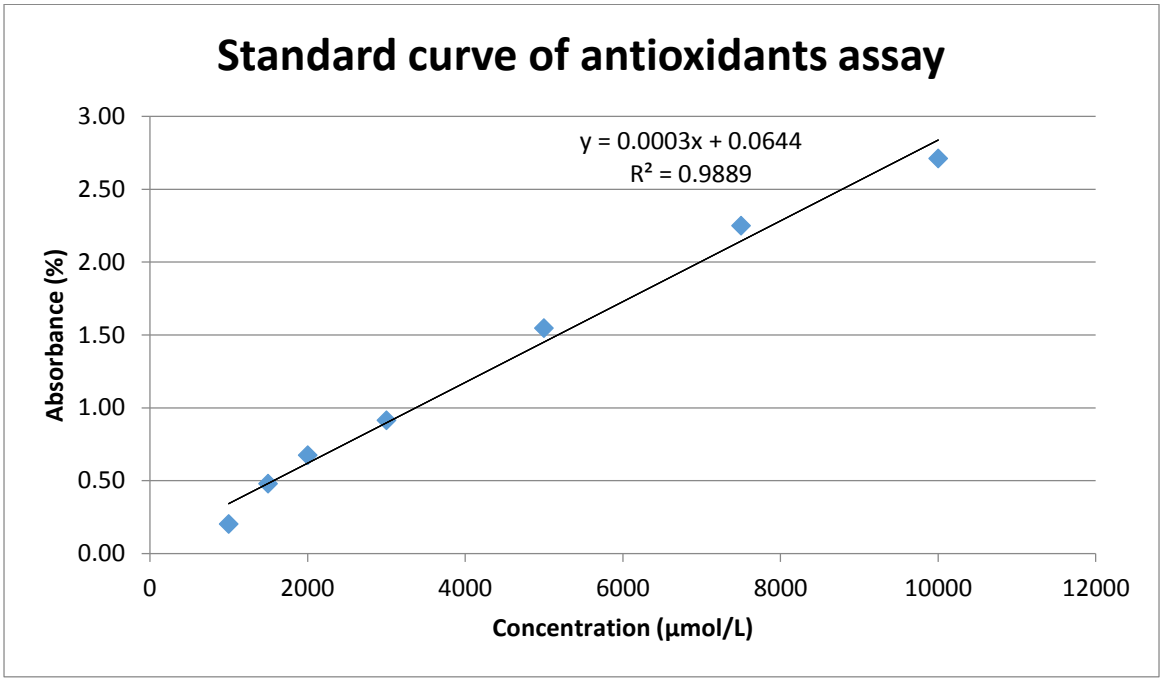
Appendix 2: Description of milk samples collected in Phase 2 experiment

Sampling period	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Date (in 2014)	5/15	6/5	6/26	7/17	8/7	8/28	9/18	10/9
Number of samples	4	4	4	3	4	4	4	4
Total	31 samples (frozen, 4 tubes/sample)							

Four types of milk were collected every three weeks from May to October, 2014. These included conventional whole milk, ecological whole milk, conventional low fat milk and ecological low fat milk.

FAT CONTENT	**TYPE**	**PERIOD**	**SAMPLE ID**	**SAMPLE no**
Whole	Conventional	1	F-1-C	**1**
Whole	Conventional	2	F-2-C	**2**
Whole	Conventional	3	F-3-C	**3**
Whole	Conventional	4	F-4-C	**4**
Whole	Conventional	5	F-5-C	**5**
Whole	Conventional	6	F-6-C	**6**
Whole	Conventional	7	F-7-C	**7**
Whole	Conventional	8	F-8-C	**8**
Whole	Ecological	1	F-1-E	**9**
Whole	Ecological	2	F-2-E	**10**
Whole	Ecological	3	F-3-E	**11**
Whole	Ecological	5	F-5-E	**12**
Whole	Ecological	6	F-6-E	**13**
Whole	Ecological	7	F-7-E	**14**
Whole	Ecological	8	F-8-E	**15**
Low	Conventional	1	L-1-C	**16**
Low	Conventional	2	L-2-C	**17**
Low	Conventional	3	L-3-C	**18**
Low	Conventional	4	L-4-C	**19**
Low	Conventional	5	L-5-C	**20**
Low	Conventional	6	L-6-C	**21**
Low	Conventional	7	L-7-C	**22**
Low	Conventional	8	L-8-C	**23**
Low	Ecological	1	L-1-E	**24**
Low	Ecological	2	L-2-E	**25**
Low	Ecological	3	L-3-E	**26**
Low	Ecological	4	L-4-E	**27**
Low	Ecological	5	L-5-E	**28**
Low	Ecological	6	L-6-E	**29**
Low	Ecological	7	L-7-E	**30**
Low	Ecological	8	L-8-E	**31**

Appendix 3: A/ Calibration curve was made from different concentrations of $FeSO_4.7H_2O$ in ferric reducing antioxidant power (FRAP) assay



B/ Results from Tukey pairwise comparisons of antioxidant activity of four types of milk with regards to production type and fat content. Ecological whole milk, conventional whole milk, ecological low fat milk and conventional low fat milk are denoted by F-E, F-C, L-E and L-C, respectively.

Milk type	Number of sample	Mean	Grouping
F-E	7	4.001	A
F-C	8	3.392	B
L-E	8	2.231	C
L-C	8	2.78	C

Appendix 3: C/ Absorbance values and calculated concentration of antioxidant activity of milk samples in Phase 2.

Sample	Absorbance	Concentration (mmol/L)
F-1-C	1.14	3.58
F-2-C	1.16	3.65
F-3-C	1.20	3.78
F-4-C	1.21	3.83
F-5-C	1.02	3.20
F-6-C	0.99	3.09
F-7-C	1.01	3.16
F-8-C	0.92	2.85
F-1-E	1.45	4.61
F-2-E	1.21	3.83
F-3-E	1.30	4.13
F-5-E	1.29	4.07
F-6-E	1.21	3.81
F-7-E	1.22	3.84
F-8-E	1.18	3.72
L-1-C	0.81	2.48
L-2-C	1.06	3.31
L-3-C	0.83	2.55
L-4-C	1.09	3.42
L-5-C	1.05	3.29
L-6-C	1.03	3.21
L-7-C	0.59	1.77
L-8-C	0.73	2.21
L-1-E	0.65	1.97
L-2-E	0.69	2.09
L-3-E	0.96	2.98
L-4-E	0.75	2.30
L-5-E	0.71	2.16
L-6-E	0.72	2.19
L-7-E	0.71	2.17
L-8-E	0.67	2.00

Appendix 4: Lipid compounds from UPLC-TOF-MS analysis in Phase 1 and *P*-values of different factors, i.e. production type (Ecological and conventional milk), period of production, geographical areas (4 domestic regions in Norway and 4 countries)

Compounds			*P-value*			
	m/z	Retention time (min)	Production type	Period	4 regions	4 countries
Sulfoglycolithocholate	495.27	3.75	0.76	0.00E+00	0.68	0.23
PG(12:0/0:0)	451.21	5.40	0.63	1.84E-09	0.36	0.37
1-(6-[3]-ladderane-hexanoyl)-2-(8-[3]-ladderane-octanyl)-sn-glycerophosphocholine	810.54	3.40	0.72	1.62E-08	0.48	0.30
22:6 Cholesteryl ester	697.59	4.22	0.76	1.32E-08	0.56	0.53
PG(O-16:0/12:0)	653.48	3.72	0.14	1.71E-08	0.99	0.05
MG(20:0/0:0/0:0)[rac]	409.33	3.37	0.80	1.48E-11	0.92	0.12
Oceanalin A	737.53	4.32	0.83	2.78E-08	0.69	0.50
Anhydrorhodovibrin	605.41	2.50	0.53	8.78E-04	0.21	0.42
Bacteriorubixanthinal	579.42	1.44	0.78	2.46E-06	0.61	0.71
Jurubine	578.41	0.56	0.68	0.001808	0.47	0.54
TG(22:5(7Z,10Z,13Z,16Z,19Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))[iso3]	1025.76	4.82	0.79	0.001984	0.73	0.84
34:6(16Z,19Z,22Z,25Z,28Z,31Z)	535.39	1.87	0.84	0.001059	0.13	0.55
Termitomycesphin A	766.54	3.46	0.77	8.67E-04	0.43	0.00
(-)-11-hydroxy-9,10-dihydrojasmonic acid 11-beta-D-glucoside	373.19	4.81	0.21	0.005991	0.18	0.80
Ketospirilloxanthin	593.44	6.87	0.86	0.004024	0.20	0.56
2-bromopalmitaldehyde	301.15	3.33	0.49	0.007359	0.20	0.19
CerP(d18:1/24:1(15Z))	766.55	5.36	0.75	0.007123	0.15	0.87
Depdecin	229.11	4.85	0.81	0.017726	0.65	0.57
C19 Sphingosine-1-phosphate	416.25	3.01	0.14	0.008262	0.58	0.38
DG(O-16:0/18:1(9Z))	603.53	4.33	0.02	0.086811	0.67	0.13
1-(2E,6E-phytadienyl)-2-(2E,6E-phytadienyl)-sn-glycero-3-phosphocholine	792.66	4.05	0.76	1.97E-04	0.93	0.03
2-arachidonoyl glycerol-d5	384.32	3.35	0.98	1.36E-06	0.94	0.04
Diketospirilloxanthin/ 2,2'-Diketospirilloxanthin	607.42	3.66	0.59	0.035282	0.43	0.03
Coenzyme Q10	901.65	3.74	0.44	0.002376	0.46	0.30
LacCer(d18:0/22:0)	970.72	4.01	0.17	0.025907	0.20	0.22
18:1 Cholesteryl ester (d5)	694.60	4.24	0.54	0.068819	0.79	0.26

Appendix 4 to be continued

Appendix 4 continued

Compounds	*m/z*	Retention time (min)	Production type	Period	4 regions	4 countries
bacteriohopane-,32,33,34-triol-35-cyclitolguanine	732.55	3.40	0.88	0.004196	0.26	0.07
MG(18:0/0:0/0:0)[rac]	381.30	3.97	0.57	0.003054	0.65	0.71
13,14-dihydroxy-docosanoic acid	395.31	4.24	0.41	0.052839	0.72	0.50
PA(O-16:0/O-16:0)	603.51	4.12	0.14	0.020472	0.89	0.03
N-ornithinyl-35-aminobacteriohopane-32,33,34-triol	682.55	3.46	0.83	0.035522	0.32	0.16
1-O-alpha-D-glucopyranosyl-(2-hexadecanoyloxy)-eicosan-1-ol	737.59	3.66	0.62	0.007133	0.91	0.67
PC(O-20:0/22:4(7Z,10Z,13Z,16Z))	852.72	4.25	0.19	0.019602	0.64	0.27
DG(13:0/13:0/0:0)	467.41	3.76	0.45	5.10E-06	0.52	0.70
Oleandomycin	670.42	5.99	0.72	0.177946	0.37	1.00
TG(14:0/16:1(9Z)/14:0) (d5)	792.65	3.88	0.92	0.003921	0.86	0.03
DG(18:2(9Z,12Z)/0:0/18:2(9Z,12Z)) (d5)	604.53	5.03	0.01	0.245428	0.31	0.23
27-Nor-5b-cholestane-3a,7a,12a,24,25-pentol	439.34	3.95	0.03	0.038154	0.49	0.06
N-stearoyl histidine	422.34	4.06	0.67	0.082442	0.39	0.99
3Z,6Z,9Z,12Z,15Z-Pentacosapentaene	381.29	3.35	0.00	0.127049	0.40	0.00
DG(21:0/22:3(10Z,13Z,16Z)/0:0)[iso2]	739.62	4.43	0.50	0.036074	0.84	0.67
1alpha-hydroxy-18-[m-(1-hydroxy-1-ethylpropyl)-benzyloxy]-23,24,25,26,27-pentanorvitamin D3 / 1alpha-hydroxy-18-[m-(1-hydroxy-1-ethylpropyl)-benzyloxy]-23,24,25,26,27-pentanorcholecalciferol	505.37	2.89	0.26	0.022151	0.29	0.44
8E-Heptadecenedioic acid	299.22	3.97	0.71	0.003881	0.59	0.19

P-value <0.001
P-value < 0.01
P-value < 0.05
No highlight Not significant difference

Appendix 5 List of 61 elements found in ICP-MS analysis with mean and standard deviation according to production type (conventional and organic milk).

Element (µg/kg)	**Conventional milk**		**Ecological milk**		***P*-value from t-test**
	MEAN	**STD**	**MEAN**	**STD**	
Li7(LR)	1.19	0.63	1.78	1.43	0.027089087
Be9(LR)	0.04	0.02	0.04	0.03	0.7113458
Se82(LR)	13.41	2.53	13.40	2.38	0.9890454
Y89(LR)	0.02	0.01	0.02	0.01	0.0098271
Zr90(LR)	0.06	0.06	0.06	0.07	0.68419015
Cd114(LR)	0.03	0.13	0.07	0.23	0.35805994
Mo98(MR)	26.99	4.52	49.18	22.60	1.71E-06
Sn118(LR)	0.03	0.03	0.08	0.16	0.13733219
In115(LR)	0.02	0.13	0.03	0.17	0.77318364
Cs133(LR)	4.68	2.32	3.96	3.57	0.29069805
Ce140(LR)	0.01	0.01	0.01	0.01	0.00477199
Pr141(LR)	0.04	0.19	0.06	0.23	0.64340204
Nd146(LR)	0.03	0.13	0.02	0.01	0.55201185
Sm147(LR)	0.11	0.31	0.18	0.38	0.4018766
Tb159(LR)	0.11	0.31	0.03	0.17	0.12030186
Dy163(LR)	0.08	0.26	0.03	0.17	0.34112746
Ho165(LR)	0.31	0.47	0.29	0.46	0.81582046
Er166(LR)	0.33	0.47	0.26	0.44	0.4794848
Tm169(LR)	0.20	0.40	0.26	0.44	0.5386249
Yb172(LR)	0.27	0.45	0.32	0.47	0.6574615
Lu175(LR)	0.13	0.34	0.03	0.17	0.06930742
Ta181(LR)	0.20	0.40	0.14	0.35	0.48421282
Hf178(LR)	0.08	0.26	0.03	0.17	0.33873144
Pt195(LR)	0.11	0.31	0.12	0.32	0.94419223
Au197(LR)	0.09	0.04	0.08	0.03	0.6630269
W182(LR)	0.07	0.19	0.25	0.34	0.006056096
Hg202(LR)	0.07	0.04	0.13	0.29	0.22526366
Tl205(LR)	0.15	0.08	0.06	0.05	5.31E-10
Pb208(LR)	0.18	0.08	0.28	0.18	0.004595566
Bi209(LR)	0.15	0.30	0.28	0.62	0.2575866
Th232(LR)	0.07	0.19	0.05	0.17	0.64327514
U238(LR)	0.04	0.05	0.04	0.06	0.8952992
B11(MR)	116.11	31.25	135.03	20.04	7.38E-04
Na23(MR)	351122.62	40008.91	347447.00	33178.14	0.6379531
Mg25(MR)	104629.78	12244.25	100983.29	9388.89	0.11507361
Al27(MR)	3.92	1.82	5.53	2.40	0.001217783

Appendix 5 to be continued

Appendix 5 continued

Element (µg/kg)	**Conventional milk**		**Ecological milk**		***P-value***
	MEAN	**STD**	**MEAN**	**STD**	
Si29(MR)	565.54	275.05	638.67	210.34	0.22065659
P31(MR)	903294.50	102286.59	920899.90	83517.74	0.37498596
S34(MR)	313298.94	36324.65	308353.60	23198.84	0.43273032
K39(MR)	1466092.80	162732.92	1465713.40	121624.93	0.98996305
Ca43(MR)	1031394.30	127719.35	1054153.20	92103.64	0.32967138
Sc45(MR)	0.11	0.22	0.08	0.16	0.582636
Ti49(MR)	0.96	0.78	0.89	0.65	0.61867326
V51(MR)	0.07	0.02	0.08	0.03	0.25091994
Cr52(MR)	0.40	0.51	0.42	0.20	0.869713
Mn55(MR)	17.18	2.48	17.55	3.03	0.54411405
Fe56(MR)	136.51	16.81	138.11	15.03	0.6385775
Co59(MR)	0.24	0.04	0.23	0.05	0.68800044
Ni60(MR)	0.24	0.12	0.35	0.20	0.004457491
Cu63(MR)	40.05	5.07	36.85	5.89	0.01006697
Zn66(MR)	3640.80	383.94	3595.91	348.60	0.568818
Ga69(MR)	0.04	0.02	0.04	0.01	0.1177718
Rb85(MR)	1805.73	519.47	1936.46	1090.62	0.5109279
Sr88(MR)	555.48	137.28	501.51	164.72	0.11248567
Ag109(MR)	0.04	0.03	0.11	0.23	0.095694
Sb121(MR)	0.12	0.06	0.10	0.06	0.1176054
Ba137(MR)	62.20	17.25	57.01	20.59	0.21996643
La139(MR)	0.02	0.01	0.02	0.01	0.07887745
Ge72(HR)	0.11	0.13	0.10	0.05	0.62722373
As75(HR)	0.14	0.06	0.18	0.04	4.63E-04
Nb93(HR)	0.06	0.18	0.09	0.23	0.6039961

P-value <0.001
P-value < 0.01
P-value < 0.05
No highlight Not significant difference

Appendix 6: List of metabolites found in GC-MS analysis with mean and standard deviation according to production type (conventional and organic milk)

		Conventional milk		Ecological milk		
Compound	**Average**	**Mean**	**STD**	**Mean**	**STD**	***P*-value**
carbodiimide	215.49	2.60	3.02	2.02	2.84	0.34
pyruvic acid	12.06	0.11	0.10	0.15	0.25	0.24
lactic acid	117.12	1.33	0.35	1.18	0.38	0.06
alanine	6.11	0.06	0.08	0.08	0.09	0.30
N-carboxymethylamine	243.37	2.60	1.71	2.63	1.78	0.94
3-hydroxybutanoic acid	128.33	1.36	0.39	1.40	0.52	0.68
heptanoic acid	346	3.65	1.56	3.79	1.80	0.68
monomethylphosphate	127.15	1.31	0.61	1.42	0.73	0.44
amine	51.50	0.54	0.22	0.57	0.24	0.62
valine	9.06	0.10	0.09	0.10	0.09	0.70
ethanolamine	93	1.06	0.35	0.94	0.40	0.13
aromatic	23.17	0.27	0.12	0.23	0.14	0.14
urea	3829.28	41.29	16.32	41.05	16.48	0.94
octanoic acid (C8:0)	26.85	0.26	0.13	0.32	0.19	0.08
glycerol	460.64	4.93	1.01	4.97	1.44	0.87
phosphoric acid	21679.27	236.15	45.94	229.86	52.18	0.54
glycine	130.35	1.44	0.61	1.36	0.66	0.54
succinic acid	163.95	1.72	0.72	1.81	0.73	0.58
glyceric acid	92.08	1.03	0.30	0.94	0.36	0.20
itaconic acid	20.22	0.16	0.25	0.28	0.33	0.06
fumaric acid	69.14	0.71	0.23	0.78	0.32	0.30
methylmaleic acid	34.21	0.33	0.28	0.41	0.30	0.23
decanoic acid (C10:0)	39.75	0.38	0.14	0.48	0.25	0.01
1-monoisobutyrin	19.65	0.18	0.12	0.24	0.15	0.05
erythrulose	17.15	0.20	0.16	0.17	0.16	0.31
malic acid	753.05	7.92	2.35	8.28	3.75	0.58
pyroglutamic acid	221.44	2.41	1.17	2.35	1.10	0.80
4-hydroxyproline	39.87	0.42	0.21	0.44	0.20	0.58
aspartic acid	34.01	0.32	0.14	0.41	0.26	0.05
4-aminobutyric acid	54.43	0.52	0.34	0.65	0.60	0.19
creatinine	47.88	0.49	0.29	0.54	0.30	0.41
2-hydroxyglutaric acid	175.86	1.81	0.51	1.98	0.65	0.16
proline (+CO2)	46	0.49	0.17	0.51	0.22	0.66
pipecolinic acid deriv	203.45	2.24	1.18	2.13	1.26	0.64
glutamic acid	696.53	7.21	2.62	7.78	3.43	0.38
phenylalanine	38.97	0.40	0.14	0.43	0.21	0.41
dodecanoic acid (C12:0)	40	0.39	0.17	0.49	0.27	0.04

xylose	58	0.68	0.24	0.56	0.27	0.04
ribose	33	0.38	0.20	0.33	0.24	0.33
arabinose	161.37	1.70	0.54	1.77	0.64	0.56
6-deoxy-mannose	16.90	0.22	0.25	0.14	0.22	0.08
orotic acid	1663.20	18.49	5.96	17.23	6.13	0.32
glycerol-3-phosphate	2303.67	26.02	8.06	23.44	9.13	0.15
ethanolaminephosphate	116	1.16	0.45	1.35	0.59	0.09
tetradecanoic acid (C14:0)	8.16	0.09	0.52	0.09	0.29	0.92
citric acid	17436.05	189.78	34.67	185.04	44.38	0.57
fructose	466.69	5.07	1.29	4.96	1.34	0.70
sugar	246.04	2.58	1.23	2.72	1.41	0.60
lysine	16	0.18	0.11	0.17	0.12	0.92
sugar	21.39	0.24	0.14	0.22	0.14	0.58
quinic acid	22	0.25	0.12	0.23	0.14	0.36
glucose	1127.76	12.48	3.90	11.75	5.65	0.47
galactose	487.18	5.47	3.70	4.99	3.44	0.52
mannose	70.45	0.78	0.23	0.74	0.28	0.44
glucuronic acid	237.35	2.66	0.76	2.44	0.85	0.20
galactitol	706	8.12	2.64	7.02	2.76	0.05
galacturonic acid	41.22	0.48	0.18	0.41	0.20	0.10
panthotenic acid	35.25	0.38	0.12	0.38	0.12	0.96
gluconic acid	76.26	0.66	0.19	1.00	1.08	0.04
hexadecanoic acid (C16:0)	345	3.50	1.11	3.94	1.74	0.15
sugar	36.66	0.44	0.18	0.35	0.17	0.02
sugar	225.81	2.59	0.58	2.26	0.55	0.01
tryptophan	192.07	2.21	0.48	1.91	0.50	0.00
myo-inositol	2008.66	22.54	4.49	20.60	5.03	0.05
N-acetyl glucosamine	564.30	6.43	1.16	5.68	1.17	0.00
uric acid	81.32	0.87	0.68	0.88	0.66	0.94
glucose oxime	41.72	0.41	0.20	0.49	0.22	0.07
galactose oxime	62.77	0.65	0.18	0.70	0.23	0.25
octadecenoic acid, 9-(Z)-(C18:1)	149	1.59	0.71	1.61	0.89	0.90
octadecenoic acid, 9-(E)-(C18:1)	10	0.11	0.11	0.11	0.12	0.82
octadecanoic acid (C18:0)	208	2.26	0.59	2.22	0.62	0.78
glycero-3-phospho-ethanolamine	212.40	2.42	0.75	2.14	0.76	0.08
fructose-6-phosphate	63.02	0.66	0.20	0.69	0.26	0.58
glucose-6-phosphate	117.05	1.22	0.35	1.30	0.46	0.37
mannose-6-phosphate	24.28	0.25	0.21	0.27	0.21	0.58
1-monomyristin	39.56	0.38	0.19	0.48	0.25	0.03
monoglyceride	42.01	0.44	0.13	0.46	0.19	0.46

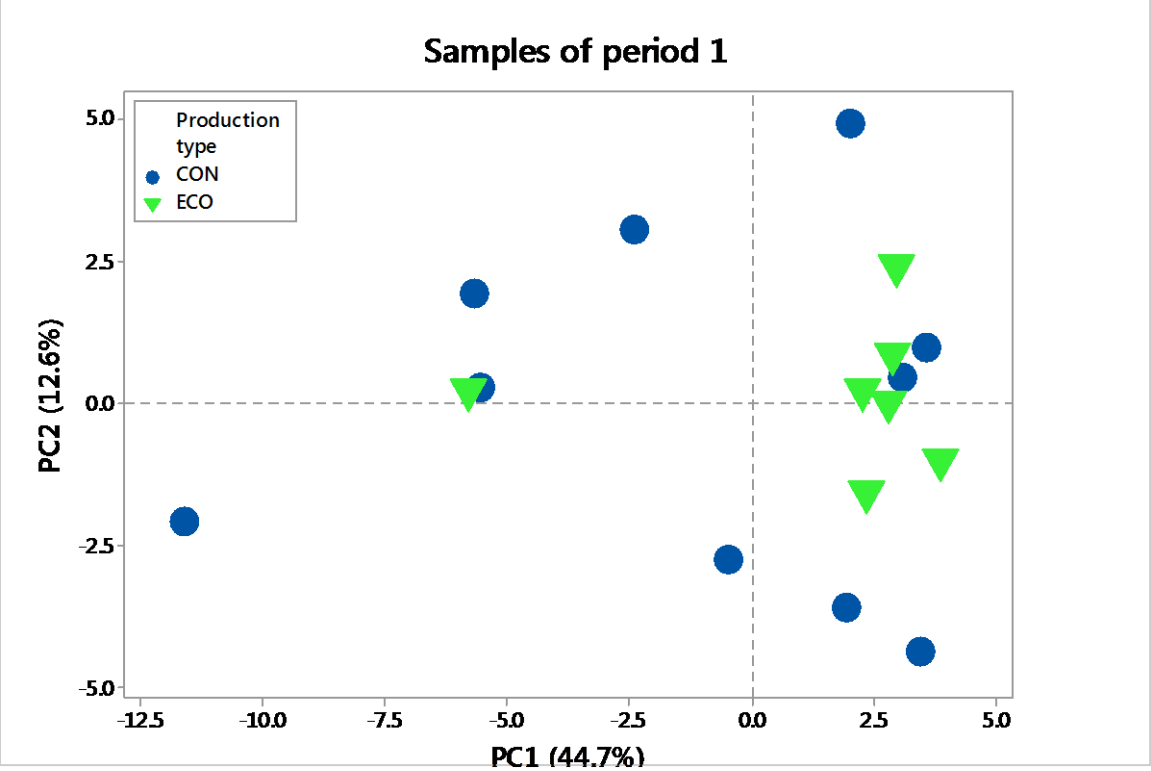
sugar deriv.	28.88	0.36	0.29	0.26	0.28	0.13
monoglyceride	21.36	0.22	0.15	0.24	0.16	0.46
1-monopalmitin	60	0.60	0.26	0.68	0.41	0.29
a-lactose	1587.05	16.92	3.75	17.22	2.09	0.63
maltose	2688.70	27.07	16.68	30.88	19.31	0.31
disaccharide	21.41	0.23	0.19	0.23	0.17	0.84
diglyceride (C16:0,C18:1)	50.96	0.52	0.23	0.57	0.34	0.43
diglyceride (C16:0,C18:0)	33.85	0.38	0.21	0.35	0.28	0.58
galactinol	671.79	7.42	2.28	7.01	2.47	0.41
sugar/lipid phosphate	50.80	0.60	0.27	0.48	0.31	0.05
sugar/lipid phosphate	41.59	0.42	0.18	0.48	0.29	0.29
cholesterol	404.32	4.47	1.76	4.22	2.08	0.53
sugar/lipid phosphate	57.84	0.62	0.32	0.62	0.34	0.94
sugar/lipid phosphate	29.96	0.31	0.24	0.34	0.22	0.60
diglyceride	33.14	0.36	0.26	0.35	0.31	0.80
diglyceride	45.00	0.46	0.21	0.51	0.47	0.50
sugar/lipid phosphate	87.82	0.88	0.34	1.01	0.61	0.21
sterol	32.90	0.36	0.16	0.34	0.25	0.68
diglyceride	53.45	0.53	0.22	0.62	0.47	0.23
sugar/phosphate	55.55	0.59	0.22	0.60	0.39	0.83
maltotriose	173	1.85	0.63	1.87	0.70	0.86
1,2-palmitin	127.67	1.34	0.57	1.40	1.05	0.73
diglyceride	75.64	0.82	0.39	0.81	0.48	0.88

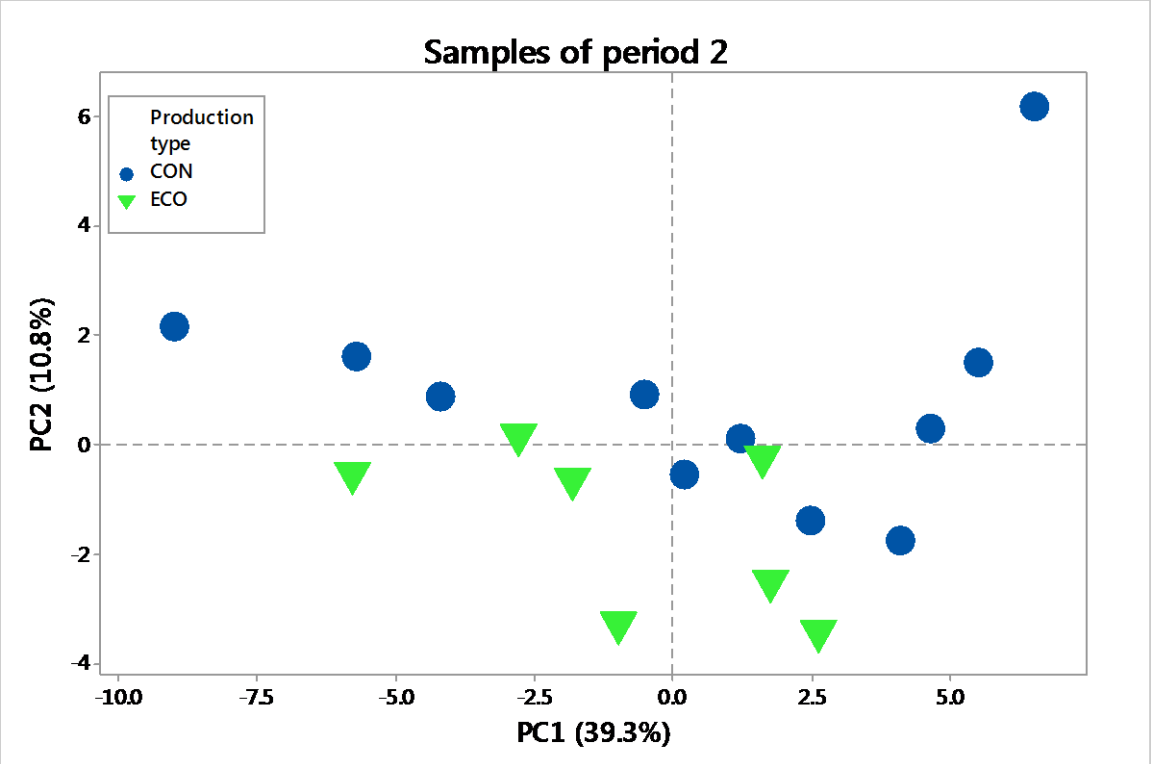
P-value < 0.01

P-value < 0.05

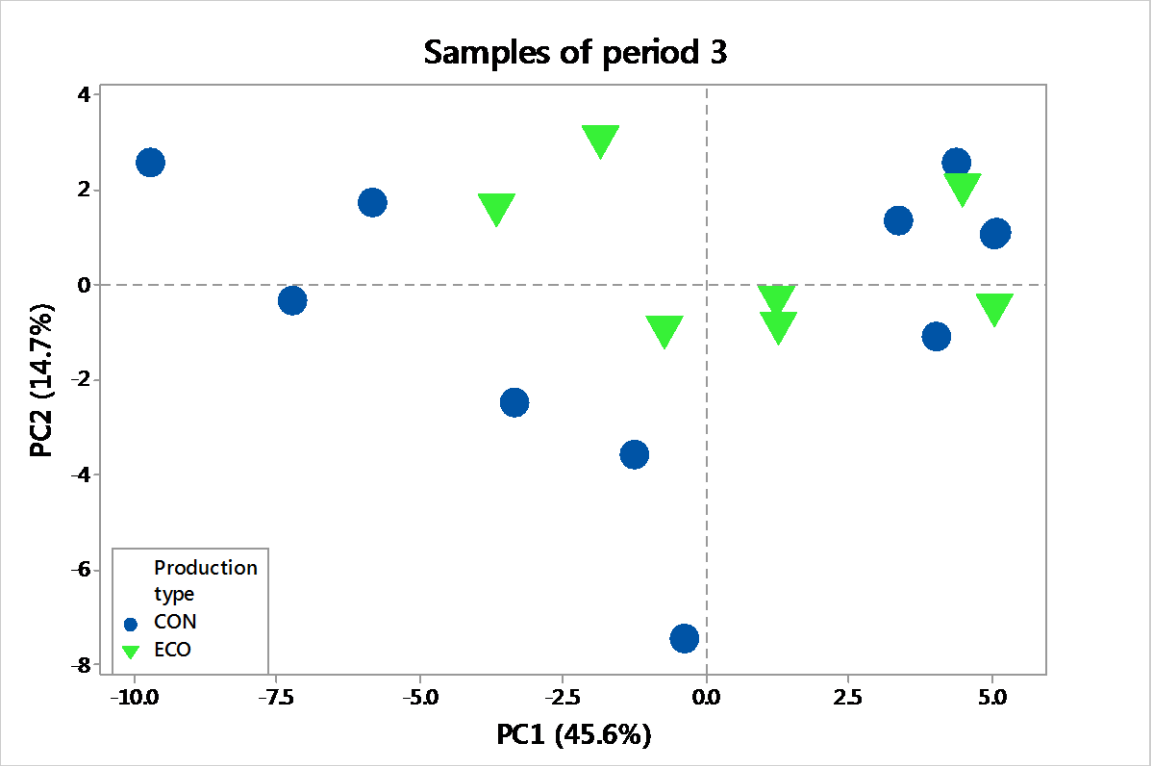
No highlight Not significant difference

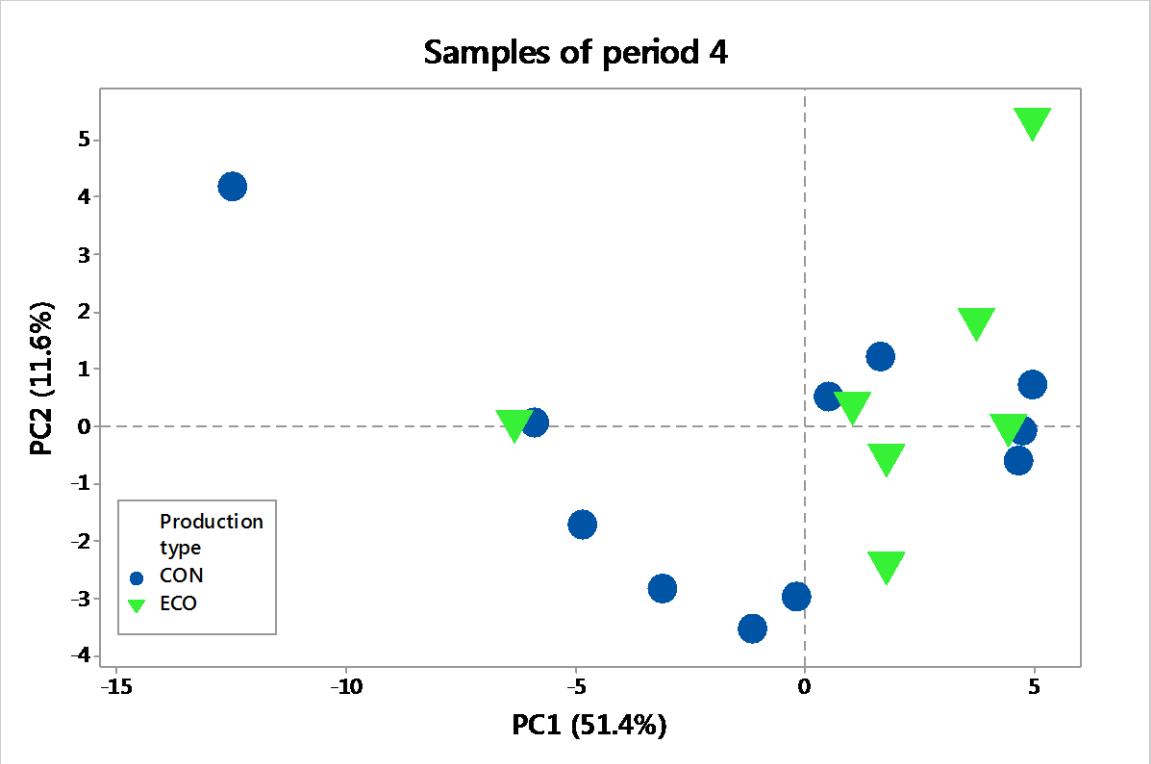
Appendix 7: Score plots from PCA of lipid compounds in milk samples of five production periods. The samples are colour grouped according to the production types, i.e. ecologically (ECO) and conventionally (CON) agricultural methods. The data was obtained from UPLC-TOF-MS analysis.





Appendix 6 ***(continued)***





Appendix 6 ***(continued)***