Fate of fatty acids during ensiling: relationship with milk fat composition of dairy cows

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Thesis

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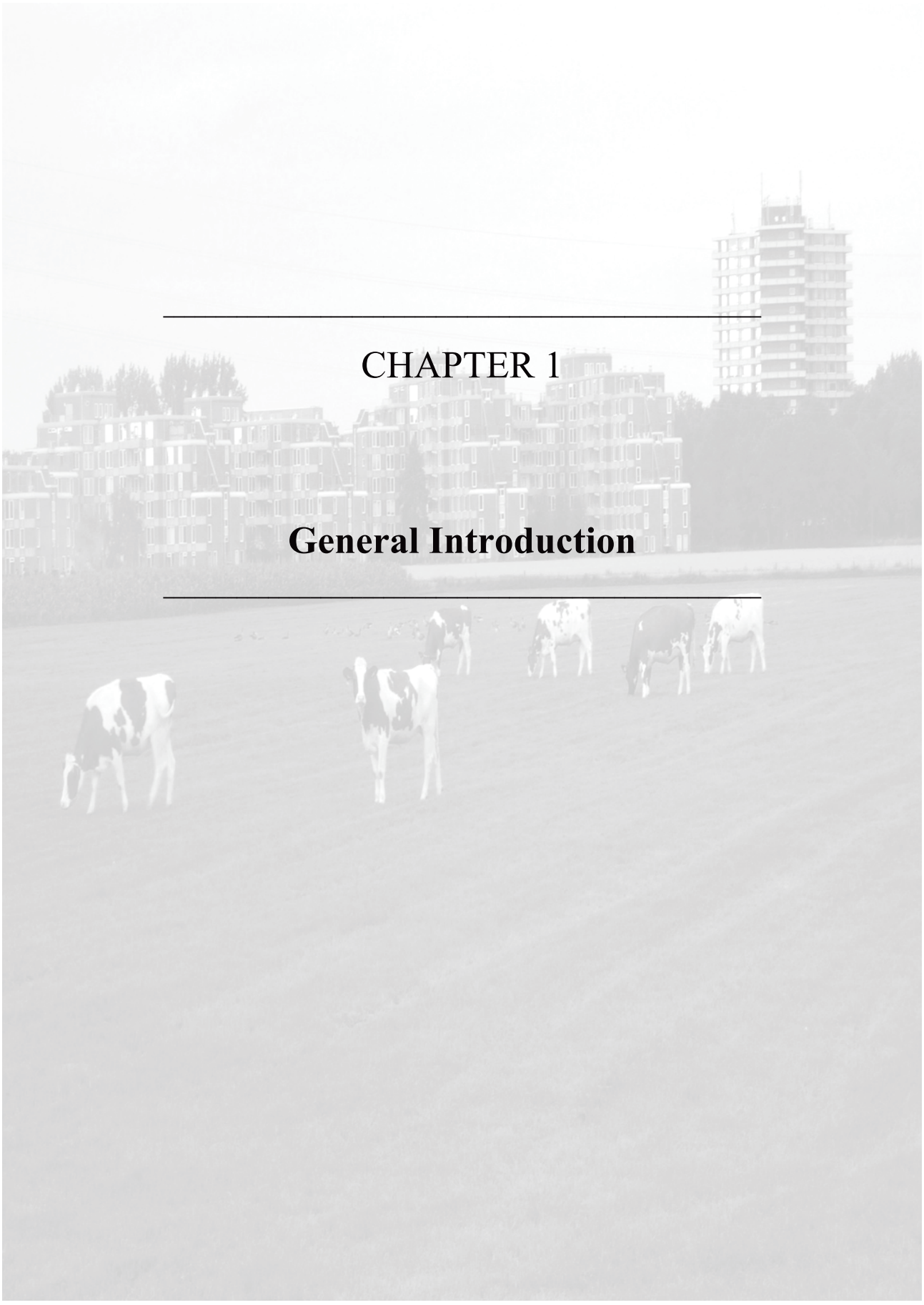
Abstract

Transition of dairy cows from grazing to silage based rations significantly increases the saturated: unsaturated fatty acids (FA) ratio and decreases the content of beneficial C18:1 *cis*-9, C18:1 *trans*-11, C18:2 *cis*-9, *trans*-11 and C18:3n-3 in milk fat. This is partly related to a lower polyunsaturated FA (PUFA) supply from ensiled forages. The aim of this thesis was to investigate the scope of increasing the content PUFA in grass and maize silages, and to establish relationships between silage quality on the one hand and the FA content and composition, post-ensiling stability of PUFA, and milk FA composition of dairy cows on the other hand. The first focus of this thesis was to quantify the variation in FA content and composition in grass (n = 101) and maize (n = 96) silages, randomly sampled from commercial dairy farms in the Netherlands, and use multivariate analysis to identify the causes of this variation. The FA content and composition of grass and maize silages were highly variable, and this variation was primarily caused by differences in plant maturity at harvest. Silages made from younger grass and maize have higher contents of C18:3n-3. Most of the variation in FA content in the ensiled forages was caused by differences in plant maturity at harvest. Changes in FA content and composition were investigated in stover (leaves and stem) and ears (cob, shank and husks) in a set of maize genotypes, grown on sandy and clay soils and harvested at 14, 42, 56, 70, and 84 days after flowering (DAF). The contents of C18:3n-3 and total FAs in the stover dry matter (DM) declined at a slow rate up to 56 DAF and then decreased rapidly during 56–84 DAF. On the other hand the content of C18:2n-6 and total FAs in the ears DM increased up to 56 DAF and thereafter remained more or less constant. The maximum amount of PUFA in silage maize can be harvested around 56 DAF. Identifying pre and post-ensiling processes that optimize the stability of PUFA was the next goal. The stability of FA were investigated in untreated and mechanically bruised perennial ryegrass (*Lolium perenne L.*), wilted under field conditions for 0, 12, 24, 36, and 48 h, or wilted under controlled climate conditions at three temperatures (15, 25 or 35 °C) and two light (dark or light) regimes to DM contents of 425, 525 or 625 g/kg. The oxidation of FAs during wilting of grass was mainly caused by the duration of the wilting, wilting temperature only provoked small differences, whereas mechanical bruising of grass and light intensity did not affect the changes in FA contents. The highly esterified lipids of forages are extensively hydrolysed in the silo. Therefore, the post-ensiling stability of FAs was investigated in grass and maize silages, with a wide range in qualities, exposed to air for 0, 12, and 24 h. Exposure of grass and maize silages to air results in a quantitatively small, but consistent decline in the contents of major unsaturated FAs with a concomitant increase in the proportion of C16:0. The final study evaluated the effects of feeding maize silages, ensiled at different maturities, in combination with a high or low degradable carbohydrate concentrate on nutrient intake, milk production, and composition of milk and milk fat in early lactating dairy cows. Maize maturity at harvest at a DM content of 300-420 g/kg fresh weight, did not affect the production performance of dairy cows, but resulted in decreased contents of C18:3n-3 and total n-3 and a decreased n-6:n-3 ratio in the milk fat of dairy cows.

Contents



CHAPTER 1

General Introduction

Lipids and fatty acids in forages

The bulk of the world's lipids are *de novo* synthesised by plants and, of these, the acyl lipids form the largest part. The principal acyl lipids in plants are triacylglycerol (storage lipids) and the glycerolipids (membrane lipids). All these molecules have fatty acids (FAs) esterified to a glycerol backbone. In photosynthetic tissues of forages, FAs are present in membranes lipids, predominantly in thylakoid membranes in the chloroplasts (Hawke, 1973). Lipids on average represent about 20% of the chloroplast and 50% of the dry matter of thylakoid membranes (Rawyler *et al.*, 1987; Joyard *et al.*, 1998). Galactolipids, phospholipids and sulfolipids are the major classes of membrane glycerolipids. Galactolipids constitute over 80% of the total glycerolipids and contain linolenic acid (C18:3n-3) as the major FA. Sulfolipids represent about 5 to 10% of the glycerolipids and contain palmitic acid (C16:0) as the major FA, whereas C18:3n-3 is present as a minor FA. Phospholipids contain linoleic acid (C18:2n-6) as a major FA, but also contain C16:0, palmitoleic acid (C16:1), steric acid (C18:0), oleic acid (C18:1n-9) and C18:3n-3 as minor FAs. Under normal growing conditions of forages, galactolipids and sulfolipids are exclusively present in membranes of chloroplasts, whilst phospholipids are the major constituent of extra-chloroplast membranes (Joyard *et al.*, 1998; Dörman, 2005; Dörman and Hölzl, 2009). The lipid and FA composition of the chloroplast membranes are highly conserved across forage species (Rawyler *et al.*, 1987; Harwood, 1997; Dörman and Hölzl, 2009). This is related to the functionality of lipids in preserving membrane structure to carryout photosynthesis. The total amount of galactolipids and C18:3n-3 present in forages, therefore, reflects the expansion of chloroplast membranes. In a number of forage species, a strong positive correlation exists between the content of chlorophyll and C18:3n-3 or total FAs content (Mayland *et al.*, 1976; Bolton and Harwood, 1978; Dierking *et al.*, 2010).

Although, over 300 different FAs have been isolated from plants, only a few are commonly present in the membrane lipids (Harwood, 1979; 1997). Saturated FAs (SFAs) have invariably an even number of carbon atoms. Lauric acid (12:0), myristic acid (C14:0), C16:0, C18:0, arachidic acid (C20:0) and lignoceric acid (C24:0) are the commonly observed SFAs in membrane lipids. In general, the membrane lipids of plants contain a high proportion of unsaturated FAs with *cis* double bonds to regulate their membrane fluidity. In membrane lipids, C16:1, C18:1n-9, C18:2n-6 and C18:3n-3 are the major unsaturated FAs. C18:3n-3 is the predominant FA in forages, followed by C16:0 and C18:2n-6 (Table 1).

Table 1. Fatty acid content and composition of temperate grasses.

Fatty acid	Content (g/kg DM)				Proportion (g/100 g total fatty acids)			
	Mean	SD[1]	Min	Max	Mean	SD	Min	Max
C16:0	3.43	1.07	1.60	6.99	16	0.4	11	25
C18:0	0.47	0.43	0.10	2.86	2	0.2	1	10
C18:1n-9	0.60	0.40	0.20	1.84	3	0.2	1	8
C18:2n-6	2.69	0.90	1.05	6.76	13	0.3	7	24
C18:3n-3	13.78	6.00	3.89	31.87	62	0.9	43	79
PUFA[2]	15.97	5.77	4.94	35.76	75	0.7	59	88
Total fatty acids	21.72	7.76	7.53	44.40	-	-	-	-

Based on literature studies (Dewhurst and King, 1998; Dewhurst *et al*., 2001, 2002; Boufaïed *et al*., 2003; Elgersma *et al*., 2003a,b; Elgersma *et al*., 2005; Palladino *et al*., 2009; Van Ranst *et al*., 2009b; Dierking *et al*., 2010).

[1] SD, standard deviation.

[2] PUFA, polyunsaturated fatty acids.

Factors affecting fatty acids in forages

The FA content and composition of forages can be distinct based on their family, species and cultivar. Grasses are higher in the contents of C18:3n-3 and lower in C16:0 and C18:2n-6 content compared to legumes (Boufaïed *et al*., 2003; Dierking *et al*., 2010). There is, however, a large variation in the FA content and composition within grass and legume species. In grass species, the highest content of C18:3n-3 and total FAs was observed in perennial ryegrass (Dierking *et al*., 2010) and annual ryegrass (Boufaïed *et al*., 2003; Lee *et al*., 2006). In legume species the highest content of C18:3n-3 and total FAs was observed in white clover (Boufaïed *et al*., 2003), and the lowest content of C18:3n-3 and total FAs was observed in alfalfa (Boufaïed *et al*., 2003; Lee *et al*., 2006). Although, differences between cultivars are less distinct and not consistent among cuts (Dewhurst *et al*., 2001; Boufaïed *et al*., 2003; Elgersma *et al*., 2003b; Van Ranst *et al*., 2009b), a higher content of polyunsaturated FAs (PUFA) was found in late heading cultivars of perennial ryegrass compared to intermediate heading cultivars (Palladino *et al*., 2009). Similar patterns in PUFA content among the cultivars of Timothy were also observed by Boufaïed *et al*. (2003). The significant genetic differences in FA content and composition in forages

provide an opportunity to further improve the PUFA content through breeding. Genetic differences in FA content are more pronounced in young growing plants, while a decrease in leaf/stem ratio, initiation of flowering and senescence become increasingly important in the more mature grasses used to make conserved products, such as silages (Dewhurst *et al.*, 2006).

There is considerable variation in the FA content and composition of forages due to season. The FA content in temperate grasses are higher during spring and autumn and decrease during the stemmy, reproductive regrowth in summer (Bauchart *et al.*, 1984; Dewhurst *et al.*, 2001). The content of C18:3n-3 follows a similar pattern, while the content of C18:2n-6 and C16:0 remain more or less constant throughout the growing season. As a consequence, C18:3n-3 as a proportion of the total amount of FAs is higher during spring and autumn and lower during the summer (Bauchart *et al.*, 1984; Dewhurst *et al.*, 2001). The common basis for seasonal effects appears to be variation in leaf/stem ratio and initiation of flowering. However, seasonal effects are also associated with variation in photoperiod and temperature (Hawke, 1973; Allakhverdiev, 2009). The effects of light on the FA content in forages are related to effects on photosynthesis and hence on the content and composition of the lipids in the chloroplast membranes (Erwin and Bloch, 1963; O'Brien and Benson, 1964; Witkowska *et al.*, 2008). Temperature is the environmental factor with the strongest influence on the degree of unsaturation of FAs in membrane lipids of forages (Nishida and Murata, 1996). A decrease in temperature during growth induces the enzymatic desaturation of FAs to regulate membrane fluidity to provide an optimal environment for photosynthesis (Allakhverdiev, 2009). Low growing temperatures (15 *vs.* 30 °C) reduced the content of chloroplast lipids in perennial ryegrass. However, the proportion of C18:3n-3 was higher at the low temperature (Hofaecker-Klett and Beringer, 1975). Falcone *et al.* (2004) showed that the FA composition of membrane lipids in *Arabidopsis* was affected by the temperature (17, 20, 29 and 36 °C) during growth. Increasing the temperature from 17 to 36 °C decreased the content of C16:3n-3 and C18:3n-3 in the total FAs, while the content of C16:0 and C18:2n-6 increased. The high content of C18:3n-3 at lower temperatures increases membrane fluidity, maintains membrane integrity and permits essential trans-membrane transport to continue, and thus minimizes the disruption of cellular functions (Uemura *et al.*, 1995).

A high level of N fertilization increased the FA content in several forage species, during vegetative and reproductive growth, under a wide range of environmental conditions (Barta,

1975; Boufaïed *et al.*, 2003; Witkowska *et al.*, 2008). Nitrogen fertilization, however, did not alter the FA composition of the total fat (Mayland *et al*., 1976; Elgersma *et al*., 2005; Arvidsson, 2009). High N availability increases DM production, leaf area and stimulates the synthesis of metabolic components including, chlorophyll of forage plants. The increase in chloroplast density with N fertilization results in a high accumulation of membrane lipids and FAs. In addition, a positive relationship exists between plant N and chlorophyll contents (Gaborcik and Paulik, 2002). The strong positive correlation between plant N content with C18:3n-3 or total FAs content (Barta, 1975; Boufaïed *et al.*, 2003; Witkowska *et al.*, 2008) is associated with the positive effect of N on photosynthetic tissues.

The FA content and proportion of C18:3n-3 in the total FAs fraction decline with increasing plant maturity (Dewhurst *et al.*, 2001; Boufaïed *et al.*, 2003; Elgersma *et al.*, 2003a; Elgersma *et al.*, 2005; Vanhatalo *et al.*, 2007; Dønnem *et al.*, 2011). Although the content of all major FAs: C16:0, C18:2n-6 and C18:3n-3 declines with advancing plant maturity. The content of C18:3n-3 declines at a preferentially faster rate. The rapid decrease in C18:3n-3 is related to the rapid decline in chloroplast lipids with advancing maturity of forages, due to a decrease in leaf/stem ratio (Bauchart *et al.*, 1984; Boufaïed *et al.*, 2003), maturation of the leaves (Hawke, 1973), as well as initiation of flowering (Dewhurst *et al.*, 2006) and senescence (Mishra and Sangwan, 2008; Yang and Ohlrogge, 2009).

The FA content of forages can be manipulated with management practices that prevent extended maturation and the decrease in leaf/stem ratio. This can be achieved by increasing the number of cuttings of grass and legumes during the growing season, which will maintain a high proportion of leaves and prevent the initiation of flowering. Frequent cutting of perennial ryegrass prevented the normal decline in FA content during the summer with a progressive increase in total FAs content (from 20.8 to 34.6 g/kg DM) during the growing season (Dewhurst *et al.*, 2002). In addition, the application of N fertilization increases the FA content in forage plants and delays the decline in FA content with advancing herbage maturity (Mayland *et al.*, 1976; Boufaïed *et al.*, 2003; Witkowska *et al.*, 2008).

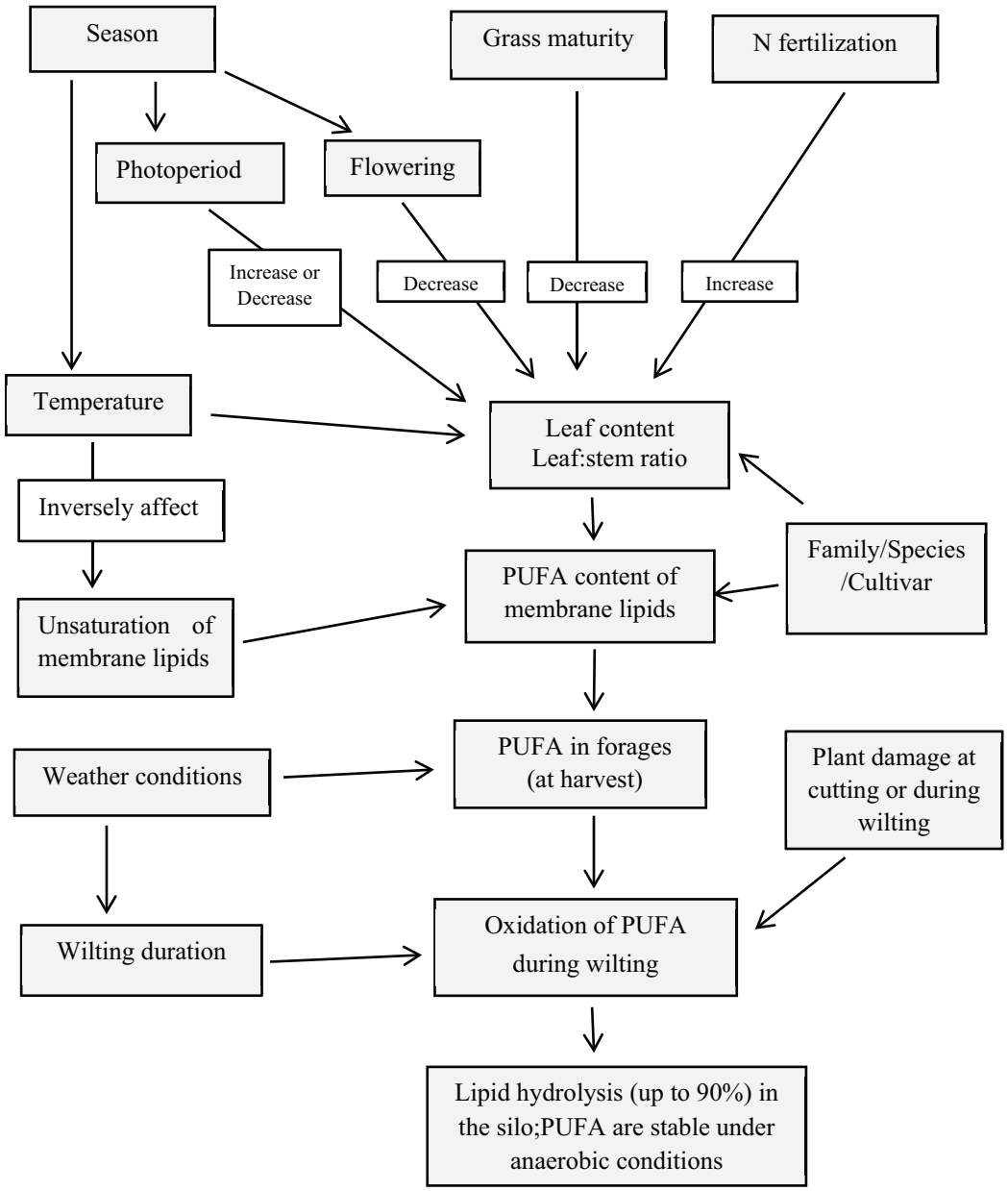


Figure 1. Summary of the major factors affecting the polyunsaturated fatty acids (PUFA) contents of grasses at harvest, and during the post-harvest wilting and ensiling process.

Nutritional modulation of milk fatty acids

Milk and milk products are an important component of the human diet in many parts of the world. Raw milk contains fat, high quality protein, lactose, essential minerals and vitamins, and serves as a valuable source of energy and nutrients. Although, milk from many mammalian species (e.g. buffalo, goat, sheep) is consumed by humans, milk from dairy cows is economically the most important. In the last few decades significant progress has been made in improving milk yield and composition of dairy cows through improved feeding and breeding. Over this period, however, the FA composition of milk fat has become less favourable for human health, mainly due to changed dairy husbandry and feeding regimes such as the shift from high grazing to more concentrate and silage based diets (Elgersma *et al.*, 2006). The saturated: unsaturated FA has generally increased, with a lower content of beneficial C18:3n-3 and C18:2 *cis*-9, *trans*-11 in milk fat (Elgersma *et al.*, 2004; Heck *et al.*, 2009). Milk fat contributes about 15-20% to the total intake of fat in a human diet (Chilliard *et al.*, 2000). However, milk fat accounts for 25-60% of the total intake of SFAs in Europe (Chilliard *et al.*, 2007), which makes milk fat a target of growing criticism from dieticians and health care professionals. Drinking milk with high proportions of SFAs is considered as a risk to human health (Givens and Shingfield, 2006). A high intake of SFAs, particularly medium chain (C12-C16) SFAs, increases the serum total and low-density lipoprotein cholesterol levels and increases the risk of cardiovascular diseases (Mensink *et al.*, 2003; Givens and Shingfield, 2006). Moreover, a high intake of SFAs is related to a reduced insulin sensitivity and a subsequent increase in type-2 diabetes (Parillo and Riccardi, 2004). However, milk fat also contains C4:0, branch-chain FAs, C18:1 *cis*-9, C18:2 *cis*-9, *trans*-11 , C18-C22 PUFA, vitamins A and D, and β-carotene that have shown positive health effects in humans (Parodi, 2001; Chilliard and Ferlay, 2004; Bauman *et al.*, 2006; Shingfield *et al.*, 2008).

This background provides an impetus for altering the composition of milk fat by decreasing the content of SFAs, particularly the medium chain (C12-C16) SFAs, and increasing the content of the beneficial unsaturated FAs. Feeding of PUFA rich feedstuffs can rapidly and favourably modulate the FA composition of milk fat in dairy cows (Chilliard *et al.*, 2001; Tamminga, 2001). The predominant PUFA in dairy cow diets are C18:3n-3 and C18:2n-6, the former derived principally from membrane glycerolipids of forages and the latter being a major component of oil seeds, including maize grain. High dietary intake of PUFA alters the FA composition of milk fat via inhibition of the *de novo* synthesis of SFAs and by

increasing the content of long chain FAs. The FAs in milk originate from two sources, namely mammary *de novo* synthesis and the uptake of preformed FAs from the peripheral circulation. All C4:0-C14:0 and about 50% of C16:0 FAs are synthesised *de novo* in the mammary gland from acetate and to a lesser extent from ß–hydroxy butrate (rumen fermentation products) by two key enzymes, acetyl-CoA carboxylase and FA synthetase (Chilliard *et al.*, 2000). Part of the C16:0 and long chain FAs (mainly C18) are derived from circulating plasma lipids. Except during early lactation, where body fat mobilization significantly contributes to the content of plasma derived long chain FAs particularly C18:0 and C18:1 *cis*-9 (Christie 1981). The content and composition of plasma derived long chain FAs in milk fat depends on their dietary supply and the subsequent biohydrogenation. Despite the extensive biohydrogenation of dietary C18:3n-3 and C18:2n-6, a higher intake of PUFA can increase the unsaturated: saturated FA ratio and increase the beneficial unsaturated FAs in milk fat (Grummer, 1991; Lourenço *et al.*, 2005b). Moreover, a higher uptake of long chain FAs (≥ 18C), particularly PUFA, by the mammary gland inhibits acetyl-CoA carboxylase and *de novo* lipogenesis of SFAs (Barber *et al.*, 1997; Chilliard *et al.*, 2000; Chilliard and Ferlay, 2004). Moreover, a higher uptake of long chain FAs decreases the content SFAs in milk fat due to the dilution effect.

The membrane glycerolipids in forages are rich in PUFA, particularly in C18:3n-3 (0.43 to 0.79 g/g total FAs) and dairy cows can consume large quantities of forage lipids without interfering with the normal rumen fermentation processes. Due to the high consumption and proportion of C18:3n-3, forages strongly contribute to the supply of C18:3n-3 in the ration of dairy cows (Arvidsson, 2009; Van Ranst, 2009), despite a low FA content (< 45 g/kg DM; Table 1). The interest in the use of high PUFA-containing forages to favourably modulate milk fat composition at a relatively low cost and in an environmentally sustainable manner has increased over the past decade (Dewhurst *et al.*, 2006; Elgersma *et al.*, 2006). Dairy cows grazing grass ingest large quantities of PUFA and produce milk fat with a high unsaturated:saturated FA ratio, and a high content of C18:3n-3 and *cis*-9, *trans*-11. However, transition of dairy cows from grazing to silage based winter diets lowers the unsaturated:saturated FA ratio as well as the content of beneficial C18:3n-3 and C18:2 *cis*-9, *trans*-11 in milk fat (Elgersma *et al.*, 2004; Heck *et al.*, 2009). This unfavourable shift in milk FA composition is partly related to the low precursor (PUFA) supply from ensiled forages due to harvesting of more mature swards for ensiling and oxidative losses of PUFA during the ensiling process. Indirect studies, suggest that a high PUFA content in grass silages could enhance the level of beneficial FAs in milk (Heck *et al.*, 2009) or muscle

(Warren *et al.*, 2008) fat. Quantifying variations in the FA content and composition of grass and maize silages and identifying causes of these variations can assist to design management strategies to increase the supply of PUFA from ensiled forages. A relatively small increase in PUFA content in silages can already lead to substantial increases in the PUFA intake of dairy cows fed on high forage diets and result in higher concentrations of beneficial FAs in milk.

Objectives and outline of the thesis

The aim of the research described in this thesis is to investigate the scope of increasing the content of PUFA in grass and maize silages, and to establish relationships between silage quality on the one hand and FA content and composition, post-ensiling stability of PUFA and milk FA composition of dairy cows on the other hand. The scientific literature is reviewed (Chapter 1) to provide insight into harvesting grass for ensiling with high content of C18:3n-3 and total FAs. In Chapter 2, the variation in FA content and composition in a large number of grass (n = 101) and maize (n = 96) silages is investigated and correlated to agronomic practices, sward quality, wilting and ensiling management, nutrient contents, feeding value and ensiling quality. For establishing the optimum harvest stage of silage maize with a high PUFA content, changes in FAs content and composition in stover (leaves and stem) and ears (cob, shank and husks) in a set of maize genotypes, grown on sandy and clay soils and harvested at 14, 42, 56, 70 and 84 days after flowering are investigated in Chapter 3. Oxidation of FAs during field wilting of herbage can cause extensive losses of PUFA. To find out environment and temporal variables that affect the losses of fatty acid during wilting. Changes in FA content and composition were compared in untreated and mechanically bruised perennial ryegrass wilted under field conditions (48 h), or wilted under controlled climate conditions at three temperatures (15, 25 or 35 °C) and two light (light and dark) regimes to DM contents of 425, 525 or 625 g/kg (Chapter 4). Lipids in forages are extensively hydrolysed in the silo with a concomitant increase in the level of free FAs of which PUFA, one of the most reactive molecules to peroxidation, are the most dominantly present. The free FAs remain stable in well-sealed and compacted silos. However, the stable environment of the silo dramatically changes after opening the silo, when silages are fed to the animals; because in the presence of oxygen plant lipoxygenases, microbes, light and pro-oxidant metal-ions can induce oxidise the free FA. Chapter-5 investigates the stability of FAs in grass and maize silages with

different qualities when exposed to air for 0, 12 and 24 h. Chapter 6 describes the effect of maize silages ensiled at targeted DM contents of 300, 340, 380 or 420 g/kg fresh matter on milk FA composition when fed to dairy cows. The final Chapter of this thesis discusses the most relevant research results presented in this thesis.

Hypotheses

The main hypotheses of this thesis were:

1. Under the current ensiling practices, FA content and composition of grass and maize silage are highly variable (Chapter 2).
2. Multivariate analysis on data related to agronomic practices, sward quality/maize harvest maturity, wilting management (grass only), chemical composition and feeding values and ensiling quality can be used to identify major causes of the variation in FA content of grass and maize silages (Chapter 2).
3. Variation in FA content and composition of grass and maize silages can be estimated from the routinely analysed data on nutrient contents, feeding value and ensiling quality (Chapter 2).
4. The post-flowering maturation of silage maize affects the FA content and composition of the stover and ear fraction of maize plants (Chapter 3).
5. Plant damage as a result of mowing-conditioning, and environmental conditions can affect the activity of lipid degrading enzymes and the rate of moisture loss, which can affect the oxidative losses of PUFA during wilting (Chapter 4).
6. During extended exposure of silages to air in the feed-out period, the free PUFA can be oxidized (Chapter 5).
7. Variation in the content and composition of carbohydrates (neutral detergent fibre:starch ratio) and FAs in maize silages, ensiled at different maturities during grain filling can affect the content and composition of milk fat of dairy cows (Chapter 6).
8. A combination of maize silages with high and low fermentable concentrate can influence the rumen environment and the transfer of FA from diet into milk.

CHAPTER 2

Causes of variation in fatty acid content and composition in grass and maize silages

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Submitted

Abstract

The aim of this study was to investigate the variation in fatty acid (FA) content and composition in grass (n = 101) and maize (n = 96) silages, randomly sampled from commercial dairy farms in the Netherlands, during 2007 and 2008. Multivariate analysis was performed on data related to agronomic practices, sward quality/maize harvest maturity, wilting management (grass only), chemical composition and feeding values of the grass and maize silages to search for variables that cause the variation in FA content and composition. The contents of all FAs, in particular the predominant polyunsaturated FAs, were highly variable in grass and maize silages. The content of C18:3n-3 showed large variations (3.57 to 20.53 g/kg DM) in grass silages, while C18:2n-6 showed large variation (6.89 to 22.41 g/kg DM) in maize silages. Redundancy analysis showed that variables related to plants maturity at harvest explained most of the variation in FA content in grass (82%) and in maize (69%) silages, with silages made of young grass and young maize having high contents of C18:3n-3. The regression analysis gave relatively good estimates for the content of C18:3n-3 ($R^2 = 0.75$) and total FAs ($R^2 = 0.65$) in grass silages, and for the content of C18:2n-6 ($R^2 = 0.64$) and total FAs ($R^2 = 0.53$) in maize silages. Among the nutrient contents and feeding values, variables related to plant maturity were the strongest "predictors" and retained in the regression equations. Bruising of grass, silage pH and ammonia-N content did not affect the FA content in the silages.

Introduction

Grass and maize silages are the major forage component in the diets of dairy cows in many countries as in the Netherlands. The membrane glycerolipids of grasses are dominated by C18:3n-3 (0.58 ± 0.16 g/g total fatty acids (FA)), whereas the storage-lipids of grains are the major lipids in maize silages, which are dominated by C18:2n-6 (0.60 g/g total FAs) (Chilliard *et al*., 2001; Van Ranst, 2009). Due to high consumption and high proportion of polyunsaturated FAs (PUFA), these basal forages are often the major source of PUFA (C18:3n-3, C18:2n-6) in dairy cow rations, despite of their relatively low FA contents (< 46 g/kg DM). As such, high PUFA-containing forages can be used to favourably modulate milk FA profile of dairy cows (Dewhurst *et al*., 2006; Elgersma *et al*., 2006; Van Ranst, 2009). Grazing dairy cows ingest large quantities of PUFA and produce milk fat with a high unsaturated:saturated FA ratio, and high content of C18:3n-3 and C18:2 *cis*-9, *trans*-11. However, transition of dairy cows from fresh grass to silage based rations significantly lower proportion of the unsaturated FA and increased proportion of saturated FA in milk fat (Elgersma *et al*., 2004; Heck *et al*., 2009). This is partly related to a lower PUFA intake from ensiled grasses, due to harvesting more mature swards for ensiling as well as oxidative losses of PUFA during ensiling (Dewhurst *et al*., 2006; Heck *et al*., 2009). Increasing the PUFA content in ensiled forages can also increase the proportion unsaturated FA, C18:3n-3 and C18:2 *cis*-9, *trans*-11 in milk fat (Heck *et al*., 2009).

Variation in FA content and composition in grass silages can originate from differences in the sward quality at mowing and in post-harvest oxidation of PUFA. In temperate grasses, the FA content and proportion of C18:3n-3 are higher during a leafy regrowth in spring and autumn, and are lower during the stemmy, reproductive regrowth in summer (Bauchart *et al*., 1984; Dewhurst *et al*., 2001). The FA content and proportion of C18:3n-3 decrease with advancing maturity of grasses (Dewhurst *et al*., 2001; Boufaïed *et al*., 2003). In addition, the FA content and composition can be distinct due to grass species or cultivars (Palladino *et al*., 2009; Van Ranst *et al*., 2009b). However, the seasonal and genetic differences can be largely manipulated by grass maturity at harvesting (Dewhurst *et al*., 2006). A high rate of N fertilization generally increase the FA content and delays the decline in FA content with advancing maturity (Mayland *et al*., 1976; Witkowska *et al*., 2008). After harvest, grass is wilted for various periods of time to reach a higher dry matter (DM) content to enhance silage fermentation quality (Steen *et al*., 1998; Wright *et al*., 2000). The wilting of grass is associated with oxidative losses of PUFA, mainly C18:3n-3, which decrease as a proportion

of the total FAs with a simultaneous increase in the proportion of C16:0 (Van Ranst *et al*., 2009a; Khan *et al*., 2011a). After ensiling, there are no or little oxidative losses of PUFA, as anaerobic conditions establish quickly in well-sealed and compacted silo.

Owing to the high DM content silage maize is not wilted, which ensure lower oxidative losses of PUFA during the ensiling process. However, silage maize is harvested at a wide range of maturity (crop DM content of 250-450 g/kg) in Northwest Europe, which can produce major changes in the FA content and composition of maize silages. During the grain filling maturation, the content of C18:1n-9 and C18:2n-6 increases in whole plant DM due to substantial growth of ears and accumulation of FAs in ears, whereas the content of C18:3n-3 decreases due to rapid senescence of the leaves (Khan *et al*., 2011b).

Some intrinsic plant components can be used to assess the FA contents of grass and maize silages. In grass and in pre-flowering maize plants, there is a strong positive relationship between chlorophyll a + b and C18:3n-3 or total FA content (Mayland *et al*., 1976; Bolton and Harwood, 1978). Similarly, a strong positive relationship exists between plant N content and C18:3n-3 or total FA content (Barta, 1975; Boufaïed *et al*., 2003; Witkowska *et al*., 2008). Quantifying the variation in FA content and composition in relation to variables that cause these variation can help to design management strategies to increase the content of PUFA in grass and maize silages. Due to high intake potential, a relatively small increase in the PUFA content in grass or maize silage can lead to substantial increase in the intake of PUFA by dairy cows. The present study was designed to investigate the variation in FA content and composition in a large number of grass and maize silages in the Netherlands. Moreover, data related to agronomic practices, sward quality, wilting and ensiling management as well as chemical composition, feeding values and ensiling quality parameters were subjected to multivariate analysis to search for variables that mark the major causes of variation in FAs content and composition.

Material and Methods

Grass and Maize Silage Samples

Samples of grass (n = 101) and maize (n = 96) silages were randomly obtained from commercial dairy farms in the Netherlands, in 2007 and 2008. Each silage-clamp/silo was sampled 8 to 10 weeks after ensiling by a trained technical assistant of a commercial

laboratory (Blgg, Oosterbeek, The Netherlands). From each grass silage, samples were collected using a hollow drill at 6 different locations (1/3, 1/2 and 2/3 of the height and width of a silo), whilst from each maize silo, samples were collected from 4 different locations (1/3 and 2/3 of the height and width of a silo). After collection, samples from each silo were pooled, placed in a polythene bag, immediately cooled and transported to the laboratory in chilly-bins. Upon arrival, individual samples were thoroughly mixed before subsamples were taken and frozen immediately at -20 °C for analysis of nutrient composition, feeding value and FAs. Data on soil type (sandy, peat, loss, clay sea, clay river and dalgrond (sandy soil mixed with bonk peat)), N fertilization (kg/year), sward type (grass species), maturity (age and regrowth period), date and number of cuttings and DM yield (kg/ha) were collected for each silage. The grass silages were prepared either from pure swards of perennial ryegrass (*Lolium perenne L.*) or mixtures of perennial ryegrass with timothy (*Phleum pratense*), smooth meadow-grass (*Poa pratensis*), field meadow-foxtail (*Alopecurus pratensis*) or Italian ryegrass (*Lolium multiforum*). Data on the proportion of grass species in case of mixed swards were recorded. Sward age was recorded as years after sowing. In addition, data on wilting and ensiling conditions were recorded for individual silages. Including plant damage at cutting to manipulate the wilting process (bruised, tedded or untreated), weather conditions (sunny, cloudy or mixed (sunny:cloudy)) at cutting, duration of wilting (in days), type of additives or acid used to improve the fermentation process, type of silo covering material and an indication of heat production during ensiling. Silage heat production was recorded subjectively (no heat, minor heat, some heat and hot). Grass silages sampled in this study were made from single cuts, and not from mixtures of two or more cuts. For maize silages, data on sowing date and maturity at harvest were collected for individual silages.

Chemical Analysis

All samples were freeze dried, ground to pass a 1 mm screen and analysed for the contents of DM, ash, crude protein (CP), crude fat (Cfat), sugar, starch (only for maize silages), acid detergent fibre (ADF), neutral detergent fibre (NDF) and acid detergent lignin (ADL). The DM content of the fresh and freeze dried samples were determined by oven drying at 103 ºC for 4 h (ISO 6496; ISO, 1999). Ash, CP, Cfat, sugar, ADF, NDF and ADL were determined using the NIRS standard calibration of a commercial laboratory (Blgg, Oosterbeek, The Netherlands). The NIRS values were calibrated according to wet chemical determinations with CP (N × 6.25) determined using the Kjeldahl method (ISO 5983; ISO,

2005), ash after incineration at 550 °C for 4 h (ISO 5984; ISO, 1978), sugars by colorimetrical determined according to the method described by Van Vuuren *et al*. (1993) and Cfat gravimetrically after 6 h extraction with petroleum ether (40/60, v/v) (ISO 6492; ISO, 1999). ADF and ADL were determined according to Van Soest (1973) and NDF according to Van Soest *et al.* (1991), with some modification as described by Khan *et al.* (2009). Starch content was determined as glucose using the amyloglucosidase method (ISO, 5914; ISO 2004) after an initial extraction of the samples with 40% ethanol (to remove the sugar fraction). Ammonia was determined according to the Berthelot method as modified by Schneider (1976). The feeding values: *in vitro* organic matter digestibility (OMD), net energy for lactation (NE), true protein digestion in the small intestine (DVE), degraded protein balance in the rumen (OEB) and structure index (SI) of the grass and maize silages were also determined using the NIRS standard calibration of Blgg. These NIRS values were calibrated using the following techniques. OMD was determined according to the method of Tilley and Terry (1963), NE for lactating dairy cows was calculated according to Van Es (1978). The Dutch protein evaluation system as described by Tamminga *et al.* (1994) was used to determine DVE and OEB. The structure index (SI) for grass and maize silages was calculated according to De Brabander *et al.* (2002).

Lipids from ground, freeze dried samples were extracted with chloroform-methanol (2:1 v/v) (1957), with some modification as described by Khan *et al.* (2009). After extractions, FAs in the residual fat were (trans) esterified, using both acid and base catalysed methods as described by Khan *et al.* (2011b). The FA methyl esters (FAMEs) were quantified by gas chromatography (GC) (TRACE GC Ultra™, Thermo Electron Corporation, Waltham, MA, USA) equipped with a flame-ionization detector (FID) and an auto-sampler. Methylated FAs were separated using a fused silica capillary column (100 m × 0.25 mm and 0.2 μm film thickness; Supelco SP-2560, Bellefonte, PA, USA), using hydrogen as carrier gas at a constant flow of 1.5 ml/min. One μl of sample was injected in the GC with a split ratio of 1:50. The following program was used for the GC: starting temperature 140 ºC, held for 4 min; increased at the rate of 4 ºC per min until 240 ºC, and then held for 20 min. The temperature of the injector and the FID was 250 and 280 ºC, respectively. Peaks were identified by comparing retention time with corresponding peaks of external standards (S37, Supelco, Bellefonte, PA, USA). Individual FAME contents were calculated from the peak area using the peak area of the internal standard (C13:0).

Statistics

Data were first graphically inspected for normality and the presence of outliers. Data were summarised by descriptive statistics and Pearson correlation coefficients were determined using the program Statistical Analysis System (SAS; 2003). To visualize the relationship between the multiple explanatory variables and FA contents, Redundancy Analysis (RDA) (Lepš and Šmilauer, 2003) were performed. Initial analysis by detrended correspondence analysis revealed that the data exhibited a linear rather than a unimodal response to explanatory variables. Redundancy analysis can be viewed as a combination of principal components analysis and multiple regressions. Compared with principal components analysis, the components of RDA maximize the variance explained by the predictor variables. Results were reproduced in a standard bi-plot ordination diagram constructed by the CanoDraw program (Ter Braak and Šmilauer, 2002), where each of the response and explanatory variables are displayed as vectors (arrows point in the direction of increasing variable values) and correlations between variables are shown by the angle between arrows. An angle of less than 90° between two arrows implies a positive correlation (the strength increases as the angle decreases from 90 to zero), whereas an angle of 90° shows no correlation and an angle above 90° shows a negative correlation (the strength increases as the angle increases from 90° to 180°). The length of an arrow depicts the strength of association between a variable and the ordination axes shown in the bi-plot.

A stepwise multiple regression procedure of SAS (2003) was used to obtain regression equations for the estimation of total and major individual FA content in grass and maize silages. Only equations with parameters contributing significantly ($P < 0.05$) to the explanation of the dependent variable were considered. Partial least square (PLS) regression analysis was carried out to calculate the maximum covariance between (all) explanatory variables and FA content of the grass and maize silages. The observations were split for model fitting (80%) and validation (20%). The models were validated through split-sample cross-validation ($n = 7$), and then tested to predict the FA content of the remaining 20 silages. Criteria used to select latent variables in equations were based on high coefficients of determination (R^2) of the model fit, predicted R^2 [based on the relationship (1:1) between predicted and measured Y values] and low standard errors of cross validation (SECV) and standard errors of estimation (SEE). SEE was calculated as the square root of the average of the squared differences between predicted and observed values.

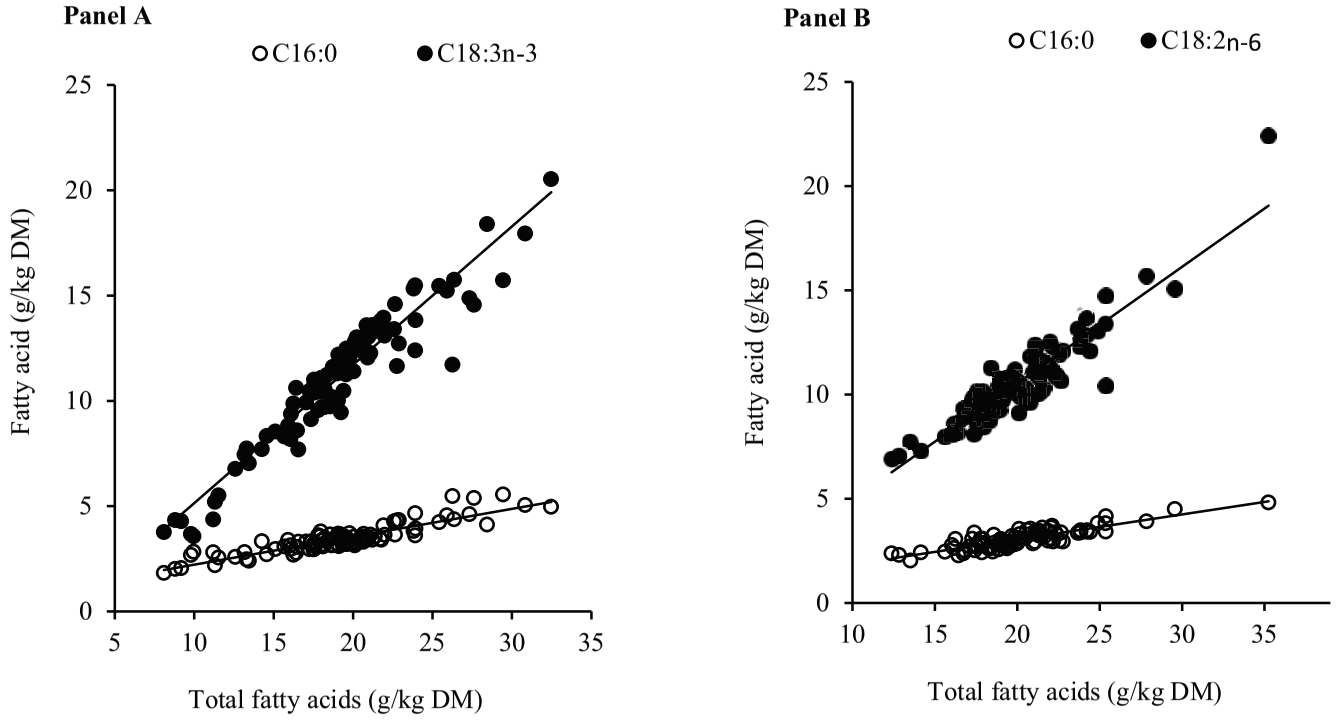

Figure 1. Change in the major polyunsaturated fatty acids and C16:0 in relation to changes in total FA content in grass (Panel A; n = 101) and maize (Panel B; n = 96) silages.

Results

Data on variation in nutrient content, feeding value, ensiling quality and FA content of grass and maize silages are summarized in Table 1. There was a high variation in the nutrient content of grass and maize silages, which was also reflected in a large variation in feeding value parameters and ensiling quality. The FA content was highly variable in grass and maize silages. In grass silages, variation in total FA content was associated with variation in the predominant FAs, C16:0, C18:2n-6 and C18:3n-3. On average, these three FAs represented 0.93 g/g of the total FAs. The content of C18:3n-3, however, showed the largest variation ranging from 3.57 to 20.53 g/kg DM. The content of C16:0 varied from 1.83 to 5.55 g/kg DM, while C18:2n-6 varied from 1.74 to 4.69 g/kg DM. Notably, the contents of C16:0 and C18:3n-3 in grass silages varied linearly with changes in the content of total FAs (Figure 1, Panel A).

In maize silages, the three major FAs; C16:0, C18:1n-9 and C18:2n-6 on average accounted for 0.90 g/g of the total FAs, and contributed predominantly to the variation in the total FAs. The content of C18:2n-6, however, showed the highest variation ranging from 3.57 to 20.53 g/kg DM. The content of C18:1n-9 varied from 2.23 to 8.83 g/kg DM while C16:0 varied from 2.03 to 4.82 g/kg DM. Unlike grass, the content of C18:3n-3 in maize silages was much lower, but showed a similarly high variation from 0.43 to 2.45 g/kg DM. The content of C16:0, and C18:2n-6 varied linearly with changes in the content of total FA in maize silages (Figure 1, Panel B).

The RDA ordination bi-plot (Figure 2; Panel A) visualizes the relationship between the multiple explanatory variables and the FA content in grass silages. The first axis of the RDA bi-plot explained 82%, while the second axis explained 4% of the variation in FA content. Axis-1 may be referred to as “plant maturity-axis”, as it correlates positively with fibre content and SI of the grass silages, whereas it correlates negatively with the contents of CP, Cfat, NE and digestibility of the grass silages. Axis-2 may be referred to as “ensiling quality-axis”, as it correlates positively with pH and sugar, whilst it correlates negatively with NH_3-N content. Hence, ensiling characteristics only have a minor influence on the variation in FA content as compared to plant maturity (4% *vs.* 82%). The contents of CP, Cfat as well as OMD, CWD, NE and DVE were positively correlated with the FA content in grass silages. In contrast, DM, NDF, ADF and ADL content, as well as a prolonged

Table 1. Nutrient composition, feeding value, ensiling quality and fatty acid contents of grass (n = 101) and maize silages (n = 96).

Parameters	Grass silages				Maize silages			
	Mean	SD	Minimum	Maximum	Mean	SD	Minimum	Maximum
Dry matter (DM)[#]	480	106	187	810	342	46	250	568
Nutrient composition g/kg DM *								
Crude protein,	163	28	81	214	71	6	58	89
Sugar	78	42	10	233	13	5	12	47
Crude fat	41	6	21	69	35	3	27	41
Neutral detergent fibre	493	45	316	610	383	36	207	467
Acid detergent fibre	278	28	179	356	210	28	16	262
Acid detergent lignin	23	6	9	40	21	28	12	296
Starch	-	-	-	-	349	56	231	691
Feeding value*								
OMD[1] (%)	75	4.0	62.1	84.2	75.2	1.8	71.1	79.3
CWD[2] (%)	69.2	5.6	49.1	79.2	-	-	-	-
DVE[3]	73.5	12.0	43.0	95.0	48.5	3.9	41.0	71.0
OEB[4]	35.2	29.2	-39.0	126.0	-30.2	4.5	-41.0	-19.0
NE[5]	6.1	4.6	4.6	7.1	6.7	0.3	6.1	8.6
Structure index[6]	3.1	0.3	2.0	3.8	1.7	0.2	0.8	2.2
Ensiling quality*								

pH	5.03	0.53	3.70	6.20	3.84	0.48	0.14	4.40
NH_3-N[7]	8.79	3.62	4.00	17.00	9.32	2.95	2.67	17.00
Fatty acids, g/kg DM								
C16:0	3.43	0.67	1.83	5.55	3.04	0.47	2.03	4.82
C18:0	0.33	0.22	0.16	1.57	0.45	0.14	0.28	1.27
C18:1n-9	0.49	0.24	0.27	2.57	4.53	1.19	2.23	8.83
C18:2n-6	3.28	0.54	1.74	4.69	10.51	2.01	6.89	22.41
C18:3n-3	11.17	3.12	3.57	20.53	1.10	0.39	0.43	2.45
Total fatty acids	19.17	4.53	8.10	32.47	19.98	3.31	12.37	35.25

SD, standard deviation; #, g/kg fresh matter ; *, Predicted through near infrared reflection spectroscopy (NIRS) by Blgg, Oosterbeek, the Netherlands.
[1] Organic matter digestibility, determined in vitro according to Tilly and Terry (1963) as modified by van der Meer (1987).
[2] Cell wall digestibility determined in vitro.
[3] Intestinal digestible protein (Tamminga *et al*., 1994).
[4] Degraded protein balance in the rumen (Tamminga *et al*., 1994).
[5] Net energy lactation (in MJ/Kg DM) calculated with VEM (feed unit lactation) system (Van Es, 1978).
[6] Structure index, in units from 1 to 5 according to De Brabander *et al*. (2002).
[7] Ammonia N g /100 g total N.

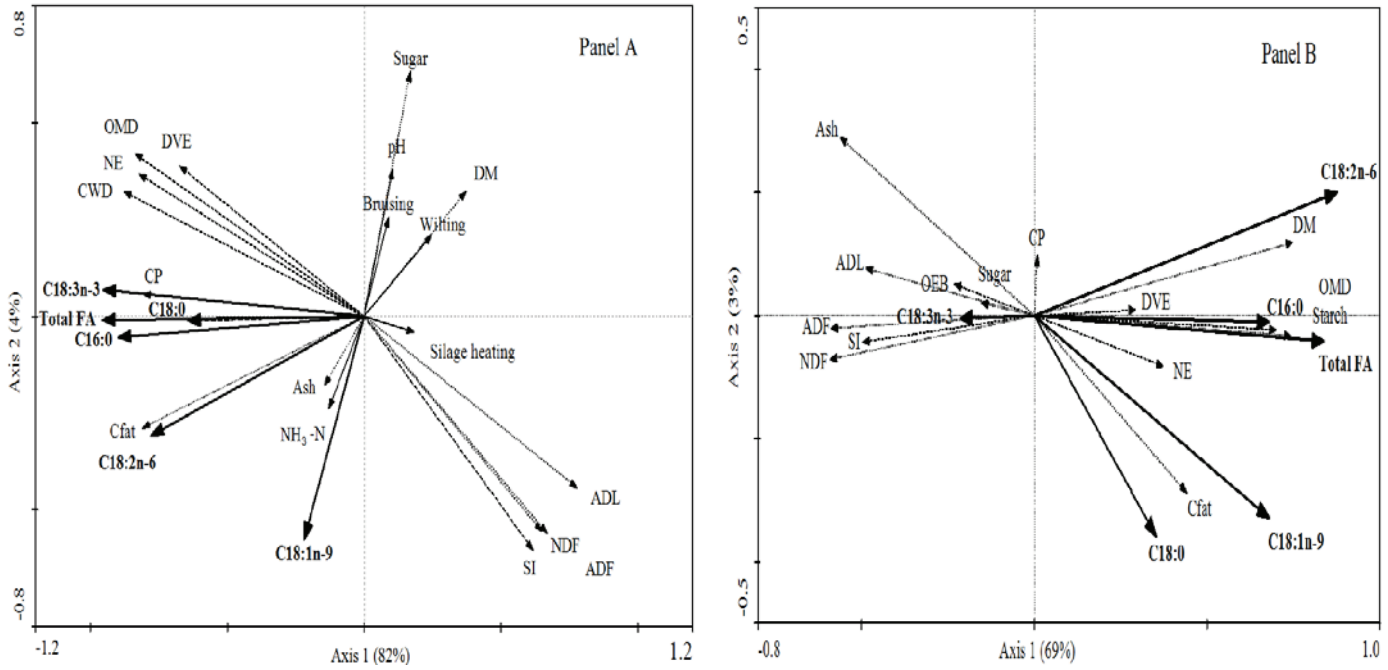

Figure 2. Redundancy analysis (RDA) ordination diagrams, visualizing the relationship between the multiple explanatory variables and fatty acids contents in grass (panel A; n=101) and maize (panel B; n=96) silages. The explanatory variables are indicated by different arrow (↑): chemical fraction (doted arrows), feeding value (dashed arrow) and, sward and wilting management as well as ensiling quality (narrow solid arrows). The fatty acid contents are represented as bold solid arrows. DM, dry matter; NDF, neutral detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin; Cfat, crude fat; CWD, cell wall digestibility; OMD, *in vitro* organic matter digestibility; DVE, metabolisable protein; OEB, degraded protein balance; NE, net energy lactation; SI, structure index; NH_3-N, ammonia N content; FA, fatty acids.

wilting period and a high SI were negatively correlated with the FA content in grass silages. Silage heating negatively affected the FA content. Bruising of grass, silage pH and NH_3-N content did not significantly affect the FA content. The Pearson correlation (full data not shown) between the chemical composition or feeding values and C18:3n-3 content was high for ADL (-0.73; $P < 0.001$), NDF (-0.66; $P < 0.001$), ADF (-0.64; $P < 0.001$), OMD (0.80; $P < 0.001$), NE (0.79; $P < 0.001$) and CWD (0.77; $P < 0.001$).

The bi-plot visualizing the relationship between multiple explanatory variables and FA content of maize silages using RDA is presented in Figure 2 (Panel B). The first two axis of RDA explained 69% and 3% of the variation in the FA content in maize silages. As in grass silages, the first axis may be referred to as "maturity-axis", as it correlates positively with the contents of DM, starch, Cfat, NE and digestibility, (indicating nutrient filling of grains during progressive maturation). Whereas, it correlates negatively with contents of fibre and SI of maize silages. The RDA shows that the content of starch, DM and Cfat, NE as well as DVE and OMD were positively correlated with the total and major individual FA content in maize silages. In contrast, the content of plant fibre, SI and OEB correlated negatively with the total and major individual FA content. However, the effect of NE, DVE and OEB on the FA content was much smaller as indicated by the small arrows (Figure 2; Panel B). The Pearson correlation analysis (full data not shown) between chemical composition or feeding values with C18:2n-6 content showed a high coefficient with starch (0.73; $P < 0.001$), DM (0.70; $P < 0.001$), Cfat (-0.67; $P < 0.001$), OMD (0.61; $P < 0.001$), NE (0.61; $P < 0.001$) and SI (-0.61; $P < 0.001$). The content of C18:3n-3 was negatively related with plant maturity and showed a negative correlation with DM (-0.49; $P < 0.001$) and starch content (-0.36; $P < 0.01$).

Table 2 shows the step-wise multiple linear regression equations and their predictive power to estimate the contents of total and major individual FAs in grass and maize silages. For grass silages, equations were obtained for the estimation of C16:0, C18:2, C18:3n-3 and total FA content, based on nutrient content or feeding value (Table 2). The equations showed a relatively high degree of fit for the estimation of C18:3n-3 ($R^2 \geq 0.72$) and total FAs ($R^2 \geq 0.63$). Equations based on nutrient composition or feeding values had similar R^2 values (Table 2), while equations based on sward and wilting management showed a lower goodness of fit (data not shown). For maize silages, regression equations were based on combined data of both nutrient content and feeding value, due to the low predictive power of separate equations based on either nutrient content and feeding values.

Table 2. Multiple regression equations, degree of fit (R^2) and standard error of estimation (SEE) for the prediction of C16:0, C18:1n-9 , C18:2n-6, C18:3n-3 and total fatty acids (FA) contents (g/kg DM) in grass and maize silages.

	Regression equation	R^2	SEE
Grass silages[1]			
Based on nutrient composition[3]			
C16:0	3.35 – 0.0596 × ADL+0.0357 × Cfat	0.43	0.65
C18:2n-6	2.22 + 0.0550 × Cfat – 0.0268 × ADL – 0.0056 × Ash	0.52	0.38
C18:3n-3	14.29 – 0.2644 × ADL + 0.2310 × Cfat – 0.0131 × NDF	0.73	1.66
Total FAs	16.93 – 0.4739 × ADL + 0.3220 × Cfat	0.65	2.74
Based on feeding values[4]			
C16:0	–1.82 + 0.0758 × CWD	0.40	0.52
C18:2n-6	–0.52 + 0.0687 × CWD – 0.0148 × DVE + 0.0037 × OEB	0.39	0.43
C18:3n-3	–39.10 + 0.7161 × OMD + 0.0229 × OEB – 0.0605 × DVE	0.72	1.70
Total FAs	–50.29 + 0.9988 × OMD + 0.2763 × OEB – 0.0915 × DVE	0.63	2.82
Maize silages[2]			
Based on nutrient composition and feeding values			
C16:0	1.267 + 0.051 × Cfat	0.17	0.38
C18:1n-9	– 2.943 + 0.141 × Cfat + 0.013 × DM – 0.0431 × ash	0.39	0.89
C18:2n-6	3.0472 + 0.0266 × DM – 0.0173 × OMD + 0.2087 × Cfat	0.64	1.14
C18:3n-3	– 4.46 – 0.0043 × DM + 0.095 × OMD + 0.0344 × Cfat – 0.0039 × Starch	0.41	0.30
Total FAs	– 0.0035 + 0.03 × DM – 3.247 × SI + 0.446 × Cfat	0.48	2.11

[1] Fatty acids (mean ± SD g/kg DM): C16:0 (3.43 ± 0.67), (3.28 ± 0.54), C18:3n-3 (11.17 ± 3.12), total fatty acids (19.17 ± 4.53).

[2] Fatty acids (mean ± SD g/kg DM): C16:0 (3.04 ± 0.47), C18:1n-9 (4.53 ± 1.19), C18:2n-6 (10.51 ± 2.01), C18:3n-3 (1.10 ± 0.39) total fatty acids (19.98 ± 3.31).

[3] Chemical composition in g/kg DM; ADL, acid detergent lignin; Cfat, crude fat; CP, crude protein; DM, dry matter; NDF, Neutral detergent fibre.

[4] CWD_{IV}, Cell wall digestibility (%, in vitro); OMD_{IV}, Organic matter digestibility (%, In vitro); DVE, intestinal digestible protein (Tamminga *et al*., 1994); OEB, degraded protein balance (Tamminga *et al*., 1994); SI, structure index (De Brabander *et al.*, 2002).

The equations showed high goodness of fit values for C18:2n-6 ($R^2 = 0.64$) and total FAs ($R^2 > 0.48$). In general the PLS model gave more or less similar R^2 values for the prediction of total and major individual FAs as stepwise multiple regressions (data not shown). However, PLS models produced lower ranges of prediction errors for both the SEE and SECV compared to stepwise regression.

Discussion

The results show that the contents of the predominant PUFA in grass and maize silages were highly variable. Dairy cows can consume large quantities of grass and maize silages, magnifying the potential variation in C18:3n-3 content of grass and C18:2n-6 content of maize silages. A consumption of 10 kg DM of grass silage with the lowest or the highest C18:3n-3 content in the present study produces a difference in intake of C18:3n-3 of 170 g/d. Similarly a consumption of 10 kg maize silage DM produces a difference in intake of C18:2n-6 of 155 g/d. The large variation in FA content in grass and maize silages highlights the need to determine the quality of individual silages in terms of their FA content and composition.

Quantitatively, grass species and cultivars, N fertilization, cutting-date/season, wilting duration and environmental conditions at cutting can all affect the FA contents of grasses silages (Dewhurst *et al*., 2006; Arvidsson, 2009). However, the multivariate analysis showed that most of the variation in FA content in grass silages was caused by difference in maturity at harvest. Grass silages made from young grass were associated with high C18:3n-3 and high total FAs content, whilst, the FA content decreased with increasing maturation of grass. A decreasing FA content during maturation of forages is attributed to a decrease in the leaf/stem ratio (Dewhurst *et al*., 2001; Boufaïed *et al*., 2003), maturation of leaves (Hawke, 1973), as well as initiation of flowering (Dewhurst *et al*., 2006) and leaf senescence (Mishra and Sangwan, 2008; Yang and Ohlrogge, 2009). Among the predominant individual FAs in grass silages, the content of C18:3n-3 showed a much larger change per unit change in the total FAs content (slope of the line) compared to C16:0 and C18:2n-6 (Figure 1, Panel A). A high content of total FAs in grass silages was associated with a high proportion of C18:3n-3, and a decreasing content of total FAs was associated with a decrease in the proportion of C18:3n-3 and an increase in the proportion of C16:0 in total FA. As a consequence, a lower content of total FAs in grass silages means a loss of quality FAs (lower C18:3n-3 g/g total FAs). Extensive research has established that with

advancing maturity of forages, the content of C16:0, C18:2n-6 and C18:3n-3 declines. However, the content of C18:3n-3 declines at a preferentially faster rate, especially during extended maturation and senesce of the plants (Mishra and Sangwan, 2008; Yang and Ohlrogge, 2009) or at the onset of reproductive growth (Dewhurst *et al*., 2006). In addition, oxidation of PUFA during wilting decreases the content of C18:3n-3 at a faster rate compared to C16:0 and C18:2n-6, causing the proportion of C18:3n-3 in the total FAs to decrease while the proportion of C16:0 and C18:2n-6 increases (Van Ranst *et al*., 2009a).

Extended wilting as well as a high DM content at ensiling had a similar negative effect on the FA content of grass silages (Figure 2 Panel A). This means that an increase in DM content of grass silages by either plant maturity or wilting decreases the FA content and composition in a more or less similar way. The level of pH ($r = 0.14$; $P > 0.05$) and NH_3 –N ($r = 0.15$; $P > 0.05$) did not affect the FA content of grass silages. In addition, silage pH and NH_3 –N were associated with the 2nd component of RDA which explained only 4% of the variation in the FA content. A number of earlier studies reported that in a well-sealed and compacted silo, FAs remain stable irrespective of herbage species, cutting date, DM content at ensiling as well as the type and extent of fermentation (Dewhurst and King, 1998b; Arvidsson *et al*., 2009; Van Ranst *et al*., 2009a). Although there is some evidence for a lower proportion of C18:3n-3 and a higher proportion of C16:0 in grass silages with high NH_3 fermentation (Dewhurst and King, 1998a). These effects were, however, quantitatively much smaller and our results did not show a significant effect of a high level of NH_3 on the FA content in grass silages. In agreement with Khan *et al.* (2011a), bruising did not affect the FA content in grass silages. This finding has a significant practical application; bruising of grass increases the rate of moisture loss and could reduce the wilting period and therefore losses of PUFA.

The variation in FA content in maize silages was mainly associated with differences in maturity of the maize plants at harvest, which varied considerably as indicated by the large variation in DM contents (Table 1). The lower content of C18:3n-3 (major FA of the membrane lipids) in maize silages show that the FA contribution from the stover (leaves and stem) part is very lower. This is related to the late harvesting of silage maize during grain filling. During post-flowering maturation the content of C18:3n-3, the major FA in stover, decreases due to decreasing proportion of the stover in the whole plant DM and the declining FA content in the stover DM (Khan *et al*., 2011b), due to a rapid senescence of green leaves (Struik, 1983). During leaf senescence, chloroplast membranes degrade and

unsaturated FAs, particularly C18:3n-3, are oxidized (Thompson *et al*., 1998; Yang and Ohlrogge, 2009). On the other hand, due the large contribution of FAs from ears (grains), the FA composition of maize silages reflects to a great extent the FA composition of that of fully matured ears, as reported by Khan *et al.* (2011b). Farmers tend to harvest maize as late as possible to obtain a high starch contents in their silages. However, this strategy is associated with a lower C18:3n-3 content. The use of "stay-green" maize genotypes, which retain thylakoid membrane longer during senescence, might allow the preservation of green leaves and the content of C18:3n-3 towards the end of the grain filling period and combine a high starch content with a high C18:3n-3 content.

The large variation in FA content in grass and maize silages highlights the need for a rapid and accurate assay to determine the quality of individual silages in terms of their FA content. The FA content and composition of ensiled forages can be determined using GC, which is an accurate method but requires relatively time-consuming extractions, derivatization and chromatography steps, and is unfeasible for routine analysis of silages at dairy farms. Currently research is on-going in our lab for direct assessment of FA content in grass and maize silages by NIRS. Alternatively, the multiple regression equations may be used to estimate the FA content in grass and maize silages from the nutrient contents and feeding values. Currently, the nutrient composition, feeding value parameters and ensiling quality of individual silage clamps in the Netherland is routinely measured using NIRS analysis. Farmers and feed manufacturers can use the standard information provided and the regression equations here to assess the FA content of the grass and maize silages. The predominant PUFA, i.e. C18:3n-3 in grass silages and C18:2n-6 may be assessed even more reliably. This can help to design management strategies to increase the PUFA content in grass and maize silages and targeted feeding regimes to increase dietary supply of PUFA to favorably modulate milk fat composition of dairy cows to benefit human health.

CHAPTER 3

Changes in fatty acid content and composition in silage maize during grain filling

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Abstract

The stage of maturity at harvest has a major effect on the fatty acid (FA) content and composition of forage plants consumed by dairy cows. The present study investigated the dynamics of FA content and composition in stover (leaves and stem) and ears (cob, shank and husks) of two maize genotypes (G2 and G6) grown on sandy and clay soils and harvested at 14, 42, 56, 70 and 84 days after flowering (DAF). In addition, the FA content and composition of six maize genotypes (G1-G6) grown on the two soil types were compared at the normal harvest time of early genotypes in the Netherlands (70 DAF). The contents of total FAs and major individual FAs in both stover and ears changed significantly ($P < 0.001$) during the grain-filling period (14-84 DAF). In stover the contents of C16:0, C18:2n-6, C18:3n-3 and total FAs declined ($P < 0.001$) while those of C18:0 and C18:1n-9 increased ($P < 0.001$) with progressive grain filling. The rate of decline in C18:3n-3 and total FAs contents was slower during 14-56 DAF as compared with 56-84 DAF. In ears, the contents of C16:0, C18:1n-9, C18:2n-6 and total FAs increased up to 56 DAF and then remained more or less constant until 84 DAF. At 70 DAF the content of polyunsaturated FAs in both stover and ears did not differ among the six genotypes. However, the average contents of C16:0, C18:3n-3 and total FAs in stover were higher ($P < 0.05$) on clay soil, whereas those of C18:0 and C18:1n-9 were higher on sandy soil. The results demonstrate that the maximum polyunsaturated FAs content in silage maize is harvested around 56 DAF, in the present study at a T_{sum} of 927 °C.d or at an ear dry matter content of 440 g/kg, which is before the onset of rapid senescence. Any further delay in harvesting will cause a rapid decline in C18:3n-3 content in maize silages.

Introduction

Dairy cows fed a maize silage-based ration produce milk with a lower C18:3n-3 content and an elevated *n*-6/*n*-3 polyunsaturated fatty acid (PUFA) ratio compared with cows fed on pasture (Schroeder *et al*., 2005), pasture plus concentrate (Schroeder *et al*., 2003; Rego *et al*., 2004; Schroeder *et al*., 2005) or grass silage (Havemose *et al*., 2004; Nielsen *et al*., 2006). In addition, replacing grass silage with increasing proportions of maize silage in the diet of dairy cows decreased the proportion of C16:0 and C18:3n-3 and increased the proportion of C18:2, causing an elevated *n*-6/*n*-3 PUFA ratio in milk (Kliem *et al*., 2008). Increasing the content of C18:3n-3 in maize silages to elevate the proportion of C18:3n-3 and reduce the high *n*-6/*n*-3 PUFA ratio in milk fat of dairy cows is an important long-term and environmentally sustainable strategy for the dairy industry.

Fatty acids (FAs) in silage maize originate from two distinct sources: (1) membrane lipids in the leaves and stem (stover) and (2) storage lipids in the kernels. The membrane lipids of stover are the mainpool of C18:3n-3, while C18:1n-9 and C18:2n-6 are predominantly present in kernels. The membrane lipids in photosynthetic tissues are mainly present in membranes of chloroplasts (Hawke, 1973; Joyard *et al*., 1998), and contain as the major FA. A strong positive relationship exists between chlorophyll a + b and total FA contents (Mayland *et al*., 1976). In developing maize leaves the content of chlorophyll increases, causing a parallel increase in C18:3n-3 and total FAs (Bolton and Harwood, 1978) Therefore silage maize harvested with a larger green leaf area will represent a high mass of chloroplasts and, as a result, membrane lipids and C18:3n-3. However, for high dry matter (DM) and starch yields and lower effluent losses from ensiled material, silage maize is generally harvested at an advanced stage of grain filling.

The progression of maturation from anthesis onwards results in a considerable increase in the mass of ears, with ears being an increased fraction of the total plant (Boon *et al*., 2005b) In the whole plant dry mass the content of C18:3n-3, the major chloroplast FA, is lowered by a decreasing mass fraction of stover. In addition, the content of C18:3n-3 in stover decreases with plant maturity owing to a decrease in green leaf area and an increase in the content of other metabolites such as cellulose, hemicellulose and lignin (Clapham *et al*., 2005). During progressive grain filling, the green leaf area of maize plants decreases as a result of leaf senescence (Pommel *et al*., 2006). Although leaf senescence in maize plants starts during vegetative growth (Pommel *et al*., 2006), it generally continues at a slow rate

during most parts of the grain-filling period and increases towards the end of the growing season (Struik, 1983; Pommel *et al*., 2006). During leaf senescence, chloroplast membranes degrade (Thompson *et al*., 1998), causing the content of chloroplast lipids and their associated FAs to decline (Harwood *et al*., 1982; Mishra *et al*., 2006). However, the content of unsaturated FAs, particularly C18:3n-3, declines at a preferentially faster rate during leaf senescence (Mishra and Sangwan, 2008; Yang and Ohlrogge, 2009).

In the Netherlands, maize plants are harvested at various stages of grain filling, at a whole plant DM content ranging from 250 to 450 g/kg. The progressive maturity during grain filling could have a major effect on the FA content and composition of maize plants. In addition, maize is ensiled directly after cutting owing to its low moisture content. In a well-sealed silo the FAs remain stable irrespective of the type and extent of fermentation, (Van Ranst *et al*., 2009a), and oxidative losses during the feed-out period are limited (Khan *et al*., 2009). Therefore any increase in the quantity of unsaturated FAs, particularly C18:3n-3, in silage maize at harvest will result in a concomitant increase in the intake of these FAs by the dairy herd. However, information on the changes in FA content and composition in maize plants during grain filling is scarce. In the present study the relationship between variables marking the progress of maturation or grain filling in silage maize and the changes in FA content and composition for a set of genotypes varying in feeding value and for two different agronomic environments were investigated.

Material and methods

Experimental Site and Crop Management

Six maize genotypes (coded G1–G6) differing in their rate of grain filling and starch degradability were sown in 48 plots on 5 May 2008 at the research facility Unifarm of Wageningen University, Wageningen, The Netherlands (51° 58′ N, 5° 40′ E, 7 m a.s.l.). Plots of 9 m × 15 m (12 rows of 15 m with a row spacing of 0.75 m) were blocked on clay and sandy soils, and all genotypes were replicated four times within each soil type in a complete randomised block design. The final plant density was 10 plants/m^2. Samples from both soil types were analysed for their nutrient profile. Based on the nutrient composition of the soils, all plots were fertilized on 28 April with 80 kg P_2O_5/ha and 300 kg K_2O/ ha. At sowing, 30 kg N/ha and 30 kg P_2O_5/ha was applied to the rows of both soil types. A week

after emergence plots on the sandy soil were also fertilised with 205 kg N/ ha, while plots on the clay soil received 215 kg N/ha. On average, plants emerged on 12 May on the sandy soil and on 13 May on the clay soil. A week after emergence, 30 kg N/ha was applied to the rows of both soil types. Complete control of weeds was achieved by applying a herbicide on 30 May. Plant development was monitored by recording weekly the number of fully grown leaves on a 1 m long strip of two randomly selected rows of each plot from the 13th leaf stage onwards. The appearance of silks (silking) was recorded every 3 d. The crops were considered flowering when 50% of the plants showed silking (Table 1).

Table 1. Time of 50% silking (in days and degree days) of six genotypes grown at two sites (clay and sandy soil) near Wageningen, the Netherlands in 2008.

Genotype	Sandy		Clay	
	Days after sowing	T_{sum} (°C.d)	Days after sowing	T_{sum} (°C.d)
G1	74	491	77	504
G2	74	491	78	511
G3	74	491	76	498
G4	75	495	79	521
G5	75	495	79	521
G6	77	504	79	521

T_{sum} , temperature sum (°C.d, with a base temperature of 10 °C).

Sample Collection and Handling

The first samples were taken on 5 August 2008, approximately 14 d after flowering (DAF), followed by four more harvests at 42, 56, 70 and 84 DAF. At each sampling time a 1 m long strip from two adjacent rows (measuring 1.5 m^2 and containing approximately 15 plants) was harvested from all plots. To ensure a representative sampling, a 3 m wide strip on all sides of each plot was not sampled, and, at subsequent harvests, a 1 m area adjacent to the previously sampled area was excluded. At every sampling, plants were cut at ground level on non-rainy days. The harvested plants from each plot were labelled, covered to protect them from light and direct air, and immediately transported to the laboratory for handling and data collection. The exact number of plants per plot was counted and the fresh

weights of whole crop, stover and ears were recorded. After weighing, three plants of uniform size per plot were subsampled and the ears (cob, shank and husks) were removed. After dissection the ears and the remaining plant portion (stover) were chopped separately at 1 cm and representative samples (~700 g fresh weight) were taken and frozen at − 20 °C until further analysis.

Weather Data

Weather data were recorded at the Meteorological Station of Wageningen University, located within 1 km of the experimental fields. The temperature sum (T_{sum}) in degree days (°C.d) was calculated from the daily average temperatures minus the base temperature, accumulated over the period from sowing onwards, using the following equation (Sibma, 1987):

$$T_{sum} = \sum [(T_{max} + T_{min})/2 - T_{base}]$$

Where T_{max} (°C) is the daily maximum temperature, T_{min} (°C) is the daily minimum temperature and T_{base} = 10 °C.

Chemical Analysis

All samples were freeze-dried and ground to pass through a 1 mm screen. Their DM content was determined by oven drying at 103 °C for 4 h. For FA analysis, lipids from the ground freeze-dried samples were extracted with chloroform/methanol (2:1 v/v; Folch *et al*., 1957) with some modifications as described by Khan *et al*. (2009). After extraction, FAs in the residual fat were (trans)esterified using both acid- and base-catalysed methods. For the basic methylation, 3 mL of 0.5 mol/L NaOH methanolate was added to the extracted fat, then the mixture was vortexed and heated for 30 min at 50 °C. After cooling, 2 mL of HCl/methanol (1:1 v/v) was added and the mixture was vortexed and heated for 10 min at 50 °C. After cooling, 2 mL of hexane and 2 mL of distilled water were added and the mixture was shaken vigorously and centrifuged at 800 × g for 5 min at 20 °C. The hexane fractions containing the fatty acid methyl esters (FAMEs) were collected and transferred to 5 mL tubes. Another 2 mL of hexane was added to the non-hexane fractions, which were then shaken vigorously and centrifuged at 800 × g for 5 min at 20 °C. The hexane layers were collected, pooled with the previous fractions and evaporated to dryness

under an N_2 flux in speed vac (Saint-Herblain, France). The residual FAMEs were dissolved in 1 mL of hexane and transferred to gas chromatograph (GC) vials. FAMEs were quantified using a TRACE Ultra™ GC (Thermo Electron Corporation, Waltham, MA, USA) equipped with a flame ionisation detector (FID) and an auto-sampler. Methylated FAs were separated on an SP-2560 fused silica capillary column (100 m × 0.25 mm, 0.2 µm film thickness; Supelco, Bellefonte, PA, USA) using He as the carrier gas at a constant flow of 1.5 mL/min. A 1 µL sample containing methylated FAs was injected into the GC with a split ratio of 1:50. The GC temperature programme was as follows: initial temperature 140 °C, held for 4 min; increased at 4 °C per min to 240 °C, held for 20 min. The injector and FID temperatures were 250 and 280 °C respectively. Peaks were identified by comparing retention times with corresponding peaks of external standards (S37, Supelco). Individual FAME contents were calculated from the peak area of the FAME in the sample relative to the peak area of an internal standard (C13:0).

Statistical Analysis

The effects of harvest date (14, 42, 56, 70 and 84 DAF), genotype (G2 and G6) and soil type (sandy and clay) on FA contents of stover and ears were determined by repeated measure analysis of variance using the PROC MIXED procedure (Littell *et al*., 2006) of Statistical Analysis System (SAS, 2003). Harvest date was considered as a repeated effect on individual plots. Soil type, genotype, harvest date and their two- and three-way interactions were considered as fixed effects and replication was considered as a random effect. The different covariance structures of repeated matrices for FA content in stover (model 1) and ears (model 2) were evaluated according to Littell *et al*. (1998) and Wang and Goonewardene (2004) using the Akaike information criterion and the Schwarz Bayesian criterion. A heterogeneous compound symmetry was the best fit for model 1, while ANTE was the best fit for model 2. At 70 DAF the effects of genotype (G1–G6) and soil type (sandy and clay) on the FA content and composition of stover or ears were analysed using the PROC MIXED procedure of SAS with soil and genotype as fixed effects and plot and replication as random effects (model 3). Assumptions for both models were evaluated by examining the distribution of residuals.

Results

Plant Growth and Weather Data During Study

Silking dates for the six genotypes did not differ much, but did differ between soil types (Table 1). The plants started to silk from 74 to 77 days after sowing on the sandy soil and from 76 to 79 days after sowing on the clay soil. T_{sum} (°C.d, with a base temperature of 10 °C) at flowering ranged from 491 to 504 °C.d and from 504 to 521 °C.d on the sandy and clay soils respectively.

Harvest dates and weather data during the preceding growth period are presented in Table-2. T_{sum} consistently declined during the grain-filling season. During 56–84 DAF the mean daily temperature declined rapidly, causing a major decrease in T_{sum}.

Table 2. Sampling dates and weather conditions during the growing interval preceding the harvest dates.

Harvesting Date	DAF	Weather during preceding 14-d period				
		Mean T (°C)	Max T (°C)	Min T (°C)	T_{sum} (°C.d)	Rain (mm)
5-8-2008	14	20.1	22.0	11.5	151	211.2
19-8-2008	28	17.2	21.9	12.4	100	65.1
2-9-2008	42	17.1	20.9	13.0	98	16.8
16-9-2008	56	15.7	19.5	11.6	76	73.6
30-09-2008	70	11.3	16.6	5.8	18	35.5
14-10-2008	84	11.7	15.6	8.1	23	28.7

DAF, days after flowering; T_{sum} , temperature sum (°C.d, with a base temperature of 10 °C).

During grain filling, the DM content of ears increased (R^2 = 0.95) linearly with T_{sum}. However, the increase in stover and whole crop DM contents with T_{sum} was quadratic (Figure 1).

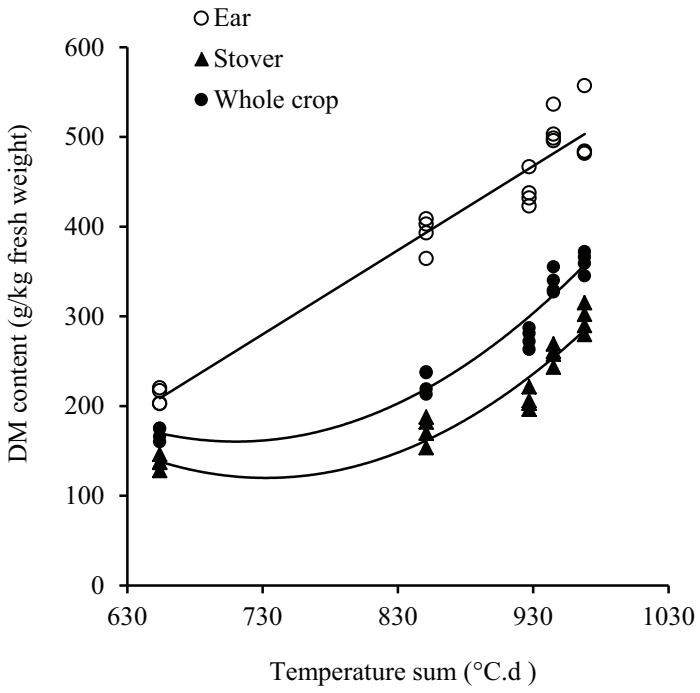


Figure 1. Relationship between the temperature sum (T_{sum}) and the DM content (g/kg fresh weight) of the whole plant and of the stover and the ears for two genotypes grown at two sites.

Regressions:

$DM_{ear} = -404.8 + 0.938 \times T_{sum}$, $R^2 = 0.95$, $P < 0.001$

$DM_{stover} = 1253 - 3.335 \times T_{sum} + 0.0024 \times (T_{sum})^2$, $R^2 = 0.92$, $P < 0.001$

$DM_{whole\ crop} = -253.65 + 7.279 \times T_{sum} + 0.011 \times (T_{sum})^2$, $R^2 = 0.95$, $P < 0.001$

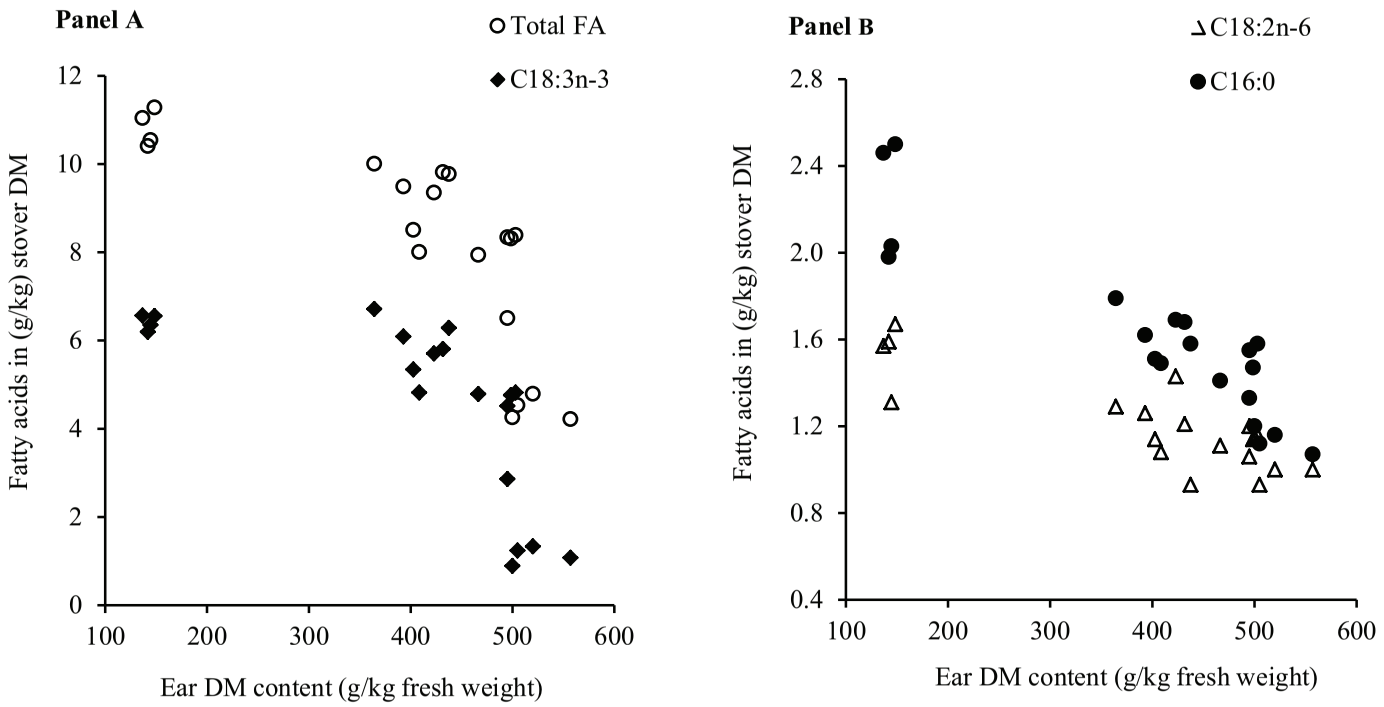


Figure 2. Relationships between the dry matter (DM) content of the ears of the maize plants and the content of total FAs, C18:3n-3 (Panel A), and C18:2n-6 and C16:0 (Panel B) in maize stovers.

Effect of harvest date, soil type and genotype on FA content in stover

The content of C16:0, C18:2n-6, C18:3n-3 and total FAs in the stover declined ($P < 0.001$) during grain filling (Table 3). During 14-56 DAF, the content of C18:3n-3 did not decline significantly and the proportion of C18:3n-3 in total FAs remained more or less constant at 0.60 g/g total FAs. During 56-84 DAF, the content of C16:0, C18:2n-6, C18:3n-3 and total FAs declined by 0.29, 0.13, 0.81 and 0.51 g/g DM, respectively. Due to the faster decrease in C18:3n-3 content during the latter phase, the proportion of C18:3n-3 decreased from 0.60 to 0.25 g/g total FAs with a simultaneous increase in *n*-6 to *n*-3 PUFA ratio from 0.21 to 0.90. Due to a linear increase in the DM content of ears with T_{sum} (Figure 1), the ear DM is an indication for the maturity of the maize plants. The contents of total FAs, C18:3n-3, C18:2n-6 and C16:0 in the stover decreased with an increasing DM of the ears (Figure 2 Panel A and B). C18:0 and C18:1n-9 were present in small quantities in the stover; their content increased ($P < 0.001$) during the grain filling period.

When averaged across harvests, the content of C18:2n-6 in stover differed significantly ($P < 0.001$) between soil types, while there was no difference in the contents of C16:0, C18:0, C18:1n-9, C18:3n-3 and total FAs between soil types (Table 3). Further comparison of the FA data within harvests revealed higher contents of C18:2n-6 ($P < 0.05$), C18:3n-3 ($P < 0.01$) and total FAs ($P < 0.05$) on the clay soil during 56–84 DAF than on the sandy soil (data not shown). The contents of C16:0 ($P < 0.05$), C18:2n-6 ($P < 0.001$) and total FAs ($P < 0.05$) differed significantly between G2 and G6. Significant harvest × genotype and harvest × soil type × genotype interactions were observed for the contents of C18:0 and C18:1n-9.

Effects of Harvest Date, Soil Type and Genotype on Fatty acid Content in Ears

The contents of C16:0, C18:1n-9, C18:2n-6 and total FAs in ears increased ($P < 0.001$) up to 56 DAF and then remained more or less constant (Table 4). C18:1n-9 and C18:2n-6 were the major FAs in ears; their proportions increased from 0.07 and 0.52 g/g total FAs at 14 DAF to 0.21 and 0.62 g/g total FAs at 56 DAF respectively and then remained more or less constant until 84 DAF.

Table 3. Content (g/kg dry matter (DM)) of individual and total fatty acids (FA) in the stover (leaves and stem) of maize plants from two different genotypes (G2, G6), grown on a clay and a sandy soil, and harvested on different days after flowering (DAF).

DAF	Soil type	Genotype	C16:0		C18:0		C18:1n-9		C18:2n-6		C18:3n-3		Total FAs	
			Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
14	Clay	G2	1.98	0.119	0.17	0.009	0.11	0.006	1.67	0.067	6.19	0.56	10.41	0.70
		G6	2.03	0.119	0.19	0.009	0.12	0.006	1.57	0.069	6.35	0.56	10.54	0.70
	Sandy	G2	2.50	0.165	0.20	0.013	0.11	0.006	1.59	0.089	6.58	0.56	11.43	0.97
		G6	2.46	0.190	0.23	0.009	0.14	0.006	1.31	0.067	6.56	0.56	11.04	0.70
42	Clay	G2	1.62	0.086	0.13	0.018	0.06	0.007	1.29	0.057	6.09	0.53	9.50	0.66
		G6	1.52	0.086	0.18	0.018	0.09	0.007	1.08	0.057	5.05	0.53	8.14	0.66
	Sandy	G2	1.79	0.086	0.11	0.018	0.07	0.007	1.26	0.057	6.71	0.53	10.17	0.66
		G6	1.49	0.086	0.16	0.018	0.08	0.007	1.14	0.057	4.81	0.53	8.01	0.66
56	Clay	G2	1.68	0.140	0.22	0.011	0.23	0.015	1.43	0.100	5.81	0.56	9.81	0.75
		G6	1.58	0.140	0.20	0.011	0.12	0.015	1.11	0.100	6.28	0.56	9.77	0.75
	Sandy	G2	1.69	0.140	0.20	0.011	0.12	0.015	1.21	0.100	5.71	0.56	9.35	0.75
		G6	1.41	0.140	0.21	0.011	0.13	0.015	0.93	0.100	4.79	0.56	7.94	0.75
70	Clay	G2	1.58	0.087	0.24	0.015	0.16	0.028	1.20	0.054	4.82	0.44	8.40	0.51
		G6	1.47	0.087	0.26	0.015	0.20	0.028	1.06	0.054	4.76	0.44	8.31	0.51
	Sandy	G2	1.55	0.087	0.33	0.015	0.20	0.028	1.16	0.054	4.51	0.44	8.34	0.51
		G6	1.33	0.087	0.28	0.015	0.25	0.028	1.14	0.054	2.86	0.44	6.50	0.51

84	Clay	G2	1.16	0.057	0.33	0.016	0.43	0.046	1.15	0.076	1.33	0.13	4.79	0.17
		G6	1.12	0.057	0.31	0.016	0.44	0.046	1.00	0.076	1.24	0.13	4.53	0.17
	Sandy	G2	1.20	0.057	0.34	0.016	0.39	0.046	1.00	0.076	0.89	0.13	4.26	0.17
		G6	1.07	0.057	0.31	0.016	0.40	0.046	0.93	0.076	1.08	0.13	4.21	0.17
Overall means DAF														
14			2.24^{a}	0.066	0.19^{c}	0.005	0.13^{c}	0.003	1.54^{a}	0.038	6.42^{a}	0.040	10.86^{a}	0.387
42			1.60^{b}	0.042	0.14^{d}	0.009	0.08^{d}	0.004	1.19^{b}	0.029	5.67^{a}	0.010	8.95^{b}	0.331
56			1.59^{b}	0.070	0.20^{c}	0.006	0.14^{c}	0.007	1.17^{bc}	0.050	5.65^{a}	0.100	9.22^{b}	0.373
70			1.48^{b}	0.044	0.28^{b}	0.007	0.20^{b}	0.014	1.14^{bc}	0.028	4.23^{b}	0.130	7.88^{c}	0.253
84			1.13^{c}	0.028	0.32^{a}	0.007	0.42^{a}	0.023	1.02^{c}	0.020	1.13^{c}	0.140	4.45^{d}	0.083
Significance														
Soil			NS		NS		NS		***		NS		NS	
Genotype			*		NS		NS		***		NS		*	
Harvest			***		***		***		***		***		***	
Harvest × Genotype			NS		**		***		NS		NS		NS	
Harvest × Soil × Genotype			NS		**		**		NS		NS		NS	

Means with different superscripts (^{abcd}) within columns differ at $P < 0.05$.

NS, not significant;*, $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 4. Contents (g/kg dry matter (DM)) of individual and total fatty acids (FA) in the ears (cob, shank and husks) of maize plants from two different genotypes (G2, G6), grown on clay or sandy soil, and harvested on different days after flowering (DAF).

DAF	Soil type	Genotype	C16:0		C18:0		C18:1n-9		C18:2n-6		C18:3n-3		Total FAs	
			Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
14	Clay	G2	2.14	0.059	0.12	0.008	0.52	0.022	3.67	0.162	0.68	0.062	7.24	0.278
		G6	2.18	0.059	0.15	0.008	0.60	0.022	4.01	0.162	0.83	0.062	7.90	0.278
	Sandy	G2	2.16	0.059	0.12	0.008	0.47	0.022	4.48	0.162	0.89	0.062	8.25	0.278
		G6	2.35	0.067	0.16	0.009	0.66	0.026	4.79	0.185	1.11	0.062	9.23	0.310
42	Clay	G2	3.36	0.109	0.29	0.012	4.95	0.211	14.15	0.542	0.51	0.115	23.30	0.871
		G6	3.69	0.109	0.34	0.012	5.41	0.211	14.82	0.542	0.50	0.115	24.79	0.871
	Sandy	G2	3.38	0.109	0.30	0.012	4.95	0.211	14.60	0.542	0.52	0.115	23.86	0.871
		G6	3.64	0.109	0.33	0.012	5.43	0.211	14.93	0.542	0.50	0.115	24.89	0.871
56	Clay	G2	3.39	0.090	0.36	0.014	5.46	0.202	16.06	0.281	0.52	0.010	25.97	0.553
		G6	3.56	0.090	0.40	0.014	5.98	0.202	16.23	0.281	0.52	0.010	26.78	0.553
	Sandy	G2	3.22	0.090	0.34	0.014	4.94	0.202	15.80	0.281	0.52	0.010	25.02	0.553
		G6	3.59	0.090	0.40	0.014	5.98	0.202	16.37	0.281	0.52	0.010	27.00	0.553
70	Clay	G2	3.14	0.085	0.36	0.015	5.00	0.212	16.07	0.410	0.46	0.017	25.11	0.722
		G6	3.26	0.085	0.48	0.015	5.99	0.212	15.21	0.410	0.44	0.017	25.51	0.722
	Sandy	G2	3.13	0.085	0.36	0.015	4.81	0.212	15.91	0.410	0.47	0.017	24.83	0.722
		G6	3.09	0.085	0.38	0.015	5.39	0.212	14.93	0.410	0.41	0.017	24.29	0.722
84	Clay	G2	3.15	0.066	0.36	0.014	4.88	0.173	15.93	0.424	0.48	0.011	24.89	0.669

Sandy	G6	3.27	0.066	0.47	0.014	6.22	0.173	15.03	0.424	0.43	0.011	25.44	0.669
	G2	3.05	0.066	0.34	0.014	4.71	0.173	15.87	0.424	0.47	0.011	24.52	0.669
	G6	3.35	0.066	0.48	0.014	6.42	0.173	16.15	0.424	0.44	0.011	26.93	0.669
Overall mean DAF													
14		2.20^{c}	0.030	0.14^{d}	0.004	0.56^{b}	0.001	4.24^{c}	0.084	0.87^{a}	0.032	8.16^{c}	0.144
42		3.52^{a}	0.054	0.31^{c}	0.006	5.18^{a}	0.105	14.62^{b}	0.271	0.51^{b}	0.006	24.20^{b}	0.436
56		3.44^{a}	0.044	0.38^{b}	0.007	5.57^{a}	0.100	16.12^{a}	0.140	0.51^{b}	0.005	26.19^{a}	0.277
70		3.16^{b}	0.042	0.39^{b}	0.007	5.30^{a}	0.106	15.53^{ab}	0.205	0.44^{c}	0.010	24.94^{ab}	0.361
84		3.20^{b}	0.033	0.41^{a}	0.007	5.56^{a}	0.086	15.75^{a}	0.211	0.45^{c}	0.010	25.45^{ab}	0.335
Statistical significance													
Soil		NS		NS		NS		NS		**		NS	
Genotype		***		***		***		NS		NS		**	
Harvest		***		***		***		***		***		***	
Harvest × Genotype		NS		***		***		NS		**		NS	
Harvest × Soil × Genotype		NS		**		NS		*		*		*	

Means with different superscripts (^{abc}) within columns differ at $P < 0.05$.
NS, not significant; *, $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

The contents of C18:1n-9, C18:2n-6 and total FAs increased with increasing ear DM content (Figure 3). Owing to the linear increase in the DM content of ears with T_{sum} (Figure 1), ear DM content is a good indicator of the stage of maturity of maize plants. Owing to the major accumulation of C18:1n-9 and C18:2n-6, the proportion of 16:0 decreased from 0.27 to 0.13 g/g total FAs during 14–56 DAF, despite an increase in the content of C16:0 from 2.20 to 3.44 g/kg DM. Soil type did not affect the FA content in ears during the grain-filling period (Table 4). The contents of C16:0, C18:0, C18:1n-9 ($P < 0.001$) and total FAs ($P < 0.01$) differed between G2 and G6. There was a significant harvest × genotype interaction for the contents of C18:0, C18:1n-9 and C18:3n-3 and a significant harvest × soil × genotype interaction for the contents of C18:0, C18:2n-6, C18:3n-3 and total FAs (Table 4).

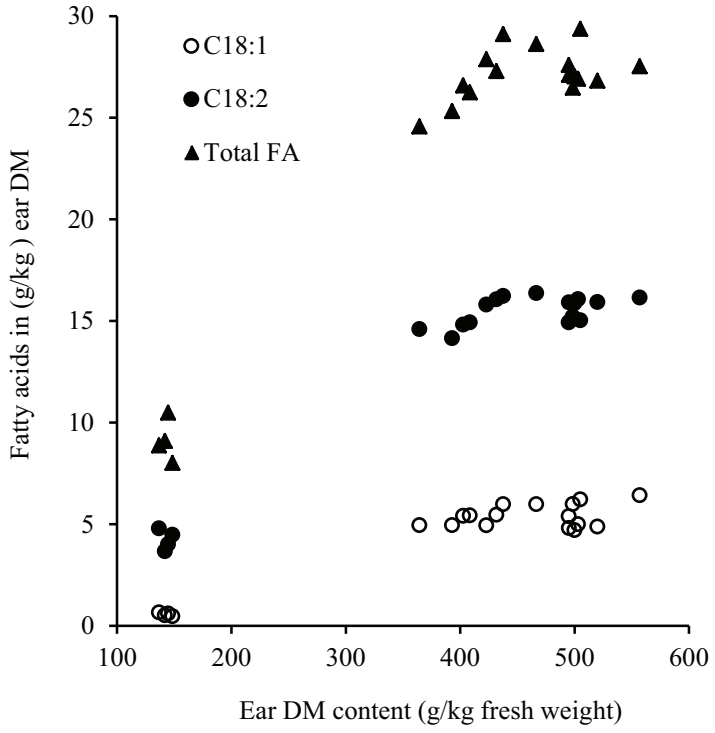


Figure 3. Relationships between the DM content of the ears of the maize plants and the content of C18:1n-9, C18:2n-6 and total FAs in the maize ears.

Effects of Soil Type and Genotype on Fatty acid Content at Normal Harvest-date

Investigating the FA content in six maize genotypes at 70 DAF (T_{sum} 945 °C.d) showed that soil type significantly affected the contents of C16:0 ($P < 0.05$), C18:0 ($P < 0.001$), C18:1n-9 ($P < 0.01$), C18:3n-3 ($P < 0.01$) and total FAs ($P < 0.05$) in stover (Table 5). Harvesting at 70 DAF represents the normal harvest time for early hybrids of silage maize in the Netherlands.

Table 5. Effect of genotype and soil type on DM content (g/kg fresh weight) and on content (g/kg DM) of individual and total fatty acids (FA) in the stover (leaves plus stem) of maize plants at 70 days after flowering.

	DM	C16:0	C18:0	C18:1n-9	C18:2n-6	C18:3n-3	Total FAs
Genotype							
G1	251	1.39	0.33^{a}	0.16^{b}	1.09^{b}	3.84	7.52
G2	256	1.51	0.28^{a}	0.17^{b}	1.14^{b}	4.50	8.27
G3	260	1.49	0.29^{a}	0.21^{b}	1.05^{b}	3.92	7.67
G4	225	1.43	0.22^{b}	0.16^{b}	1.00^{b}	4.43	7.88
G5	253	1.36	0.28^{a}	0.28^{a}	1.38^{a}	3.97	7.95
G6	259	1.35	0.26^{a}	0.22^{b}	1.06^{b}	3.67	7.30
SEM		0.056	0.012	0.026	0.053	0.367	0.434
Soil							
Clay	244	1.47^{A}	0.24^{B}	0.16^{B}	1.09	4.63^{A}	8.22^{A}
Sandy	257	1.37^{B}	0.31^{A}	0.24^{A}	1.14	3.48^{B}	7.31^{B}
SEM		0.032	0.011	0.015	0.053	0.217	0.250
Significance							
Genotype		NS	***	*	**	NS	NS
Soil		*	***	**	NS	**	*
Genotype × Soil		#	NS	NS	NS	#	*

Means with different superscripts (^{ab} or ^{AB}) within a column differ at $P < 0.05$.
NS, not significant; #, $P < 0.10$; *, $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Averaging across the six genotypes, the contents of C16:0, C18:3n-3 and total FAs were higher on the clay soil than on the sandy soil, while the contents of C18:0 and C18:1n-9 were higher on the sandy soil than on the clay soil. The contents of the two major chloroplast FAs, C16:0 and C18:3n-3, did not differ between the six genotypes. Although there were significant genetic differences in the contents of C18:0 ($P < 0.001$), C18:1n-9 ($P < 0.05$) and C18:2n-6 ($P < 0.01$) in stover, these differences were quantitatively small. Three major FAs of stover, C16:0, C18:2n-6 and C18:3n-3, made up 0.18, 0.14 and 0.52 g/g total FAs in stover respectively.

For ears, soil type significantly affected the contents of C16:0 ($P < 0.05$), C18:0 ($P < 0.01$), C18:1n-9 ($P < 0.01$) and total FAs ($P < 0.05$) (Table 6). Averaging across the six genotypes, the contents of C16:0, C18:0, C18:1n-9 and total FAs in ears were higher ($P < 0.05$) on the clay soil than on the sandy soil. There were differences in the contents of C18:0 ($P < 0.001$) and C18:1n-9 ($P < 0.01$) between genotypes. However, the contents of C16:0, C18:2n-6, C18:3n-3 and total FAs in ears did not differ among the six genotypes. The three major FAs, C16:0, C18:1n-9 and C18:2n-6, on average accounted for 0.13, 0.21 and 0.62 g/g total FAs in ears respectively, together representing 0.96 g/g total FAs in ears.

Discussion

Unlike grass, maize silages are rich in C18:1n-9 and C18:2n-6 but low in C18:3n-3, resulting in a lower C18:3n-3 content and an elevated *n*-6/*n*-3 PUFA ratio in milk fat (Chilliard *et al*., 2001; Kliem *et al*., 2008). Extensive research has established that membrane lipids in forages are rich in PUFA, particularly C18:3n-3 (Hawke, 1973; Clapham *et al*., 2005), and forages can be used as a sustainable strategy for enhancing the content of PUFA in milk fat (Dewhurst *et al*., 2006). The present study explored the relationship between maturation and the changes in FA content and composition of maize plants in order to provide information for harvesting a high level of C18:3n-3 (the major chloroplast FA) and PUFA in maize silages.

The progressive maturity during the grain-filling period has a major influence on the content and composition of FAs in the stover and ear fractions of maize plants. The content of C18:3n-3, the major FA in photosynthetic tissues (Hawke, 1973; Joyard *et al*., 1998), in whole crop DM was synergistically decreased by the decreasing proportion of stover and the

Table 6. Effect of genotypes and soil type on DM content (g/kg fresh weight) and on content (g/kg DM) of individual and total fatty acids (FA) in the ears (cob, shank plus husks) of maize plants at 70 days after flowering.

	DM	C16:0	C18:0	C18:1n-9	C18:2n-6	C18:3n-3	Total FAs
Genotype							
G1	515	3.07	0.34^{c}	4.62^{ab}	14.60	0.40	23.14
G2	499	2.94	0.35^{c}	4.37^{b}	14.23	0.96	23.00
G3	510	3.11	0.45^{ab}	5.70^{a}	17.39	0.45	27.29
G4	507	3.42	0.41^{b}	5.76^{a}	16.45	0.43	26.58
G5	523	3.03	0.49^{a}	5.26^{a}	15.65	0.49	25.03
G6	517	3.20	0.43^{ab}	5.73^{a}	15.17	0.43	25.06
SEM		0.108	0.016	0.286	0.866	0.209	1.060
Soil							
Clay	507	3.22^{A}	0.43^{A}	5.54^{A}	16.05	0.58	25.84^{A}
Sandy	517	3.04^{B}	0.39^{B}	4.92^{B}	15.12	0.48	24.19^{B}
SEM		0.051	0.011	0.150	0.460	0.121	0.554
Significance							
Genotype		NS	***	**	NS	NS	NS
Soil		*	**	**	NS	NS	*
Genotype × Soil		NS	NS	NS	NS	NS	*

Means with different superscripts (^{abc} or ^{AB}) within a column differ at $P < 0.05$.
NS, not significant; *, $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

declining content of C18:3n-3 in stover. The stover fraction decreased from 0.90 g/g whole crop DM at 14 DAF to 0.40 g/g whole crop DM at 84 DAF (data not shown). In addition, during 14–84 DAF the contents of C16:0, C18:2n-6, C18:3n-3 and total FAs in stover declined by 0.50, 0.33, 0.82 and 0.59 g/g respectively. Although the contents of total and major individual FAs declined consistently during the grain-filling period, their rates and patterns of decline differed markedly. The decline in C18:3n-3 and total FA contents occurred more or less parallel and showed biphasic rates, with a period of relatively low decrease during 14–56 DAF, where the proportion of C18:3n-3 remained more or less constant (0.60 g/g total FAs). However, in the period from 56 to 84 DAF the contents of C18:3n-3 and total FAs in stover declined rapidly. In the latter period the content of

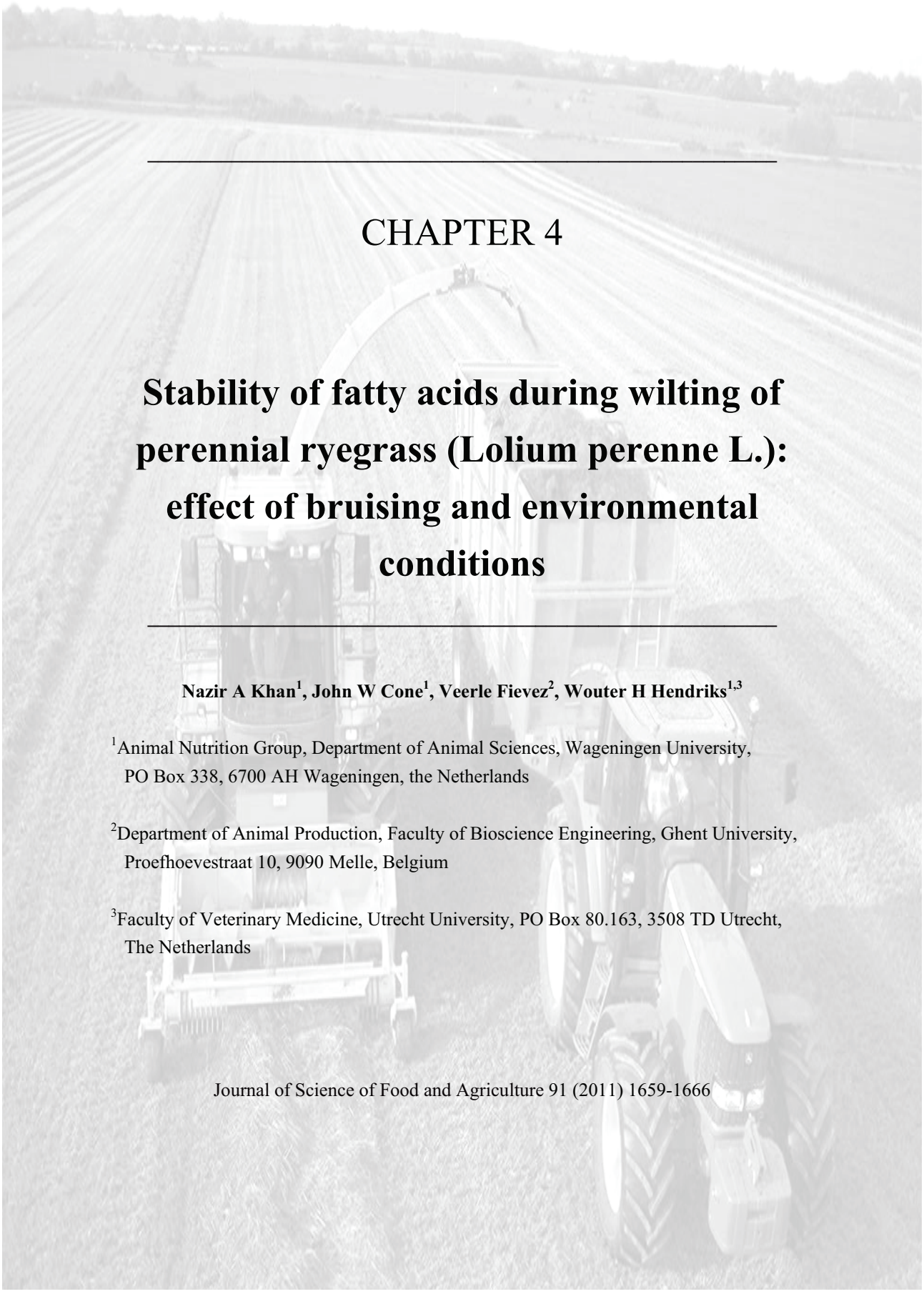
C18:3n-3 declined by 0.81 g/g DM, causing the proportion of C18:3n-3 to decrease from 0.61 to 0.25 g/g total FAs, with a concomitant increase in *n*-6/*n*-3 PUFA ratio from 0.21 to 0.90. The biphasic rates of decline in C18:3n-3 content could be explained partly by a similar pattern of decrease in the green leaf area during the grain-filling period. In maize plants the leaf area increases vertically from the bottom leaves until leaf 11 and then declines again. The middle canopy (leaves 8–12 from the bottom) around the ear represents around 50–60% of the total leaf area in maize (Dwyer *et al*., 1992). Leaf senescence starts at leaf 1 at the bottom during vegetative growth (Boon *et al*., 2005a) and progresses acropetally to the adjacent leaves during early grain filling. Owing to the senescence of smaller leaves at the bottom, the decrease in total green leaf area occurs at a relatively slow rate during early grain filling. The large leaves in the middle of maize plants stay green for a longer period. Towards the end of grain filling, leaf senescence also progresses from the tassel towards the ear leaf. In the last 4 weeks of the grain-filling period the green leaf area declines at a more rapid rate under Dutch environmental conditions (Struik, 1983). During this period, leaf senescence advances from both top and bottom and leaves with larger area senesce, increasing the rate of loss of green leaf area (Valentinuz and Tollenaar, 2004).

The decrease in green leaf area, initiation of reproductive growth (Boufaïed *et al*., 2003) and increasing content of cell wall components (cellulose, hemicellulose and lignin) (Clapham *et al*., 2005) during plant maturation decrease the contents of C16:0, C18:2n-6, C18:3n-3 and total FAs in a number of forages (Dewhurst *et al*., 2001; Boufaïed *et al*., 2003; Vanhatalo *et al*., 2007). In addition, during leaf senescence the membrane lipids of the chloroplasts degrade (Thompson *et al*., 1998), causing a decline in the contents of C16:0, C18:2n-6, C18:3n-3 and total FAs (Harwood *et al*., 1982; Wanner *et al*., 1991; Mishra *et al*., 2006). The content of C18:3n-3, however, decreases at a much faster rate than the contents of C16:0 and C18:2n-6 during prolonged leaf senescence (Mishra and Sangwan, 2008; Yang and Ohlrogge, 2009), which could explain the rapid decrease in C18:3n-3 content compared with C18:2n-6 and C16:0 contents in the present study. In contrast to C18:3n-3, the content of C18:2n-6 in stover declined at a decreasing rate while the content of C16:0 declined at a more or less constant rate during the grain-filling period. The proportion of C16:0 increased from 0.17 to 0.25 g/g total FAs and the proportion of C18:2n-6 increased from 0.12 to 0.22 g/g total FAs during the latter phase, despite the decline in their contents. This could be explained by the preferentially faster and quantitatively larger decline in the content of C18:3n-3 in the second phase.

Both G2 and G6 flowered earlier on sandy soil than on clay soil and showed high contents of C16:0 and C18:3n-3 (major chloroplast FA) during the first harvest on sandy soil. In contrast, at 56–84 DAF the contents of C16:0, C18:3n-3 and total FAs were higher on clay soil. The latter trend was also observed for the six genotypes at 70 DAF. This discrepancy in the FA content during the grain-filling period on the two soil types could partly be explained by a faster growth and accumulation of high stover (green leaves) mass at the first harvest on sandy soil and to a subsequent rapid decline in stover dry mass mainly due to leaf senescence and shedding of dead leaves during 56–84 DAF (data not shown). In the present study the rate of stover DM remobilisation, as calculated from the difference in stover dry mass at 14 DAF and at the subsequent harvests, was faster on sandy soil during 56–84 DAF, particularly during the final 14 days. The increased rate of remobilisation of stover DM could explain the concomitant increase in leaf senescence and lower C16:0, C18:3n-3 and total FA contents on sandy soil (Valentinuz and Tollenaar, 2004). The significant interactions between genotype and harvest date during grain filling and between soil and genotype at 70 DAF demonstrate the importance of constant factors such as soil characteristics as well as more variable factors such as selection of suitable genotypes and management of harvest date for harvesting high C18:3n-3 and total FA contents in silage maize.

Although the maximum accumulation of FAs in ears was recorded at 56 DAF (T_{sum} 927 °C.d, ear DM content 440 g/kg), the rate of FA deposition was much faster during the period from 14 to 42 DAF. In this period the contents of C16:0, C18:1n-9, C18:2n-6 and total FAs increased by 0.60, 0.83, 2.45. and 1.97 g/g ear DM respectively. Rapid synthesis of FAs occurs only during a brief period early in the development of oil-bearing seeds. Weber (1969) reported a major deposition of lipids in the developing kernels during 15 to 45 days after pollination, which coincides with the findings of our study, as nutrients in the ears are mainly stored in the maize kernels. The proportions of C16:0, C18:1n-9 and C18:2n-6 in total FAs increased up to 56 DAF and then remained more or less constant until 84 DAF. Ear DM content increased linearly with T_{sum} (R^2 = 0.95) and is therefore a good indicator of the stage of maturity of maize plants. As the contents of C16:0, C18:2n-6, C18:3n-3 and total FAs in stover decreased during maturation, an inverse relationship between stover FA content and ear DM content was observed. Furthermore, ear DM content was a strong variable to estimate the increase in contents of C18:1n-9, C18:2n-6 and total FAs in ears with maturity.

The maximum amount of PUFA in whole crop was harvested before the onset of rapid senescence around 56 DAF (T_{sum} 927 °C.d, ear DM content 440 g/kg). The current study shows that a delay in cutting at the end of the grain-filling period should be avoided in order to harvest high amounts of green leaves and C18:3n-3 in maize silages. Alternatively, maize hybrids with a high green leaf area index, and which stay greener towards the end of the grain-filling period, can be expected to maintain a high content of C18:3n-3 at harvest. Unlike grass, which needs an extended period of wilting before ensiling, the lower moisture content of silage maize enables its direct ensiling after cutting. Unsaturated FAs remain stable during a short exposure to air between harvest and ensiling (Arvidsson *et al*. 2009; Ueda *et al*., 2002). Furthermore, in a well-sealed and compacted silo, anaerobic conditions are established within about 30 min of ensiling (Woolford, 1990), and the content of FAs remains stable irrespective of the type and extent of fermentation (Van Ranst *et al*., 2009a). The oxidative losses of unsaturated FAs during feed-out are quantitatively small (< 0.05 g/g total FA; Khan *et al*., 2009). Therefore any increase in the quantity of unsaturated FAs, particularly C18:3n-3, in silage maize at harvest will result in a more or less similar increase in the intake of these FAs by the dairy herd. Moreover, owing to the high ingestion of maize silages by dairy cows, a small increase in the content of PUFA in silage will result in a relatively large increase in their intake. Dietary supply of PUFA is the major environmental factor influencing both the content and relative proportion of PUFA in milk fat of dairy cows (Chilliard and Ferlay, 2004).



CHAPTER 4

Stability of fatty acids during wilting of perennial ryegrass (Lolium perenne L.): effect of bruising and environmental conditions

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Abstract

Oxidation of fatty acids (FA) during field wilting of herbage could cause extensive losses of polyunsaturated FA. Recent studies showed a variable effect of wilting on the losses of FA in grasses. This suggests that environment and management conditions influence the loss of FA during wilting. The present study investigated the stability of FA in untreated and mechanically bruised perennial ryegrass, wilted under field conditions for 0, 12, 24, 36 and 48 h, or wilted under controlled climate conditions at three temperatures (15, 25 or 35 °C) and two light (dark or light) regimes to dry matter (DM) contents of 425, 525 or 625 g/kg. During 48 h of field wilting, the total FA content declined (15.2 to 11.9 g/kg DM) consistently, despite an increase in herbage DM content (197 to 676 g/kg). Under controlled climate conditions, the herbage total FA content declined (15.1 to 11.7 g/kg DM) mainly during the prolonged (56 to 62 h) initial drying to a DM content of 425 g/kg and did not decline with further drying to DM contents of 525 and 625 g/kg. The decline in total FA was mainly caused by a parallel decline in C18:3n-3 content under field (9.15 to 6.36 g/kg DM) and controlled (9.12 to 6.15 g/kg DM) conditions. Concomitantly, the proportion of C18:3n-3 in total FAs decreased, whilst the proportion of C16:0 and C18:0 increased. Lower losses of FA ($P < 0.05$) were observed at 15 °C compared to 25 and 35 °C. Light did not affect the losses of FA during wilting. The duration of the wilting period mainly affected the changes in FA content and composition. Stability of FA in herbage could be increased by minimizing the duration of wilting.

Introduction

The membrane lipids in photosynthetic tissues of forages are rich in polyunsaturated fatty acids (PUFA; C18:3n-3, C18:2n-6) (Hawke, 1973; Clapham *et al*., 2005), and strongly contribute to the supply of C18:3n-3 in the ration of dairy cows. In many temperate countries, perennial ryegrass is the main herbage fed to dairy cows in fresh (grazed) or ensiled form. Milk fat from dairy cows grazing on the fresh grass has a high proportion of C18:2 *cis*-9, *trans*-11 and C18:3n-3 and a lower proportion of saturated FA compared to cows fed silage-based rations during the winter (Heck *et al*., 2009) The positive effect of herbage lipids on the milk FA profile is diminished by ensiling, which is related to the oxidation of PUFA during field wilting and hydrolysis of membrane lipids in the silo (Dewhurst *et al*., 2006) After harvesting, grass is wilted for various durations of time to reach a DM content of 250–500 g/kg at ensiling to enhance silage fermentation quality (Wright *et al*., 2000). However, wilting of grass can cause oxidative loss of PUFA. In response to the stress of cutting, the membrane bound plant lipases are activated and hydrolyse FA from the membrane lipids (Thomas, 1986). These free FA are dominated by PUFA, which are one of the most oxygen sensitive molecules in nature (Spiteller, 2003), and make a favourable substrate for plant lipoxygenases, by which these are oxidised into a number of volatile compounds (Fall *et al*., 1999; Feussner and Wasternack, 2002).

The duration of wilting has been shown to have a marked effect on the extent of FA loss. Extended (68 h) wilting of ryegrass (Dewhurst and King, 1998) to a DM content of 230 g/kg, and wilting of red clover (Van Ranst *et al*., 2009a) for 5 days, to a DM content of 300 g/kg led to high losses of FA, particularly C18:3n-3. However, Udea *et al*. (2002) found no differences in FA content and composition between direct cut silage (no wilting) of legumes and the fresh material. Moreover, a wilting process shorter than 24 h to a DM content of 330–350 g/kg, did not have any effect on the proportions of FA in timothy (Arvidsson *et al.*, 2009). Similarly, forced artificial drying of fresh grass for 24 h (50 °C) did not affect FA composition (Fievez *et al*., 2004). On the other hand there is some evidence that the change in herbage DM content during wilting affects the loss of FA. Timothy wilted to a DM content of 450 g/kg within a few hours (Boufaïed *et al*., 2003) and ryegrass ensiled after wilting to a DM content of 700 g/kg for 24 h showed reduced FA content and proportion of C18:3n-3 (Elgersma *et al*., 2003).

Although former studies suggest that the duration of wilting or the change in DM content during wilting could influence the losses of FA during wilting, the importance of both factors could not be quantified from the comparison of different experiments. Moreover, the variable responses in the former studies might have been induced by differences in environmental factors such as temperature, light, humidity etc. and in the extent of mechanical damaging of plant tissues at cutting (Van Ranst, 2009). In many temperate countries, including the Netherlands, grasses are often bruised to increase the rate of moisture loss. Bruising of grass involves a mechanical abrade of plant tissues. This is a severe type of wounding and therefore could multiply the response of plant enzymes to oxidise PUFA (Spiteller, 2003). During cutting and bruising, the thylakoid membranes could be disrupted, and chlorophyll pigments tend to remain associated with hydrophobic component such as membrane lipids. The presence of chlorophyll with the highly unsaturated membrane lipids could induce photo-oxidation (Foote, 1976; Makoni *et al.,* 1993).

The aim of this study was to investigate the influence of bruising and environment conditions on stability of FA during wilting of perennial ryegrass. Changes in FA content and composition were studied in untreated and mechanically bruised perennial ryegrass: wilted under field conditions for 0, 12, 24, 36 and 48 h, or wilted under controlled climate conditions at three temperature (15, 25 or 35 °C) and two light (light *vs*. dark) regimes to a DM contents of 425, 525 or 625 g/kg. In order, to determine environment and management variables that relate to the variation in stability of FA during wilting of perennial ryegrass.

Material and methods

Plant Material and Sampling

The study used a homogenous stand of perennial ryegrass (*Lolium perenne* L.), sown in September 2005, at the research fields of Wageningen University in Wageningen, the Netherlands (51° 58′ N and 5° 40′ E, 7 m a.s.l.). The sward consisted of the cultivars Elgon and Veritas (70 and 30%). During the growing years, the fields were fertilised in spring (April) with 90 kg N/ha as NH_4NO_3, 20–25 kg P/ha as P_2O_5, and 25–27 kg K/ha in the form of K_2O. In addition, 81 kg N/ha as $(NH_4)_2SO_4$ was applied prior to each re-growth. The grass was cut for ensiling at intervals of 4–6 weeks over the growing seasons (April to

September). To prepare sward for the current study, grass grown on eight plots (24 m × 25 m) were cut on 27 May and fertilised with 81 kg N/ha on 3 June 2009. For the experimental trials, the grass was harvested on 1 July, after 34 d of re-growth and at a DM yield of 2000 kg/ha. Grass on four plots was harvested with a mechanical plot harvester (Haldrup, Løgstør, Denmark), with mowers set at a height of 6 cm. The plot harvester is designed to handle the harvested material gently. Grass on the other four plots was harvested with a conditioner mower (John Deere-1365; John Deere, Moline, IL, USA) which removes the waxy layer of leaves and stems of the grass with some additional crimping.

Immediately after harvest, representative samples of the fresh herbage (0 h wilting) were collected from each plot. To ensure representative sampling, grass from each plot was collected from four randomly selected 1 m^2 spots. Each sampling spot was sub-divided into six squares of equal size and samples of about 500 g of fresh herbage were collected from each square. The samples from each plot were pooled and carried in bags to the laboratory for further analysis. The pooled samples was thoroughly mixed and a representative sample of about 700 g was immediately stored in a freezer at − 20 °C, representing the 0 h samples. The weather data (Table 1) were recorded at the Meteorological Station of Wageningen University, located within 300 m of the experimental fields.

Table 1. Weather data during the wilting period, starting at 9 am, July 1, 2009.

Hours	Temperature °C			Irradiation W/m²			Humidity (%)	Wind m/sec
	Mean	Min.	Max.	Long wave*	Short wave#	Total		
0-12	24.9	24.1	25.5	369	494	863	65.2	1.88
12-24	19.5	18.9	20.4	353	138	490	87.2	1.48
24-36	27.8	27.0	28.6	393	470	863	50.7	2.13
36-48	21.9	21.3	22.7	366	133	499	85.4	2.03

*, Radiation > 3 µm.
#, Radiation between 0.4 and 3 µm.

Wilting

In the field, both untreated and bruised grass was wilted for 48 h. The John Deere-1365 conditioner mower harvested a 3 m wide strip of grass and made a windrow of 1–1.5 m wide (of bruised grass), while in case of the plot harvester, the harvesting strip and windrows of untreated grass were more or less of similar width (1.2–1.5 m).The windrows of bruised grass were much thicker than the windrows of untreated grass during the first 12 h. However, the grass was turned/tedded with a mechanical turner after 12, 24, 36 and 48 h which spread the grass uniformly for both bruised and untreated grass. The plots were sampled after 12, 24, 36 and 48 h of harvesting. Samples from each plot were pooled, mixed and 700 g of the herbage per plot was sub-sampled and frozen immediately. The mean daily temperature was 27 °C and the wilting days were sunny. The average temperature during the night was 20 °C with no sunlight for approximately 9 h and 30 min.

Parallel to the field wilting, the fresh (0 h wilted) samples from the untreated and bruised herbage were wilted in climate chambers. The bruised or untreated grass was assigned in triplicate to six drying conditions according to a factorial combination of three temperatures (15, 25 and 35 °C) and two light regimes (light and dark). For each combination of temperature and light a separate climate chamber was used. The humidity was fixed at 60–65% and the air flow was maintained at 0.2 m/s. The light was emitted by white cold lamps (SON-T AGRO 400; Philips, Eindhoven, the Netherlands), set at a height of 1 m and illumination of ca. 792 W/m^2. The grass was turned manually at 9 am and 9 pm daily. Each replicate contained about 1 kg of untreated or bruised grass, which was spread in a tray (37 × 56.5 cm and 15 cm high) making a 5 cm thick layer. The trays contained holes of about 0.6 cm in diameter on the side walls. Moreover, each tray was divided into three equal sub-parts through a wire netting. Grass from one of the three sub-parts was collected as sample after reaching the target DM content of 425, 525 or 625 g/kg fresh matter. This ensured a uniform sampling without affecting the wilting process of the remaining herbage. In the climatic chambers, sampling at the target DM content was achieved by determining the rate of moisture loss from the grass over time under the six different wilting conditions in a pilot study. Herbage samples were taken every 2 h and dried to a constant weight in an oven at 103 °C. In addition to the information from the pilot study, in the final study one of the three replicate trays with a known weight and known amount of herbage (around 1 kg fresh material) was not sampled. This tray was weighed with progressing wilting, to determine the extent of moisture loss of the herbage. The DM content of the fresh herbage was

determined in an oven at 103 °C for 4 h at the start of the wilting period. The actual DM contents were close to the target DM contents at 35, 25 and 15 °C. At the target DM content of 425 g/kg, the actual DM contents were 446, 414 and 392 g/kg at 35, 25 and 15 °C, respectively. At the target DM content of 525 g/kg the actual DM contents were 551 (at 35 °C), 530 (at 25 °C), 517 (at 15 °C) g/kg, while at the target DM content of 625 g/kg the actual DM contents were 633 (at 35 °C), 626 (at 25 °C) and 601 g/kg (at 15 °C).

Chemical Analysis

All samples were freeze dried and ground in a wiley mill to pass a 1 mm screen. The DM content of the freeze dried samples was determined after 4 h oven drying at 103 °C. For FA analysis, lipids from the freeze dried and ground samples were extracted with chloroform–methanol (2:1 v/v; Folch *et al.* 1957) with modifications as described by Khan *et al.* 2009) After extractions, FAs in the residual fat were (trans)esterified, using both acid and base catalysed methods. For the basic methylation, 3 mL of 0.5 mol/L NaOH–methanol was added to the extracted fat, vortexed and heated for 30 min at 50 °C. After cooling, 2 mL HCl–methanol (1:1, v/v) was added to the mixture, vortexed and heated for 10 min at 50 °C. After cooling, 2 mL of hexane and 2 mL of distilled water was added to the mixture, shaken vigorously, and centrifuged at 800 × *g* for 5 min at 20 °C. The hexane fractions containing the FA methyl esters (FAMEs) were collected and transferred to 5 mL tubes. Another 2 mL of hexane was added to the non-hexane fractions, shaken vigorously, and centrifuged at 800 × *g* for 5 min at 20 °C. The hexane layers were collected, pooled with the previous fractions and evaporated to dryness under a N_2 flux speed vac (Saint-Herblain, France). The residual FAMEs were dissolved in 1 mL of hexane and transferred to gas chromatograph (GC) vials. The FAMEs were quantified by GC (TRACE GC Ultra™; Thermo Electron Corporation, Waltham, MA, USA) equipped with a flame-ionisation detector (FID) and auto-sampler. Methylated FAs were separated using a fused silica capillary column (100 m × 0.25 mm and 0.2 µm film thickness; Supelco SP-2560, Bellefonte, PA, USA), using He as carrier gas at a constant flow of 1.5 mL/ min. One microlitre of sample was injected in the GC with a split ratio of 1:50. The following programme was used for the GC: starting temperature 140 °C for 4 min, increasing with 4 °C per min until 240 °C, and held for 20 min. The temperature of the injector and the FID was 250 and 280 °C, respectively. Peaks were identified by comparing retention time with corresponding peaks of external standards (S37; Supelco, Bellefonte, PA, USA). Individual

FAME contents were calculated from the peak area of the FAME from samples using the peak area of the internal standard (C13:0) as described by Khan *et al*. (2009).

Statistical Analysis

In the field wilting trial, the effect of wilting duration (0, 12, 24, 36 and 48 h), and pre-treatments (bruised and untreated) on herbage FA content and composition was determined by repeated measure analysis of variance using the PROC MIXED procedure of the Statistical Analysis System (SAS, 2003; Littell *et al.,* 2006). Individual plots were considered as experimental units, and included in the model as subjects for repeated effect of wilting duration. Pre-treatment, wilting duration and their interaction were fixed effects (Model 1). Replicates were considered as random effects.

$$Y_{ijk}=\mu + T_i + W_j + T_{i\times} W_j + e_{ijk} \qquad \text{(Model 1)}$$

where Y_{ijk} is the dependent variable; μ the general mean; T_i is the fixed effect of treatment (i = 1, 2: bruised *vs.* untreated grass); W_j is the fixed effect of repeated measures of wilting duration (j = 1-5), and e_{ijk} are the residual. The different covariance structures of the repeated matrixes for the changes in FA content and composition of herbage were evaluated according to Littell *et al*. (1998) and Wang and Goonewardene (2004) using the Akaike information criterion (AIC) and the Schwarz Bayesian criterion (BIC). A heterogeneous compound symmetry was the best fit.

For the wilting in climate chambers, the effect of drying to different DM contents (425, 525 and 625 g/kg DM), pre-treatment (bruised and untreated), temperature (15, 25 and 35° C) and light regime (light and dark) on the FA content and composition of herbage was analysed with the PROC MIXED procedure of SAS (Littell *et al*., 2006). Individual trays were considered as experimental units and included in the model as subjects to test the repeated effect of drying to different levels of DM content. Pre-treatments, wilting to the target DM contents, temperature, light and their two way interactions were considered as fixed effects (Model 2). Replicates trays were considered as random effects. A heterogeneous compound symmetry was the best fit according to AIC and BIC values.

$$Y_{ijklm} = \mu + T_i + T_j + L_k + W_l + T_i \times W_l + t_j \times W_l + L_k \times W_{l+} e_{ijklm} \qquad \text{(Model 2)}$$

where Y_{ijklm} is the dependent variable; μ the general mean; T_i is the effect of treatment (i = 1, 2: bruised *vs.* untreated grass); t_j is the effect of wilting temperture ($_j$ = 1, 2, 3); L_k = is the effect light regime ($_k$ = 1,2: light *vs.* dark); W_l is the effect of repeated measures of drying to target DM content ($_l$ = 1-4);and e_{ijklm} are the residual.

Results

Changes in Fatty Acid Content and Composition During Field Wilting of Perennial Ryegrass

The mean content of FA consistently declined ($P < 0.001$) with progressive wilting of grass Table 2. The decline in total FAs was mainly associated with a decrease in the contents of C18:3n-3 (9.15 to 6.36 g/kg DM) and C18:2n-6 (2.34 to 1.89 g/kg DM). The contents of saturated and mono-unsaturated FA, however, did not change ($P > 0.05$) with progressive wilting (data not shown). Due to the major decline in C18:3n-3 content, the proportion of C18:3n-3 in total FAs decreased whilst the proportion of C16:0 and C18:0 increased. The DM content of the herbage increased (197 to 676 g/kg) with progressive wilting. Despite of the high rate of moisture loss in untreated grass during the first 12 h, the moisture loss during 48 h was higher for bruised than for untreated grass. Moisture loss was much higher during the daytime compared to the subsequent 12 h wilting during the night. Bruising did not affect ($P > 0.05$) the FA content and composition during field wilting (Table 2).

Changes in Fatty Acid Content and Composition of Perennial Ryegrass Wilted Under Controlled Climatic Conditions

The changes in total FAs content and proportion of individual FA in the grass wilted to a DM content of 425, 525 and 625 g/kg at 15, 25 or 35 °C are presented in Table 3. Wilting to a higher DM content decreased ($P < 0.001$) the content of total FAs at all temperatures. The main change in FA content and composition, however, occurred during the prolonged initial wilting period to DM content of 425 g/kg. The FA content and composition of herbage did not change further during wilting of grass to a DM content of 525 and 625 g/kg. The grass was wilted for 56 h at 25 and 35 °C and for 62 h at 15 °C to the target DM content of 425 g/kg. The grass was wilted for 82 and 110 h at 25 or 35 °C to reach the target DM content of 525 and 625 g/kg, respectively. Whilst, the grass was wilted for 90 and 120 h at 15 °C to reach the target DM content of 525 and 625 g/kg, respectively. The

Table 2. Contents of dry matter (DM), total fatty acids (FA) and proportion of individual FAs in bruised and untreated perennial ryegrass wilted for 0, 12, 24, 36 and 48 h under field conditions.

Wilting time (h)	Pre-treatments	DM (g/kg)		Total FAs (g/kg DM)		Proportion of individual FA (g /100g total FAs)									
						C16:0		C18:0		C18:1n-9		C18:2n-6		C18:3n-3	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
0	Bruised	190	15.6	15.2	0.35	17.4	0.79	1.16	0.097	2.26	0.546	15.4	0.606	61.3	1.24
	Untreated	205	15.6	15.2	0.35	17.5	0.79	1.29	0.097	2.48	0.546	15.7	0.606	59.8	1.24
12	Bruised	372	19.6	14.9	0.48	17.9	0.38	1.48	0.063	3.21	0.708	15.0	0.230	56.3	1.22
	Untreated	483	19.6	14.1	0.48	18.0	0.38	1.24	0.063	3.28	0.708	15.4	0.230	56.9	1.22
24	Bruised	420	16.9	13.1	0.62	18.8	0.28	1.63	0.073	2.55	0.330	15.3	0.301	55.1	0.84
	Untreated	480	16.9	13.5	0.54	18.3	0.24	1.46	0.062	2.68	0.286	15.2	0.270	55.5	0.73
36	Bruised	638	12.3	12.6	0.63	18.8	0.37	2.15	0.348	2.63	0.931	15.8	0.193	54.6	1.08
	Untreated	671	12.3	12.4	0.54	18.6	0.32	1.56	0.301	2.60	0.081	16.4	0.180	54.1	0.94
48	Bruised	676	28.4	11.8	0.37	19.9	0.40	1.75	0.048	2.61	0.086	15.8	0.255	52.9	0.81
	Untreated	676	28.4	12.0	0.37	19.6	0.40	1.72	0.048	2.70	0.086	16.1	0.255	54.1	0.81

Overall mean (wilting time)														
0	197^{c}	11.4	15.1^{a}	0.26	17.4^{b}	0.56	1.23^{c}	0.068	2.37	0.386	15.5^{a}	0.441	60.6^{a}	0.89
12	428^{b}	14.2	14.5^{a}	0.35	17.9^{b}	0.28	1.36^{bc}	0.045	3.24	0.500	15.2^{b}	0.193	56.6^{b}	0.88
24	450^{b}	12.4	13.3^{ab}	0.42	18.6^{b}	0.20	1.54^{b}	0.048	2.61	0.219	15.2^{b}	0.227	55.3^{c}	0.58
36	654^{a}	9.2	12.5^{b}	0.42	18.7^{ab}	0.25	1.85^{a}	0.230	2.62	0.062	16.1^{a}	0.168	54.3^{bc}	0.74
48	676^{a}	20.3	11.9^{b}	0.27	19.7^{a}	0.29	1.73^{a}	0.038	2.65	0.061	15.9^{a}	0.208	53.5^{c}	0.60
Statistical significance														
Wilting time	***		***		***		***		NS		***		***	
Pretreatment	**		NS		NS		#		NS		NS		NS	
Wilting * Pretreatment	NS		NS		NS		NS		NS		NS		NS	

Means with different superscripts (^{abc}) within columns differ at $P < 0.05$.
NS, not significant; # $P < 0.1$; *, $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 3. Changes in the contents (g/kg dry matter (DM)) of total fatty acids (FA) and proportion of individual FAs in perennial ryegrass wilted at 15, 25 and 35 °C to a DM content of 425, 525 and 625 g/kg.

DM ($g\ kg^{-1}$)	Temperature (°C)	Total FAs (g/kg DM)		Proportion of individual FA (g /100g total FAs)									
				C16:0		C18:0		C18:1n-9		C18:2n-6		C18:3n-3	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Fresh		15.1	0.12	17.9	0.298	1.28	0.031	2.38	0.325	15.5	0.225	60.4	0.28
	35	11.1	0.27	20.2	0.301	1.76	0.055	2.60	0.220	16.8	0.263	52.2	0.72
425	25	10.8	0.27	20.5	0.301	1.78	0.055	2.96	0.220	16.4	0.263	51.7	0.72
	15	12.4	0.27	19.0	0.301	1.44	0.055	2.54	0.220	15.9	0.263	55.0	0.72
	35	10.5	0.28	20.5	0.295	1.81	0.093	2.50	0.074	17.0	0.243	51.1	0.76
525	25	10.8	0.28	20.5	0.295	1.74	0.093	2.59	0.074	16.7	0.243	51.5	0.76
	15	12.7	0.28	19.2	0.295	1.57	0.093	2.30	0.074	16.0	0.243	55.9	0.76
	35	11.5	0.41	20.2	0.450	1.71	0.059	2.54	0.092	17.0	0.277	50.9	1.03
625	25	11.4	0.41	20.4	0.450	1.70	0.059	2.49	0.092	17.1	0.277	51.8	1.03
	15	12.4	0.41	19.3	0.450	1.51	0.059	2.35	0.099	16.1	0.277	55.1	1.10
Overall mean temperature													
35 °C		12.1^{A}	0.14	19.7^{A}	0.236	1.64^{A}	0.037	2.51	0.110	16.6^{A}	0.162	53.6^{B}	0.413
25 °C		12.0^{A}	0.14	19.8^{A}	0.236	1.63^{A}	0.037	2.60	0.110	16.5^{A}	0.162	53.8^{B}	0.413
15 °C		13.1^{B}	0.15	18.8^{B}	0.237	1.46^{B}	0.037	2.40	0.110	15.9^{B}	0.163	56.6^{A}	0.423

Overall mean DM												
Fresh	15.1[a]	0.067	17.9[b]	0.233	1.28[b]	0.025	2.40	0.191	15.6[b]	0.161	60.4[a]	0.233
425	11.4[b]	0.153	19.9[a]	0.231	1.66[a]	0.036	2.70	0.132	16.3[a]	0.180	53.0[b]	0.449
525	11.3[b]	0.161	20.1[a]	0.228	1.70[a]	0.056	2.46	0.057	16.6[a]	0.170	52.8[b]	0.470
625	11.7[b]	0.242	20.0[a]	0.297	1.76[a]	0.038	2.45	0.066	16.8[a]	0.188	52.6[b]	0.630
Statistical significance												
DM	***		***		***		NS		***		***	
Temperature	***		***		***		NS		***		***	
DM * Temperature	***		*		***		NS		NS		***	

Means with different lowercase or capital letters within a column differ at $P < 0.05$.
NS, not significant; *, P < 0.05; ** P < 0.01; *** P < 0.001.

decrease in total FA was mainly caused by a major decrease in the C18:3n-3 content. As a consequence the proportion of C18:3n-3 in total FAs decreased with a concomitant increase in the proportion of C16:0 and C18:0. Wilting temperature affected ($P < 0.001$) the decline in total FA content and the changes in proportion of C16:0, C18:0, C18:2n-6 and C18:3n-3 in total FAs (Table 3). On average, the loss in total FAs content was lower ($P < 0.05$) when

the grass was wilted at 15 °C compared to 25 and 35 °C. In addition, a higher ($P < 0.05$) proportion of C18:3n-3 were maintained when the grass was wilted at 15 °C. The total FAs content and the proportion of individual FA did not differ between wilting at 25 and 35 °C. Bruising did not affect the changes in FA content and proportion of individual FA during wilting (data not shown). In the fresh grass the bruised grass had a numerically high content of total FAs and proportion of C18:3n-3 compared to untreated grass. In addition, there were no significant differences in the content of total FAs and in the proportion of individual FA, when the grass was wilted in a light or a dark environment (data not shown).

Discussion

The lower content of total FAs and lower proportion of C18:3n-3 in fresh grass compared to the reported values in earlier studies (Elgersma *et al*., 2003a; Dewhurst and King 1998) could be related to the mature summer re-growth used in the present study. Extensive research has established that the FA content in perennial ryegrass is lower during summer compared to spring and autumn (Elgersma *et al*., 2003b; Dewhurst *et al*., 2001; Van Ranst *et al*., 2009b). Seasonal variation in FA content is associated with changes in canopy characteristics such as leaf to stem ratio as well as climate variables such as day length, light intensity and temperature (Hawke, 1973). In perennial ryegrass, the summer re-growth, particularly around early July, is stemmier and flowering could be initiated. In addition, the longer re-growth (34 d) interval in the present study could have further re-enforced the increase in ratio of stems to leaves (Elgersma *et al*., 2003b; Barata, 1975) and results in a lower FA content in the fresh herbage.

Recent research focusing on the stability of FA during wilting showed that despite of some intrinsic differences in FA susceptibility to oxidation among herbage species (Van Ranst *et al*., 2009a; Van Ranst *et al*., 2009c) the duration of wilting (Dewhurst and King, 1998; Dewhurst *et al*., 2002; Van Ranst *et al*., 2009a) and change in DM content of herbage during wilting (Elgersma *et al*., 2003a; Boufaïed *et al*., 2003) could be manipulated to

minimise the loss of FA. Indirect comparison of earlier studies, however, demonstrated a variable effect of the duration of wilting and the change in herbage DM content on the loss of FA. Van Ranst (2009) suggests that the variation in FA oxidation during wilting among and within studies could be caused by the difference in environmental conditions (such as temperature and light) as well as to the extent of plant damage at cutting. A direct comparison in the present study, however, showed that the length of wilting has the most marked effect on the losses in FA compared to changes in environmental conditions, plant damage at cutting (bruising *vs.* untreated) and the rate and extent of increase in herbage DM content. During the 48 h field wilting, the total FA content of the herbage declined consistently at a more or less constant rate, without any influence of the consequent increasing DM content of the herbage from 197 to 676 g/kg. In addition, despite the higher moisture loss during the day compared to night, there were no differences in the extent of FA loss during day or night.

In the climate chambers, the drying of grass was slower compared to the field conditions. The grass was wilted for 56 h at 25 and 35 °C and for 62 h at 15 °C to achieve the initial target DM content of 425 g/kg. At all temperatures, the content of total FAs and C18:3n-3 declined during the prolonged initial drying period. The fatty acid content and composition, however, did not change with further drying to a DM content of 525 and 625 g/kg. Combining the data from the field and climate chamber experiments, it is clear that the loss of FA was affected by the wilting period without any influence of the increasing DM content of the herbage. This supports the findings by Dewhurst and King (Dewhurst and King, 1998), who observed major loss of FA during 68 h of wilting of grass despite only small increases (79 g/kg) in the DM content. On the other hand, in red clover there was a marked decline in total FAs when the grass was wilted for 5 d under greenhouse conditions with permanent light to a DM content of 250 g/kg, but, the content of total FAs did not change during drying from 250 to 500 g/kg DM at 35 °C in hot air oven for 8 h (Van Ranst *et al*., 2009a). In the study by Boufaied *et al.* (2003) there was no further decline in the content of total FAs and C18:3n-3 when grass wilted (400 g/kg DM) for a few hours was further dried to hay (850 g/kg DM) for 3 d. In the present study, the major changes in FA content and composition occurred during the initial (60) hours after harvesting and then ceased during further wilting.

The effect of wilting temperature was not related to the speed of drying. Although drying to 425 g/kg took longer at 15 °C, the FA content as well as the proportion of C18:3n-3 was

higher at 15 °C compared to 25 and 35 °C. In contrast, an early study by Czerkawaki (1967) showed that in perennial ryegrass the losses of FA were lower when dried (750–900 g/kg DM) for 12 h at 100 °C than for 18 h at 50 °C or for 26 h at room temperature. In the previous study, the effect of temperature was mainly related to the duration of wilting; wilting at a high temperature for short time (12 h) minimised the loss of FA. Furthermore, in the previous study there was no further decline in FA content and composition during 13 months of storage. In our study the drying of samples in climate chambers took a longer time (56–62 h) to reach an initial DM content of 425 g/kg. The extended duration of wilting might have minimised the effect of temperature. The lower losses of FA at 15 °C could be caused by the lower activity of plant enzymes at the lower temperature.

Damaging plant tissues by bruising did not affect the changes in FA content and composition during wilting. In contrast, in red clover, a higher oxidation of C18:3n-3 was observed when the herbage was more severely damaged by freezing and thawing compared to being crushed or undamaged (Van Ranst *et al*., 2009b). This discrepancy in the extent of oxidation stimulated by plant damage could be due to the different types of plants and methods used for damaging of plants in the two studies. Bruising of the grass increased the moisture loss during 48 h of field wilting without affecting the FA content and composition. The high moisture loss in untreated grass during the first 12 h could be explained by the fact that windrows of bruised grass were much thicker than the windrows of untreated grass during the first 12 h. Moreover, the lower moisture losses from 12 to 24 h (night wilting) as well as from 36 to 48 h (night wilting) could be due to the moisture added by dew. Although chlorophyll act as an efficient sensitising agent and light energy could induce photo-oxidation of the free PUFA (Foote, 1976), the FA content and composition of herbage wilted under artificial light did not differ from dark conditions. During cutting and bruising of grass the thylakoid membranes are disrupted and chlorophyll pigments tend to remain associated with hydrophobic components such as membrane lipids (Makoni *et al*., 1993; Nelson, 1993; Rontani, 2001). The presence of chlorophyll within such a hydrophobic micro-environment of highly unsaturated membrane lipids could increase the photo-oxidative effects (Foote, 1976; Rontani, 2001). The lack of differences in oxidative loss of FA in grass wilted under dark or light condition could be due to the low intensity of the light (792 W/m^2), compared to sunlight. This light intensity probably did not raise oxygen to an exited state. In addition, it is well documented that in plants enzymatic oxidation of PUFA via lipoxygenase is very active compared to non-enzymatic oxidation (photo-oxidation) (Zhuang *et al*., 2002). The latter could have masked the effect of photo-

oxidation as both photo-oxidation and plant lipoxygenase use free PUFA as substrate (Feussner and Wasternack, 2002).

Conclusions

This study investigated the influence of bruising and environmental conditions on stability of FA during wilting of perennial ryegrass. Herbage FA content and composition were mainly affected by the duration of the wilting and occurred independently of the changes in herbage DM content. The major changes in FA content and composition occurred during the initial wilting period (60 h) and ceased during further wilting. Wilting temperature (15, 25 or 35 °C) provoked only small differences in FA losses. Mechanical bruising of grass at cutting and light intensity during wilting did not affect the changes in FA content and composition. Stability of fatty acid in herbage could be increased by minimising the duration of wilting.

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CHAPTER 5

Stability of fatty acids in grass and maize silages after exposure to air during the feed out period

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Abstract

Lipids in forages are extensively hydrolysed in the silo with a concomitant increase in the level of free fatty acids (FFA). After opening of the silo, exposure of the FFA to air and light with, a concomitant increase in pH and microbial growth, could induce oxidization. The present study investigated the stability of FA in grass and maize silages exposed to air for 0, 12 and 24 h. Eight maize silages were selected with varying dry matter (DM) contents, being very wet, wet, normal and dry. In addition, eight grass silages were chosen on the basis of ammonia (NH_3) content and pH level. Grass and maize silages were sampled 8–10 weeks after ensiling and anaerobically transported to the lab in cooled plastic bags. After mixing, each sample was divided into three subsamples and exposed to air for 0, 12 or 24 h. Contents of individual FA were quantified by gas chromatography. Among the investigated silages, contents of total FAs varied greatly and ranged from 16.4 to 23.9 and 9.5 to 21.6 g/kg DM in grass and maize silages, respectively. Exposure to air up to 24 h lowered ($P < 0.01$) the contents of C18:3n-3, C18:2n-6, C18:1n-9 and total FAs in maize silages. In grass silages, 24 h exposure to air decreased ($P < 0.05$) contents of C18:3n-3, C18:2n-6 and total FAs ($P < 0.01$). In both grass and maize silages a decline in contents of major unsaturated FA (UFA) was associated with a concomitant increase ($P < 0.01$) in the proportion (g/g total FAs) of C16:0. The relative decrease in total FAs after 24 h exposure to air was higher in maize silages with a high moisture content, and progressively decreased with increasing DM content. In contrast, pH and NH_3 levels of grass silages had no effect on the stability of FA during feed out. The present study demonstrates that extended exposure of silages to air during feeding increased the proportion (g/g total FAs) of C16:0 and lowered the content of polyunsaturated FAs.

Introduction

The growing interest in forage lipids stems from their potential to favourably modulate the fatty acids (FA) profile of cow milk and benefit human health. Lipids in the photosynthetic tissues of plants are rich in polyunsaturated FA (PUFA; 0.60 to 0.85 g/g total FAs) and strongly contribute to the supply of C18:3n-3 in the ration of dairy cows (Dewhurst *et al*., 2001; Elgersma *et al*., 2003b; Clapham *et al*., 2005; Noci *et al*., 2007). Milk from dairy cows fed fresh green herbage, particularly grazed grass, has high proportions of C18:3n-3 and C18:2 *cis*-9, *trans*-11conjugated linoleic acid. However, ensiling reduces the positive effects of herbage lipids on the FA composition of milk due to extensive hydrolysis of forage lipids in the silo, as well as oxidation of FA in the period from plant cutting to ensiling (Dewhurst *et al*., 2006; Elgersma *et al*., 2006; Chilliard *et al*., 2007).

Plant lipases release free FA (FFA) from damaged tissues after cutting (Thomas, 1986), or during ensiling, of herbage (Steele and Noble, 1983; Chow *et al*., 2004). The FFA can be further oxidized by plant lipoxygenases (Fall *et al*., 1999; Feussner and Wasternack, 2002). However, if herbage is ensiled directly after cutting (Ueda *et al*., 2002), or wilted for only a short time (<24 h), the contents of FA remain relatively stable (Arvidsson *et al*., 2009). Wilted silages resulted in a higher milk PUFA content compared to unwilted silages despite a more extensive oxidation, and lower C18:3n-3 contents, caused by the longer wilting period (Noci *et al*., 2007). Wilting increases the dry matter (DM) contents at ensiling, which restricts fermentation, reducing in silo lipolysis (Van Ranst *et al*., 2009a). The lower proportion of FFA could explain the high transfer of PUFA from wilted silages into milk.

The quality of silages, and in silo lipolysis, therefore affects post ensiling stability of FA. Compared to fresh material, contents of FFA as a proportion of total FAs in ryegrass increased from 2 to as high as 27 to 73 (Elgersma *et al*., 2003a), in timothy from 14.8 to 55.8 and in red clover from 8.2 to 45 after ensiling (Vanhatalo *et al*., 2007). Despite large variation among silages, herbage lipids are extensively hydrolysed in the silo. In a well-sealed and compacted silo, anaerobic conditions are established within about 30 min of the start of ensiling (Sprague, 1974), and contents of FA remain stable irrespective of type and extent of fermentation (Van Ranst *et al*., 2009a). However these stable conditions dramatically change during opening and feeding of the silages when the FFA are exposed to air. The presence of oxygen, light, microbes and plant lipoxygenases can all induce oxidation.

The objective was to evaluate the stability of FA in grass and maize silages, with varying ensiling qualities, over the length of the feeding period, in order to assess opportunities to increase the transfer of PUFA from silages into milk.

Materials and methods

Sample Collection and Handling

Eight maize silages were chosen on the basis of DM contents from commercial farms in The Netherlands, ranging from very wet (DM < 280 g/kg), wet (DM = 280–320 g/kg), normal (DM = 320–360 g/kg) to dry (DM > 360 g/kg). Similarly, eight grass silages were chosen on the basis of pH and NH_3 level (g/100 g total N) and categorized as LNH_3 LpH (NH_3 < 8, pH < 4.5), HNH_3 LpH (NH_3 > 11, pH < 4.5), LNH_3 HpH (NH_3 < 8, pH > 4.8) and HNH_3 HpH (NH_3 > 11, pH > 4.8). Two silages from each category of grass and maize were sampled 8–10 weeks after ensiling by an experienced employee of a commercial laboratory (Blgg, Oosterbeek, The Netherlands). From each silage, approximately 700 g of sample was taken from the middle (in height) at the back of the silage clamp with a hollow drill (2 cm diameter). After collection, each sample was put in a polythene bag, flushed with N_2 closed with a zip and transported to the laboratory in a cooled box filled with N_2. Individual samples from the same silage were mixed thoroughly and divided into three equal subsamples. One subsample was immediately stored at −20 °C under N_2. The other two subsamples were spread on 30 cm × 20 cm trays, making a thin layer at room temperature (*i.e.* 20 °C) to mimic feedout for 12 and 24 h, respectively. After exposure to air, the 12 and 24 h samples were stored in a freezer at −20 °C under N_2.

Chemical Analysis

All samples were freeze dried and ground to pass a 1 mm screen. The DM contents of freeze dried samples were determined by oven drying at 103 °C for 4 h. Crude fat (CFat) was determined directly with petroleum ether as described by Elgersma *et al*. (2003a) for ensiled materials. Contents of crude protein (CP), acid detergent fiber (ADF), neutral detergent fiber (NDF), starch, ash, and grass silage NH_3 and pH (Table 1) were determined using near infrared reflectance spectrometry (NIRS). The ADF values used for calibration of NIRS were analysed according to Van Soest (1973) and NDF was analysed according to Van Soest *et al*. (1991), without the use of sodium sulphite. For NDF a slightly modified

method was used, in that after boiling with neutral detergent, the residue was incubated in 60 ml Na–K–phosphate buffer (pH, 7.0) for 15 min at 40 °C with 15 mg amylase (Thermamyl 120, NOVO Nordisk, Bagsvaerd, Denmark) and 0.25 ml protease (Alcalase 2.4 L, NOVO Nordisk, Bagsvaerd, Denmark) to remove residues of starch and protein. ADF and NDF values were expressed inclusive of residual ash.

For FA analysis, lipids from freeze dried samples were extracted with chloroform–methanol (2:1, v/v) (Folch *et al*., 1957). Tridecanoic acid (C13:0, 3 mg/20 ml chloroform–methanol) was added as internal standard. Homogenized extracts were filtered, water was added and centrifuged at 800 × *g* for 5 min at 20 °C. The upper phase was removed thoroughly, using repeated washing with a solution containing 30 ml chloroform, 480 ml methanol and 470 ml NaCl solution (7.3 g/l water). From the bottom phase, containing lipids, approximately 3 ml was collected. Solvents were evaporated by vacuum centrifugation and residual fat was collected and FA were (trans)esterified using both acid and base catalyzed methods. For basic methylation, 0.5 ml of 0.5 N NaOH methanolate was added. The mixtures were vortexed and heated for 10 min at 80 °C. After cooling, 0.5 ml of boron trifluoride (14 g/100 ml methanol) was added for methylation of FFA. The mixtures were vortexed and heated for 2 min at 80 °C. The fatty acid methyl esters (FAMEs) were collected in 1 ml of hexane. For a clear separation of the hexane layer, a saturated salt solution (400 g NaCl/l water) and centrifugation at 800 × g for 5 min at 20 °C were used. Residues were dissolved in 1 ml of hexane and transferred to GC vials. FAMEs were quantified using GC (Carlo Erba 8560 HRGC, Rodano, Italy) with a fused silica capillary column (100 m × 0.25 mm and 0.2 µm film thickness; Superlco; SP2560, St. Louis, MO, USA) using He as the carrier gas. The following program was used for the GC: starting temperature 140 °C for 4 min, increased by 4 °C per min till 240 °C; and left for 20 min at 240 °C. Temperature of the injector was 250 °C and the detector 280 °C. FAMEs were identified using external standards (S37, Supelco, Poole, Dorset, United Kingdom). Individual FAME contents were calculated from the peak area of the FAME and the peak area of the internal standard. Individual FAME contents were used to calculate the contents of individual FA in the silages as:

FA g/kg DM = ((Weight of IS in mg × PA of FA)/ PA of IS)/Sample weight in mg × 1000

where: PA= Peak area; IS= Internal standard.

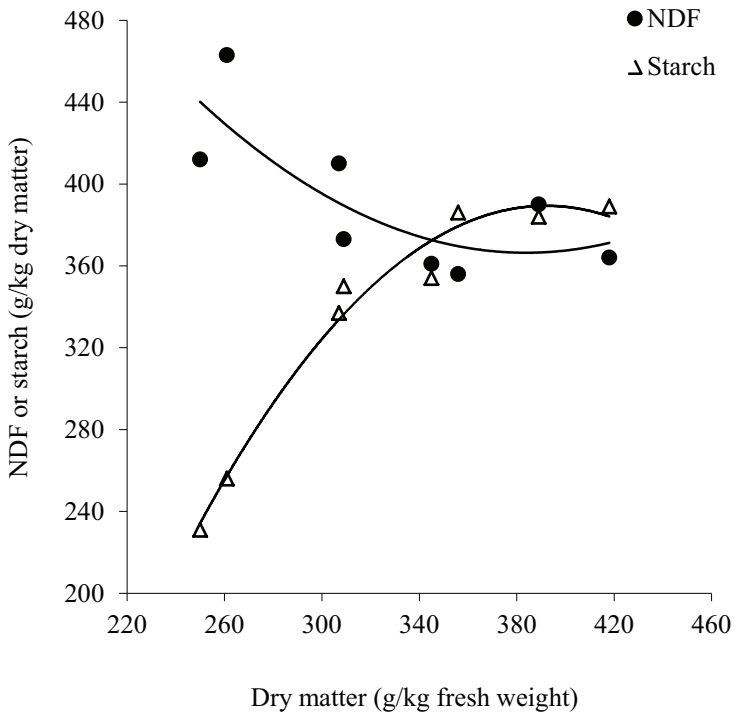

Figure 1. Relationship and polynomial regression equations of aNDF and starch content (g/kg DM) with the DM contents (g/kg) of maize silages:
NDF = 0.0041 × DM^2 - 3.1645 × DM + 973.65, R^2 = 0.41, P < 0.05
Starch = -0.0077 × DM^2 + 6.0336 × DM- 793.15, R^2 = 0.83, P < 0.001

Statistical Analysis

Effects of the quality of grass or maize silages on chemical composition were analysed with the PROC GLM procedure of SAS (2003) using the model:

$$Y_{ij} = \mu + T_i + e_{ij}$$

where Y_{ij} is the dependent variable under examination, μ is the general mean, T_i is the fixed effect of quality of maize silages (wet, very wet, normal and dry) or grass silages (LNH_3 LpH, HNH_3 LpH, LNH_3 HpH and HNH_3 HpH) and e_{ij} is the residual.

Since multiple measurements over the length of exposure time on the FA profile of individual silages cannot be regarded as independent units of observation, repeated measures of analysis of variance (PROC MIXED (Littell *et al*., 2006) of SAS (2003)) was performed for the contents and proportions of FA with individual silage as a repeated effect. Quality of grass or maize silages, exposure time to air and interaction between quality and exposure time were fixed effects. A first order-autoregressive structure (AR (1)) was the best fit and used in the model to account for within silage variation. Assumptions for both models were evaluated by examining the distribution of residuals. Values are presented as least square means with the standard error of the means.

Results

Quality of the Maize and Grass Silages

There were large differences in the chemical composition of the maize silages, with the DM content ranging from 255 in the very wet to 404 g/kg in dry silages (Table 1). Conversely, the contents of CP declined from 86 in the very wet to 65 g/kg DM in dry silages. Average contents of starch increased from 244 in the very wet to 387 g/kg DM in the dry silages (Figure 1). The content of NDF in DM decreased from very wet to normal silages. However, from normal to dry silages there was no further decline in NDF contents (Figure 1). Among the grass silages, pH and NH_3 content varied from 2.7 to 5.8 and from 2 to 18 g/100 g total N, respectively. The four categories of grass silages varied considerably in their chemical composition. Stepwise regression (data not shown) revealed no relationship between pH and NH_3 levels and the chemical composition of the grass silages.

Table 1. DM content (g/kg), chemical composition (g/kg DM), pH and NH_3 content (g/100g total N) of the four categories of grass and maize silages.

	DM	NH_3	pH	CP	Cfat	NDF	ADF	Ash	Starch
Maize Silages[1]									
Very wet	255^{d}	-	-	86^{a}	27	437^{a}	237	53^{a}	243^{b}
Wet	308^{c}	-	-	76^{ab}	34	391^{ab}	222	38^{b}	343^{b}
Normal	351^{b}	-	-	68^{b}	30	359^{b}	195	36^{b}	370^{a}
Dry	404^{a}	-	-	65^{b}	30	377^{ab}	207	37^{b}	387^{a}
SEM	11.6	-	-	3.5	2.6	24.2	18.7	2.9	15.2
Grass silages[2]									
LNH_3LpH	347^{B}	7^{B}	3.8^{A}	146	39	513	299	119	-
HNH_3LpH	342^{B}	14^{A}	4.2^{A}	149	38	528	327	132	-
LNH_3HpH	623^{B}	4^{B}	5.5^{B}	181	38	483	257	103	-
HNH_3HpH	477^{AB}	16^{A}	5.0^{B}	173	36	478	275	150	-
SEM	79.3	2.1	0.35	23.2	6.0	33	20	24	-

Means with different superscripts (^{abcd} or ^{AB}) within column differ ($P < 0.05$).

[1] Very wet, DM < 280 g/kg; wet, DM content of 280–320 g/kg; normal, DM content of 320–360 g/kg; dry, DM > 360 g/kg.

[2] LNH_3LpH, low NH_3 low pH; HNH_3LpH, high NH_3 low pH; LNH_3HpH, low NH_3 high pH; HNH_3 HpH, high NH_3 high pH.

Fatty Acid Profile of the Maize Silages after Exposure to Air

Exposure to air lowered ($P < 0.01$) the mean contents of C18:3n-3, C18:2n-6, C18:1n-9 and total FAs in DM of maize silages (Table 2). Furthermore, exposure to air also affected the FA composition of the total fat. The proportion of C18:2n-6 ($P < 0.05$) and C18:3n-3 ($P < 0.01$) per unit total FAs decreased with a concomitant increase ($P < 0.001$) in C16:0 (Table 4). The relative decrease in total FAs after 24 h exposure to air was higher in silages with lower DM contents and decreased linearly with increasing DM contents of the silages (Figure 2). Mean contents of total FAs increased from 16.8 in the very wet to 22.2 g/kg DM

Table 2. Effect of exposure to air for 0, 12 and 24 h on the contents (g/kg DM) of total fatty acid (FA), and selected individual FAs in very wet, wet normal and dry maize silages.

Quality[1]	Exposure time	Total FAs	C16:0	C18:0	C18:1n-9	C18:2n-6	C18:3n-3
Very Wet	0	16.78	3.07	0.34	3.18	8.14	1.73
	12	15.72	3.09	0.33	2.88	7.49	1.61
	24	15.52	3.09	0.33	2.91	7.32	1.54
Wet	0	20.89	3.24	0.44	4.25	11.36	1.29
	12	20.16	3.23	0.45	4.17	10.77	1.23
	24	19.62	3.22	0.44	4.06	10.58	1.17
Normal	0	21.57	3.12	0.46	4.68	11.85	1.14
	12	21.28	3.22	0.45	4.62	11.58	1.09
	24	20.69	3.19	0.45	4.60	11.15	0.99
Dry	0	22.21	3.37	0.46	4.64	12.49	0.97
	12	21.74	3.31	0.45	4.57	12.23	0.90
	24	21.81	3.34	0.46	4.55	12.33	0.86
Pooled SEM[2]		1.525	0.139	0.046	0.637	0.794	0.221
Average across all silages							
	0	20.36[a]	3.20	0.42	4.19[a]	10.96[a]	1.28[a]
	12	19.73[b]	3.22	0.42	4.06[b]	10.51[b]	1.21[b]
	24	19.47[b]	3.22	0.42	4.03[b]	10.35[b]	1.14[c]
Pooled SEM[3]		0.762	0.070	0.022	0.318	0.397	0.11
Significance							
Quality		NS	NS	NS	NS	NS	NS
Exposure time		**	NS	NS	**	**	**
Quality x Exposure time		NS	NS	NS	NS	NS	NS

Means with different superscripts ([abc]) within column differ ($P < 0.05$).

**, $P < 0.01$; NS, non-significant $P > 0.05$.

[1] Very wet, DM content < 280 g/kg; wet, DM content of 280–320 g/kg; normal, DM content of 320–360 g/kg; dry, DM content > 360 g/kg.

[2] Estimated for the least square means values of exposure time between qualities.

[3] Estimated for least square means values of exposure time across all silages.

Fatty Acid Profile of the Grass Silages after Exposure to Air

Exposure to air decreased ($P < 0.05$) the mean contents of C18:3n-3, C18:2n-6 and total FAs in grass silages (Table 3). Furthermore, a decrease in the contents of the major unsaturated FAs was associated with an increase ($P < 0.01$) in the proportion of C16:0 expressed per unit total FAs (Table 4). However, the proportion of other FA was not affected. Among grass silages, there were large differences in contents of total FAs and the main individual FA (C16:0, 18:2, C18:3n-3). Stepwise regression of the relative decrease of the main individual and total FAs after 24 h of exposure to air on pH, NH_3 and DM level of the silages revealed an effect of silage DM on the decrease in C18:3n-3.

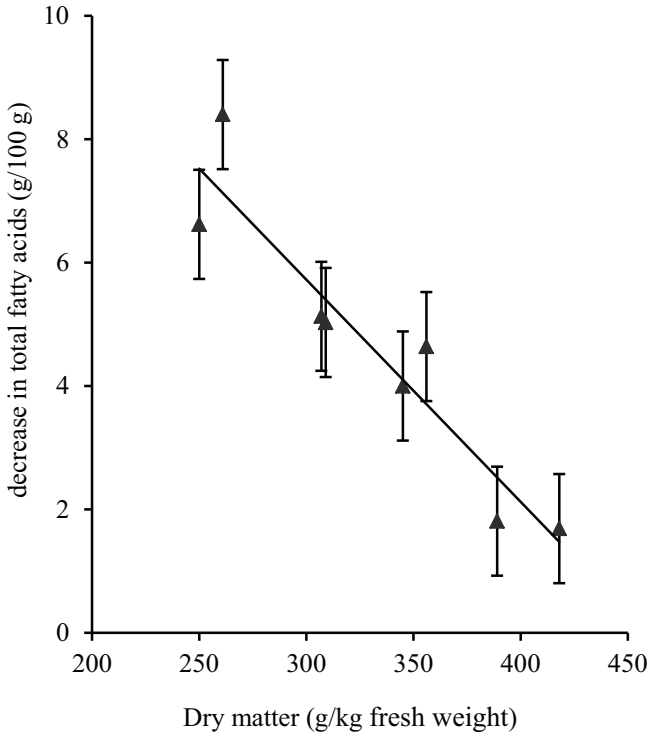

Figure 2. The relative decrease (g/100 g) in total fatty acids in the dry matter (DM) after 24 h exposure to air in maize silages varying in DM contents: relative decrease in total FAs = -0.04 × DM + 16.53, SE = 0.82, $R^2 = 0.86$, $P < 0.001$.

Table 3. Effect of exposure to air for 0, 12 and 24 h on the mean contents (g/kg DM) of total fatty acid (FA) and selected individual FAs in the four categories of grass silages.

Quality[1]	Exposure time	Total FAs	C16:0	C18:0	C18:1n-9	C18:2n-6	C18:3n-3
NH_3LpH	0	17.52	3.41	0.34	0.50	3.31	9.70
	12	17.17	3.36	0.34	0.47	3.27	9.47
	24	16.74	3.38	0.34	0.45	3.13	9.23
HNH_3LpH	0	13.77	3.01	0.30	0.33	2.71	7.34
	12	13.01	2.92	0.30	0.30	2.48	6.93
	24	12.66	2.95	0.30	0.30	2.34	6.69
LNH_3HpH	0	21.03	3.50	0.44	0.65	3.66	12.69
	12	20.54	3.52	0.41	0.54	3.13	12.42
	24	20.41	3.53	0.41	0.55	3.32	12.23
HNH_3HpH	0	17.85	3.21	0.34	0.39	2.73	10.85
	12	17.00	3.24	0.32	0.35	2.52	10.28
	24	17.08	3.30	0.33	0.33	2.51	10.33
Pooled SEM[2]		2.75	0.385	0.034	0.146	0.506	1.970
Average across all silages							
	0	17.61[a]	3.29	0.35	0.47	3.10[a]	10.15[a]
	12	16.93[b]	3.26	0.34	0.41	2.89[b]	9.77[b]
	24	16.72[b]	3.29	0.34	0.41	2.83[b]	9.61[b]
Pooled SEM[3]		1.376	0.193	0.016	0.073	0.253	0.985
Significance							
Quality		NS	NS	NS	NS	NS	NS
Exposure time		*	NS	NS	#	*	*
Quality x Exposure time		NS	NS	NS	NS	NS	NS

Means with different superscripts ([ab]) within column differ ($P < 0.05$).
* = $P < 0.05$; # = $P < 0.1$; NS = non-significant $P > 0.1$.
[1] LNH_3LpH, low NH_3, low pH; HNH_3LpH, high NH_3 low pH; LNH_3HpH, low NH_3 high pH; HNH_3 HpH, high NH_3 high pH.
[2] Estimated for the least square means values of exposure time between qualities.
[3] Estimated for least square means values of exposure time across all silages.

Table 4. Effect of exposure to air for 0, 12 and 24 h on the proportion (g/100 g total FAs) of major individual fatty acids in maize and grass silages.

	Exposure time	Maize silages			Grass silages		
		C16:0	C18:2n-6	C18:3n-3	C16:0	C18:2n-6	C18:3n-3
Overall average	0	15.8 [a]	53.6	6.6	19.4[a]	18.0	56.6
	12	16.6 [b]	53.0	6.4	20.0[b]	17.5	56.5
	24	16.8 [b]	52.8	6.1	20.4[b]	17.3	56.4
Pooled SEM		0.31	0.52	0.11	1.21	1.09	2.32
Significance							
Quality		*	*	NS	NS	NS	NS
Exposure time		***	*	*	**	#	NS

Means with different superscripts ([ab]) within column differ (P < 0.05).
*, P < 0.05; **, P < 0.01; ***, P < 0.001; #, P < 0.1, NS, non-significant.

Discussion

Recent research on lipid metabolism and stability of FA in ensiled forages, has been focused on changes occurring during wilting and ensiling. The FFA produced during ensiling, as a result of extensive hydrolysis of lipids (Steele and Noble, 1983; Elgersma *et al*., 2003a; Vanhatalo *et al*., 2007), remain stable irrespective of the type and extent of fermentation in a well-sealed silo (Van Ranst *et al*., 2009). However, the stable environment of the silo dramatically changes during opening and feeding of the silages, when the FFA are exposed to air, because the presence of oxygen, light, microbes and plant lipoxygenases all can induce oxidation.

Ensiling conditions, such as rate and extent of fermentation, influence the degree of lipolysis in silages (Van Ranst *et al*., 2009; Lourenço *et al*., 2005b; Lee *et al*., 2008). Contents of FFA, together with the quality of the silages, could in turn influence the stability of FA during feed out. To account for these variations, grass and maize silages with a wide range of qualities were selected in the present study. DM contents of maize

silages reflect the maturity stage at harvest (Di Marco *et al*., 2002) which directly influence the properties of fermentation and aerobic stability during feed out period (Bal *et al*., 1997; Bal, 2006). Unlike maize, the grass silages were selected on the basis of pH and NH_3 levels. DM contents of grass silages, as well as its effect on FA contents and in silo lipolysis, are influenced by multiple factors such as plant species, cutting date and extent of wilting (Van Ranst *et al*., 2009a; Dewhurst and King, 1998; Elgersma *et al*., 2003b). Among grass silages, there were large variations in the chemical composition, although stepwise regression analysis revealed no relationship between pH and NH_3 with any chemical component.

Although quantitatively small (< 0.06 g/g total FAs), there was a consistent decline in the contents of the major unsaturated and total FAs in both maize and grass silages after 24 h of exposure to air. The decline in the contents of unsaturated FA, with a concomitant increase in the proportion (g/g total FAs) of C16:0, could partly be related to oxidation of FFA. It is unclear whether the oxidation of FA was of microbial origin, induced by light or by plant lipoxygenases. During the feed out period, air can penetrate into the silages, promoting growth of aerobic, acid-tolerant microorganisms and consequently the oxidation of fermentation products present in the silages (Danner *et al*., 2003). Furthermore, in the presence of oxygen, plant lipoxygenase can reactivate and oxidize part of the FFA. Plant enzymes can stay functional in silages, even though the activity of plant enzymes generally declines after wilting and ensiling. A high lipoxygenase activity could be envisaged in the high pH silages in particular (Lourenço *et al*., 2005a). Furthermore, chlorophyll acts as an efficient sensitizing agent causing photo-oxidation of the FFA (Foote, 1976), and could have contributed to the decreased content of unsaturated FA. During ensiling the thylakoid membranes are disrupted and chlorophyll pigments tend to remain associated with hydrophobic components such as membrane lipids (Makoni *et al*., 1993; Nelson, 1993; Rontani, 2001).

The presence of chlorophyll within such a hydrophobic micro-environment of highly unsaturated membrane lipids could have strongly amplified its photo-oxidative effects (Foote, 1976; Rontani, 2001). High losses of FA in the very wet maize silages versus dry silages could be due to prolonged fermentation and extensive lipolysis in the high moisture silages (Van Ranst *et al*., 2009a). The same effect of DM content has been found for proteolysis (Muck, 1987). Another plausible explanation might be high effluent losses in the high moisture silages. Unlike maize, contents of C18:1n-9 and C16:1 in grass silages

remained stable during feed out. A plausible explanation is that the content of these FAs was lower in grass silages and the affinity for oxidation decreased with a decreased level of unsaturation (Gray, 1978).

The pH level and NH_3 content in grass silages did not affect the oxidative stability of the FA. In the present study, where the silages were well spread in the trays, volatile FA from the silages could have evaporated quickly thereby marginalizing effects of pH on aerobic stability of FA. The extent of fermentation, or the rate of pH decreases effect in silo lipolysis and proteolysis (Van Ranst *et al*., 2009a; Muck, 1987), although final pH did not reflect the rate of pH decrease. Furthermore, with the increase in DM content at ensiling from 300 to 500 g/kg in ryegrass, lipolysis was reduced from 57 to 32% (Van Ranst *et al*., 2008). This lower lipolysis could explain the lower oxidative losses in silages with high DM contents.

In agreement with Shingfield *et al*. (2005a), the FA composition of maize silages was dominated by C18:2n-6 and C18:1n-9 and, on average, accounted for 0.54 and 0.21 g/g of the total FAs respectively. The increase in total FAs contents from 16.8 in the very wet to 22.2 g/kg DM in the dry silages was associated with an increase in the proportion of C18:2n-6 from 0.48 to 0.56 g/g total FAs. The growing ears of the maturing maize could explain the increase in C18:2n-6 and total FAs contents (Di Marco *et al*., 2002) as maize kernels contain high proportions of C18:2n-6 and C18:1n-9 per unit total FAs and their contents in the kernels continue to increase for 6–7 weeks after pollination (Tan and Morrison, 1979). In contrast, the proportion of C18:3n-3 declined from 0.10 in very wet to 0.04 g/g total FAs in dry silages. Contents of C18:3n-3 decline progressively with maturity in maize plant. Furthermore, in maize, senescence starts before the leaves are fully developed and progresses at an increasing rate during grain filling (Eik and Hanway, 1965; Muchow and Carberry, 1989; Sadras *et al*., 2000). Metabolism of membrane lipids during senescence reduces the content of C18:3n-3 (Thompson *et al*., 1998). Large differences in contents of total and individual FA among grass silages was associated with variation in the proportion of C18:3n-3 (from 0.44 to 0.66 g/g total FAs). Recent findings by Clapham *et al*. (2005) shown that the FA composition remains constant across grass species, suggesting that differences in FA composition could be related to variations in managemental practices such as wilting conditions, duration of wilting, as well as the ensiling process (Dewhurst *et al*., 2001; Boufaïed *et al*., 2003; Clapham *et al*., 2005).

Conclusions

Although quantitatively small (< 0.06 g/g total FAs), there was a consistent decline in contents of the major unsaturated and total FAs in both maize and grass silages during 24 h exposure to air. This decline in the content of unsaturated FA was associated with a concomitant increase in C16:0 per unit total FAs. The present study demonstrates that silages should not be exposed to air for longer periods than needed in order to avoid losses of unsaturated FA by oxidation.

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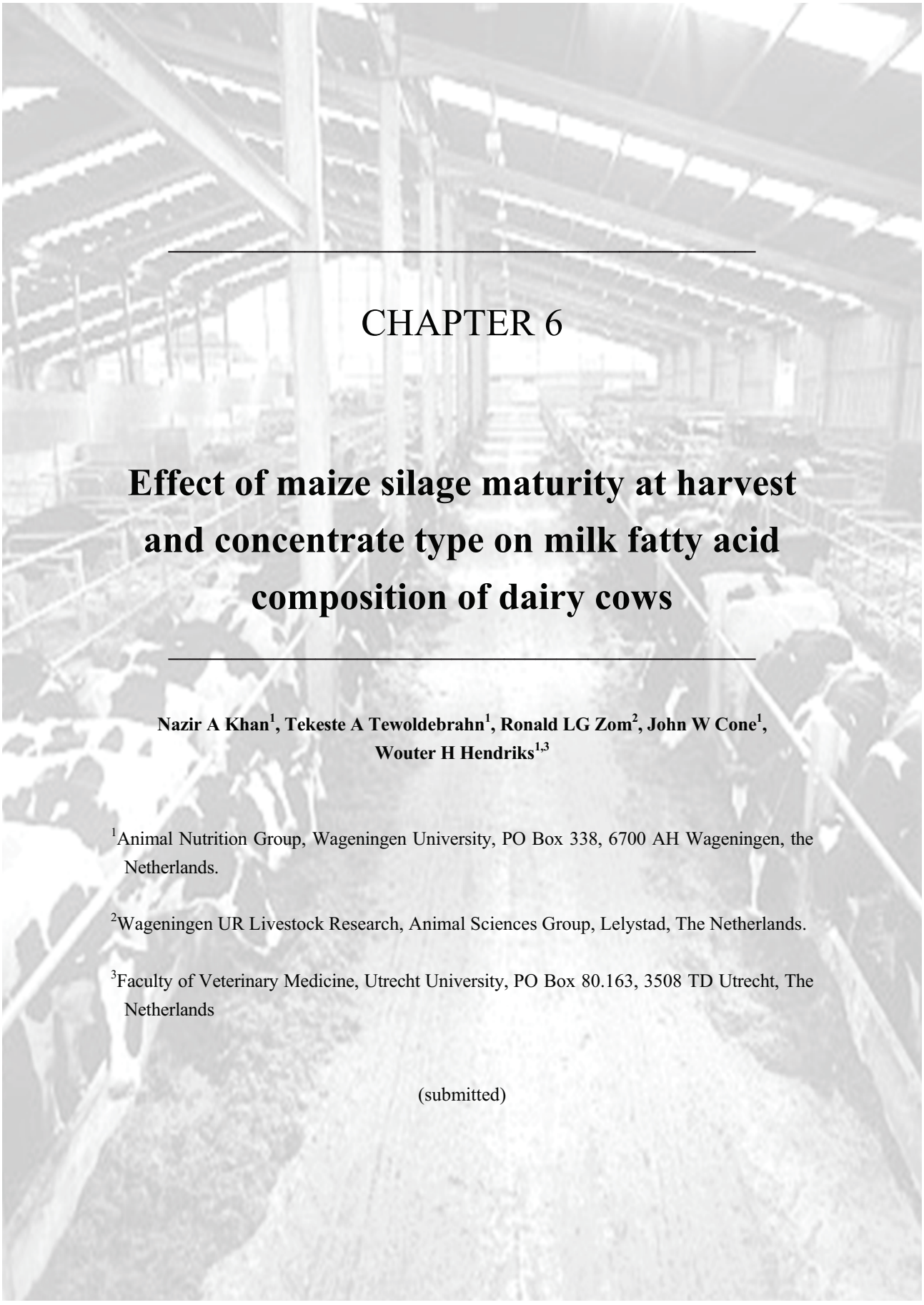

CHAPTER 6

Effect of maize silage maturity at harvest and concentrate type on milk fatty acid composition of dairy cows

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Abstract

The variation in maturity at harvest during grain-filling has a major effect on the carbohydrate composition (starch:NDF ratio) and fatty acid (FA) content of maize silages, and can alter the FA composition of milk fat in dairy cows. This study evaluated the effect of silage maize (cv. *Atrium*) harvested and ensiled at targeted dry matter (DM) contents of 300, 340, 380 and 420 g/kg fresh weight and fed to dairy cows in a combination with a high degradable carbohydrate (HC) or low degradable carbohydrate (LC) concentrate, on the nutrient intake, milk yield and composition of milk and milk fat. Sixty-four multiparous Holstein-Friesian dairy cows in their first week of lactation were assigned to the eight dietary treatments according to a randomized complete block design. The eight dietary treatments consisted of a factorial combination of the four maize silages and the two concentrates. Maize silages were offered *ad libitum* as part of a basal roughage mixture, while the concentrates were given at the rate of 8.5 kg DM/cow/day during the 15 week experimental period. DM, CP and energy intakes did not differ across the maize silages. However, the intake of starch increased, and those of NDF and C18:3n-3 decreased with increasing maturation. Milk yield and composition were not different across the maize silages. Yield (kg/d) of milk, protein and lactose was higher for LC compared to HC concentrate fed groups. Increasing maturity of maize silages decreased the content of C18:3n-3, total n-3 and n-6:n-3 ratio in milk fat. Concentrate type significantly altered the composition of all *trans* FAs. Inclusion of the HC concentrate in the diets increased the contents of all C18:1 *trans* isomers, C18:2 *cis*-9, *trans*-11 and C18:2 *trans*-10, *cis*-12 conjugated linoleic acid in milk fat. Milk fat composition was strongly influenced by the stage of lactation (week 3 to 10). The content of all even-short and medium chain FAs changed with lactation, except C8:0 and C10:0. The content of C12:0, C14:0 and C16:0 and total saturated FAs increased and the content of C18:0, C18:1 *cis*-total and total *cis*-monounsaturated decreased with lactation. Maturity of the maize silages at harvest did not affect the production performance of dairy cows, but resulted in a decreased content of C18:3n-3 and total n-3 and a decreased n-6:n-3 ratio in the milk fat of dairy cows.

Introduction

Silage maize is a major forage component in the ration of dairy cows, under most dietary regimes. The crop has a relatively stable yield, high energy content, good ensiling characteristics, and inclusion of maize silages in grass or grass silage based diets can increase feed intake, milk yield and milk protein content (Phipps *et al.,* 1995; O'Mara *et al.,* 1998; Phipps *et al.,* 2000). As a result, like many other European countries, the area used for silage maize production in the Netherlands has increased from 5.0 x 10^3 ha in 1970 to 2.4 x 10^4 ha in 2004 (Schroeder 1998; Barrière *et al.,* 2006). Due to their high consumption, forages in fresh or ensiled form are also major sources of polyunsaturated fatty acids (PUFA: C18:3n-3, C18:2n-6) in dairy cow rations, and high PUFA-containing forages can be used to favourably modulate milk fatty acids (FA) composition (Dewhurst *et al.,* 2006; Elgersma *et al.,* 2006).

Maize silages are higher in starch and C18:2n-6 (0.52 ± 0.10 g/g total FAs), whereas grass silages are higher in NDF and C18:3n-3 (0.58 ± 0.16 g/g total FAs). Inclusion of maize silages in grass based rations of dairy cows increases the level of *trans* FAs, mainly at the expense of their *cis*-isomers and lowers the content of beneficial C18:3n-3 causing an elevated n-6:n-3 PUFA ratio in milk fat (Havemose *et al.,* 2004; Shingfield *et al.,* 2005a; Nielsen *et al.,* 2006; Kliem *et al.,* 2008). Under normal rumen conditions, hydrogenation of C18:2n-6 in maize silage mainly results in an increased concentration of C18:2 cis-9, trans-11 conjugated linoleic acid (CLA) and C18:1 *trans*-11 (Chilliard *et al.,* 2001), which are considered beneficial to human health. Moreover, a combination of maize silages with high degradable carbohydrate concentrates further increases the content of *trans*-FAs and shifts the rumen biohydrogenation pathway towards the production of C18:1 *trans*-10 at the expense of C18:1 *trans*-11 (Piperova *et al.,* 2000; Nielsen *et al.,* 2006). A high level of *trans*-FAs, particularly with *trans-trans* double bonds have been reported to increase the risk of coronary heart disease and diabetes (Ascherio *et al.,* 1999; Lemaitre *et al.,* 2002). In addition, increasing the content of n-3 PUFA and decrease the n-6:n-3 PUFA ratio in milk fat of dairy cows fed maize silages, may be beneficial for long term human health (Kliem *et al.,* 2008).

In the Netherlands, but also elsewhere in Europe (Phipps *et al.,* 2000), silage maize is harvested at a wide range of maturation, with the whole crop dry matter (DM) content ranging from 250 to 450 g/kg fresh weight (FW). These differences in maturity at harvest during

grain filling result in a considerable variation in FA content (Khan *et al.,* 2011b) and carbohydrate composition (starch:NDF ratio) of maize silages (Bal *et al.,* 2000; Phipps *et al.,* 2000). These changes can influence both the rumen environment and microbial hydrogenation of unsaturated FAs (Shingfield *et al.,* 2005a; Nielsen *et al.,* 2006), and as a consequence the milk FA composition of dairy cows.

The aim of this experiment was to evaluate the effect of maize silages ensiled at different maturities in combination with concentrates with a high or low degradable carbohydrate content on nutrient intake, milk yield, milk composition, and milk FA composition in early lactating dairy cows, in order to develop practical nutritional strategies to improve milk FA composition of dairy cows fed maize silages. We hypothesised that the variation in FA composition of maize silages as well as the amount and composition of carbohydrates in the different diets can affect milk FA composition of dairy cows.

Materials and Methods

Silages

Maize silages were prepared from a single crop (*cv.* Atrium; Force Limgrain Nederland BV, Rilland, The Netherlands), sown on clay soil on April 20, 2009, at a density of 100,000 seeds/ ha (10 plants/m^2) and rows spacing of 0.75 m, at the research facility of Wageningen University and Research Center, Lelystad, The Netherlands (52° 5′ N and 5° 5′ E). The crop was fertilized with 50 tons of cattle slurry/ha (containing 4 kg N/ton and 1.3 kg P_2O_5/ton), 30 kg N/ha and 30 kg P_2O_5/ha as ammonium phosphate. The maize was harvested and ensiled at a target DM contents of 300 (MS30), 340 (MS34), 380 (MS38) and 420 (MS42) g/kg FW. No additives were used to improve the ensiling process. To determine the targeted harvest DM, 5 plants from 5 randomly selected spots in each cross section of each plot were sampled twice weekly, chopped, and dried in an oven at 103 °C for 24 h. The frequency was increased to daily sampling when the difference with the target DM content was less than 30 g/kg. The actual DM content of the crop was close to the targeted DM contents (Table 1). All silages were made with the same precision chop harvester (John Deere 7750) using the identical machine settings. Theoretical length of cut was 6 mm and roll-clearance of the kernel processor was 1 mm, to ensure that all kernels were sufficiently crushed. The maize silages were stored in bunker silos and compacted

Table 1. Harvest dates, dry matter (DM) content and yield of silage maize at the targeted harvest DM contents of 300 (MS30), 340 (MS34), 380 (MS38) and 420 (MS42) g/kg fresh weight, and temperature (in °C and °C.d) during the preceding period after flowering.

	Date (dd-mm-yy)	Harvest parameter				Temperature after flowering			
		DAF[1] (d)	DAS[2] (d)	DM[3] (g/kg)	Yield (ton DM/ha)	Mean (°C)	Max (°C)	Min (°C)	T_{sum} [4] (°C.d)
MS30	14-9-2009	64	146	296	16.28	17.6	22.7	12.5	500
MS34	23-9-2009	73	155	341	17.28	15.1	20.0	10.0	546
MS38	05-10-2009	85	167	396	17.02	12.4	16.7	8.1	574
MS42	14-10-2009	94	176	421	17.08	10.8	15.0	6.6	581

[1] Days after flowering.
[2] Day after sowing.
[3] Crop DM content at harvest (n = 10).
[4] Temperature sum (°C.d, with a base temperature of 10 °C; Sibma *et al*., 1987) after flowering.

with a heavy weight tractor and a wheel loader. The silages were airtight sealed with two layers of 0.15 mm polyethylene plastic sheets, and covered with a 20 cm thick sand load. The total silage-clamp was covered with a protection sheet being held down with sand bags.

The grass silage was prepared from first cut perennial ryegrass (*Lolium perenne* L.) cultivars (BG3; Barenbrug, OosterhoutThe Netherlands), mowed on May 1, 2009 with a disc mower and conditioner. The mower-conditioner gently removes the waxy layer of leaves and stems of the grass with some additional crimping to enhance the drying process. The grass was wilted for 36 h with 20 h of sun, and tedded twice in the field. The average day temperature was 20.4 °C and the average night temperature was 7 °C. Grass was ensiled in bunker silos compacted and sealed as described for maize silages.

Experimental Design, Animals and Diets

Sixty-four multiparous Holstein-Friesian dairy cows were assigned to eight dietary treatments (n = 8 cows per dietary treatment), according to a randomized complete block design with repeated measures. Cows were distributed over the eight blocks to balance for parity, milk yield during previous lactation, body weight and DM intake among blocks. The

eight dietary treatments consisted of a factorial combination of the four maize silages (MS30, MS34, MS38 and MS42) and two types of concentrate: a high degraded carbohydrate (HC; low NDF, high water soluble carbohydrates (WSC)) and low degradable carbohydrate (LC; high NDF and low WSC) concentrate. Cows were adapted to the experimental diets and feeding regimes just after calving and data collection started the second week after calving until 17 weeks after calving (March 30 to August 27, 2010). The four maize silages were offered *ad libitum* as part of a roughage mixture which contained 61% maize silage, 28% grass silage, 10% soybean meal, 0.45% mineral mixture (190 g Ca, 45 g Na, 120 g mg, 1200 mg Cu, 2500 mg Zn, 3000 mg Mn, 120 mg I and 34 mg Se on a DM basis) and 0.34% salt (NaCl; 380 g Na and 570 g Cl on a DM basis) on a DM basis. The concentrates (Table 2) were given at a rate of 8.5 kg DM/cow/day.

The roughage mixtures were prepared each day using a self-propelled mixer equipped with a cutter loader system and an electronic weighing unit. The roughage mixtures were fed in individual weighing troughs containing data loggers, which recorded the roughage intake after each visit for individual cows. The troughs were continuously accessible except during the milking period. The concentrates were fed individually using three transponder-controlled concentrate dispensers and dispensed at a rate of 0.3 kg/min. The total daily allowance of the concentrates was partitioned over six consecutive time windows of four hours each. The eight dietary treatments were formulated to be iso-nitrogenous and iso-energetic and only differed in maize silage maturity and carbohydrate degradability of the concentrates. The nutrient requirements of the cows were calculated according to CVB (2007) estimates.

Data Recording and Sampling

Fresh roughage mixtures were sampled daily, the ingredients of the roughage mixtures twice a week and concentrates once after delivery of a new batch for DM analysis. For chemical and FA analysis, samples of individual feedstuffs and the roughage mixture were taken weekly and frozen immediately at -20 °C. DM intake and milk yield of individual cows was recorded daily throughout the experiment with cows milked twice daily at 06:00 and 18:00 h. Milk samples were taken weekly from four consecutive milkings.

Table 2. Ingredient composition of the concentrates.

Ingredient (g/kg)	Concentrate[1]	
	LC	HC
Wheat	0.00	35.71
Corn gluten meal	75.00	25.00
Wheat middlings	43.89	50.00
Wheat gluten meal	113.99	100.00
Beet pulp, 15-20% sugar	126.42	100.00
Citrus pulp	50.00	162.19
Beet vinasses, CP > 250 g/kg	66.64	80.00
Beet vinasses, CP < 250 g/kg	39.20	10.00
Cane molasses	0.00	29.20
Soya hulls	55.33	0.00
Soybean meal, solvent extracted	124.37	116.49
Extracted linseed	11.79	10.00
Coconut expeller	50.00	50.00
Canola meal	27.49	69.13
Canola meal, formaldehyde treated	24.92	21.14
Palm kernel expeller	175.0	128.65
Magnesium oxide	3.88	4.23
Mineral and vitamin mixture [2]	3.92	3.92
Calcium carbonate	5.91	2.17
Salt	2.27	2.16

[1] Concentrate with low (LC) and high (HC) degradable carbohydrates.
[2] Contained per kg of concentrate: 6.5 g Ca, 5 g Mg, 3.5 g Na, 13.4 g K, 4.9 g P, 3.2 g S, 3.6 g Cl, 1.6 mg Co, 0.3 mg Se, 2.3 mg Fe, 0.71 mg Mo, 10000.000 IU vitamin A, 2000.000 IU vitamin D, 10.000 IU vitamin E.

The morning and evening milk samples were pooled (1:1 ratio) separately to obtain two composite milk samples. The samples were stored at 4 °C until analyzed for fat, protein, lactose and milk somatic cell count. For FA analysis, sub-samples of milk from week 3, 5 and 10 were taken and immediately stored at -20 °C. To measure changes in body weight, the pre-calving body weights were recorded weekly whereas post-calving body weights were recorded automatically twice a day at the entrance of the milking parlor. The body condition score of each cow was recorded weekly by an experienced observer on a scale from 1 (thin) to 5 (fat) with 0.25 point intervals (Edmonson *et al.,* 1989).

Chemical Analysis

All feed samples were freeze-dried and ground to pass through a 1 mm screen, and analyzed for DM, ash, CP, crude fat, NDF, ADF, ADL, starch, sugars, and FA content. DM content was determined by oven drying at 103 °C for 24 h (ISO 6496; ISO, 1983), ash after incineration at 550 °C (ISO 5984; ISO, 1978) and CP (N × 6.25) was determined using the Kjeldahl method (ISO 5983; ISO, 2005). ADF and ADL was determined according to Van Soest (1973). NDF was analysed according to Van Soest *et al*. (1991) with some modification as described by Khan *et al.* (2009). Crude fat was determined using the Berntop method with pre-acid hydrolysis (ISO 6492; ISO, 1999). Sugars were determined as described by Van Vuuren (1993). The starch content was determined as glucose using the amyloglucosidase method (ISO 5914; ISO, 2004) after an initial extraction of the samples with 40% ethanol (to remove the sugar fraction). Ammonia was determined according to the Berthelot method as modified by Schneider (1976). The feeding values: *in vitro* organic matter digestibility (OMD), net energy for lactation (NE), true protein digestion in the small intestine (DVE) and degraded protein balance in the rumen (OEB) were determined using near infrared reflectance spectrometry (NIRS) by a commercial laboratory (Blgg, Oosterbeek, The Netherlands). These NIRS values were calibrated using the following techniques: OMD was determined according to the method of Tilley and Terry (1963), NE for lactating dairy cows was calculated according to Van Es (1978). The Dutch protein evaluation system as described by Tamminga *et al.* (1994) was used to determine DVE and OEB.

For FA analysis of feedstuffs, lipids from freeze dried, grounded samples were extracted with chloroform-methanol (2:1 v/v) (Folch *et al.,* 1957), with some modification as described by Khan *et al.* (2009). After extractions, FAs in the residual fat were (trans)-esterified, using both acid and base catalyzed methods as described by Khan *et al.* (2011a). Milk FA extraction and methylation were performed as described by Jacobs *et al.* (2011), except that 30 ml of composite morning and evening (1:1) samples were used. The FA methyl esters (FAMEs) were quantified by gas chromatograph (GC) (TRACE GC Ultra™, Thermo Electron Corporation, Waltham, MA, USA), equipped with a flame-ionization detector and an auto-sampler. Methylated FAs were separated using a fused silica capillary column (100 m × 0.25 mm and 0.2 µm film thickness; Restek RT-2560, Bellefonte, PA, USA) using hydrogen as carrier gas at a constant flow of 1.2 ml/min. One µl of sample was

injected in the GC with a split ratio of 1:100 for feedstuffs and 1:50 for milk. The following program was used for the GC: starting temperature 100 ºC for 4 min, increasing with 3 ºC per min until 240 ºC, held for 10 min at 240 ºC. The temperature of the injector was 225 and the flame-ionization detector was 250 ºC. Peaks were identified by comparing their retention time with those of the corresponding FAMEs standards S37 (Supelco, Bellefonte, PA, USA); odd and branched chain FAs, C18:1 trans-11, C18:2 cis-9,trans-11 and C18:2 trans-10, cis-12 (Larodan Fine Chemicals AB, Malmö, Sweden). FAs C18:1 trans-6+7+8, C18:1 trans-9, C18:1 trans-10, C18:1 trans-11, C18:1 trans-12, C18:1 trans-16, C18:1 cis-12, C18:1 cis-13, C18:1 cis-14, C18:1 cis-15, C18:2 trans-11, cis-15 were identified according to the elution sequence reported by Loor et al. (2004) and Shingfield et al. (2006).

Statistical Analysis

The effects of maize silage maturity, concentrate type and lactation stage on intake of nutrients, milk yield, milk composition, body condition and milk FAs composition were determined by repeated measure analysis of variance using the PROC MIXED procedure (Littell *et al.,* 2006) of the Statistical Analysis Systems (SAS®, 2003). Weeks of lactation were considered as a repeated effect on individual cows. Maize silage maturity, concentrate type and lactation stage were fixed effects and block was considered as a random effect. Interactions were either non-significant or not relevant and therefore excluded out of the model,

$$Y_{ijkl} = \mu + \mathrm{M}_i + \mathrm{C}_j + \mathrm{W}_k + e_{ijkl}$$

where Y_{ijkl} is the dependent variable; μ the general mean; M_i is the fixed effect of maize silage (i = MS30, MS34, MS38 and MS42), C_j is the fixed effect concentrate types (j = HC and LC), W_j, is the fixed effect of the repeated measures of lactation weeks (k = 1-15 for all variables except milk FAs; for milk FAs, k = 3, 5, 10) and e_{ijkl} is the residual. The different covariance structures of repeated matrices were evaluated according to Littell *et al*. (1998) and Wang and Goonewardene (2004) using the Akaike information criterion (AIC) and the Schwarz Bayesian criterion (BIC). Based on the AIC and BIC values the unstructured covariance structure or ANTE (1) covariance structure were used in the models.

Table 3. Chemical composition, feeding value and fatty acid composition of roughage ingredients and concentrates.

Parameter	Roughage mixture[1]						Concentrate[2]	
	Maize silage[3]				Grass silage	Soybean meal	LC	HC
	MS30	MS34	MS38	MS42				
Chemical composition, g/kg DM								
Dry matter (DM), g/kg	319	324	361	387	286	879	897	897
Crude protein	74	77	79	78	136	385	207	196
Crude fat	35	35	36	36	42	32	54	41
WSC [4]	4.2	4.3	4.0	4.0	58	113	90	119
Starch	381	396	415	433	-	23	94	86
Neutral detergent fibre	366	350	345	341	487	146	386	312
Acid detergent fibre	212	202	198	196	289	96	213	191
Acid detergent lignin	19	19	19	19	17	6	41	38
pH	3.8	3.9	4.2	4.01				
NH_3-N, g /100 g total N	7.7	9.5	8.1	9.5	8.4	-	-	-
Feeding value [5]								
DVE [6]	50.8	51.8	51.5	50.8	48.2	208	122	123
OEB [7]	-32.3	-31.5	-29.0	-30.7	39.4	133.0	41.0	38.0
NE[8], MJ/kg DM	6.57	6.55	6.52	6.54	6.31	7.96	7.29	7.27
OMD [9] (%)	75.9	76.1	75.7	75.6	77.7	89.9	82.10	84.30
Fatty acid, g/kg DM								
C12:0	0.05	0.04	0.05	0.03	0.10	0.35	6.95	5.92
C14:0	0.08	0.12	0.18	0.12	0.09	0.15	2.49	2.08

C16:0	2.99	3.01	2.89	2.76	2.86	3.72	4.48	4.47
C16:1	0.05	0.04	0.07	0.12	0.11	0.03	0.07	0.01
C18:0	0.46	0.47	0.47	0.48	0.22	0.08	0.92	0.82
C18:1cis-9	4.45	4.45	4.33	4.35	0.31	3.56	7.22	5.97
C18:2n-6	12.2	12.4	11.9	11.9	2.35	11.6	10.7	9.07
C18:3n-3	1.56	1.32	0.78	0.58	10.40	1.50	3.25	3.11
C20:0	0.12	0.12	0.13	0.14	0.05	0.06	0.10	0.06
C20:1	0.05	0.05	0.05	0.04	-	0.03	0.01	0.07
C22:0	0.06	0.06	0.06	0.07	0.11	0.09	0.06	0.05
C24:0	0.10	0.16	0.14	0.11	0.07	0.05	0.07	0.07
Unidentified	0.38	0.11	0.56	0.17	0.12	0.76	0.82	0.45
Total PUFA[10]	13.8	13.7	12.7	12.5	12.7	13.90	13.9	12.2
Total fatty acids	22.5	22.4	21.6	20.9	16.8	22.0	38.1	33.2

[1] Roughage mixture contained (DM basis): 61% maize silage, 28% grass silage, 10% soybean meal, 0.45% mineral and vitamin mixture and 0.34% salt (NaCl).

[2] Concentrates with low degradable carbohydrates (LC) or high degradable carbohydrates (HC).

[3] DM content of 300 (MS30), 340 (MS34), 380 (MS38) and 420 (MS42) g/kg fresh weight.

[4] Water soluble carbohydrates.

[5] Calculated according to CVB (2007).

[6] Intestinal digestible protein (Tamminga *et al*., 1994).

[7] Degraded protein balance in the rumen (Tamminga *et al*., 1994).

[8] Net energy lactation calculated with VEM (feed unit lactation) system (Van Es, 1978).

[9] Organic matter digestibility determined in vitro according to Tilly and Terry (1963) as modified by van der Meer (1987).

[10] Polyunsaturated fatty acids.

Results

Data on chemical composition, feeding value and FA contents of roughage mixture ingredients and the concentrates are summarized in Table 3. The starch content of the maize silages increased (381 to 433 g/kg DM), while the NDF content decreased (366 to 341 g/kg DM) consistently in silages made from the successive harvests. The content of CP, NE and OMD were similar across the maize silages. Maturation of the silage maize decreased the content of C16:0, C18:3n-3, PUFA and total FAs (Table 3) with the content of C18:3n-3 showing the largest decline (1.65 to 0.58 g/kg DM) with maturity. The FA composition of maize silages, soybean meal and concentrates was dominated by C18:1 *cis*-9 and C18:2n-6, whilst in grass silage, C18:3n-3 was the predominant FA.

Nutrient Intake and Animal Performance

Intake of DM, CP and NE did not differ ($P > 0.05$) with advancing maturity from MS30 to MS42 (Table 4). The intake of starch increased ($P < 0.01$), and those of NDF and ADF decreased ($P < 0.05$) with increasing maturation. The intake of DM and NE did not vary due to concentrate type (Table 4). However, the intake of WSC was higher ($P < 0.001$) and that of NDF was lower ($P < 0.001$) on the HC compared to the LC concentrate. The intake of total FAs ($P < 0.01$), PUFA ($P < 0.01$) and C18:3n-3 ($P < 0.001$) linearly decreased with increasing maturation of the maize silages. The intake of C18:1 cis-9 and C18:2n-6 was higher ($P < 0.001$) in the LC compared to the HC rations. However, the intake of C18:3n-3 did not differ due to concentrate type. Lactation stage significantly ($P < 0.001$) changed the intakes of all nutrients and FAs (data not shown).

No difference in milk yield and milk composition was found between the maize silages, except for yield of fat, which significantly ($P < 0.05$) lower in the MS42 compared to the MS34 and 38 (Table 5). Yield (kg/d) of milk, protein and lactose was higher ($P < 0.05$) on the LC ration compared to the HC ration. Advancing lactation significantly ($P < 0.001$) affected the milk yield and composition. The percentage of fat and lactose in milk was higher ($P < 0.05$) on the HC ration compared to the LC ration. Body weight and BCS over the 15 weeks lactation period did not differ due to the maturity of maize silages and carbohydrate degradability concentrates.

Table 4. Effect of maize silages (MS) ensiled at different maturities in combination with a low (LC) or high (HC) degradable carbohydrate concentrate (Con) on nutrient intake of dairy cows.

Parameter	Maize silage[1]					Concentrate			Significance[2]		
	MS30	MS34	MS38	MS42	SEM[3]	LC	HC	SEM	MS	Con	Week
Intake, kg /d											
MS DM	9.7	10.0	10.0	9.5	0.38	9.9	9.7	0.35	ns	ns	***
Roughage DM	15.8	16.4	16.2	15.5	0.62	16.1	15.9	0.57	ns	ns	***
Total DM	23.2	23.5	23.7	22.8	0.68	23.4	23.1	0.64	ns	ns	***
CP	3.40	3.47	3.53	3.40	0.089	3.52	3.38	0.085	†	***	***
Crude fat	0.92	0.93	0.94	0.92	0.026	0.98	0.87	0.024	ns	***	***
Starch	4.42	4.71	4.89	4.89	0.162	4.68	4.68	0.150	**	ns	***
WSC[4]	1.27	1.27	1.29	1.24	0.028	1.16	1.38	0.026	†	***	***
NDF	8.58	8.45	8.39	8.06	0.238	8.72	8.02	0.233	*	***	***
ADF	5.00	4.93	4.88	4.70	0.139	5.01	4.75	0.130	*	**	***
DVE[5]	1.95	1.99	1.97	1.93	0.049	1.97	1.95	0.046	ns	ns	***
NE_L[6], MJ/d	160	162	159	156	4.4	161	159	4.1	ns	ns	***
Fatty acid intake, g/d											
C8:0	3.94	3.91	3.97	3.94	0.040	3.75	4.12	0.039	ns	***	***
C10:0	3.37	3.76	3.79	3.84	0.098	4.88	5.06	0.092	ns	***	***
C12:0	51.6	51.3	51.8	51.5	0.29	55.4	47.7	0.25	ns	***	***
C14:0	18.2	18.5	19.2	18.5	0.23	20.2	17.0	0.23	***	***	***
C16:0	80.4	75.3	80.9	77.1	2.05	78.8	78.1	1.95	***	ns	***
C18:0	11.9	12.1	12.2	12.0	0.28	12.5	11.6	0.26	ns	***	***
C18:1 *cis*-9	98.8	99.4	99.3	96.7	2.35	104	93.3	2.25	ns	***	***
C18:2 n-6	221	225	221	214	6.3	228	213	5.9	†	***	***
C18:3 n-3	86.2	85.1	80.7	75.7	2.47	83.0	80.8	2.32	***	#	***
Total PUFA[7]	307	310	302	289	8.8	311	294	8.3	**	***	***
Total fatty acids	590	592	592	566	14.8	606	654	14.0	**	***	***

[1] DM contents of 300 (MS30), 340 (MS34), 380 (MS38) and 420 (MS42) g/kg fresh matter.
[2] Ns, not significant; #, P < 0.1; *, P < 0.05, **, P < 0.001;***, P < 0.001.
[3] Standard error of the mean.
[4] Water soluble carbohydrates.
[5] Intestinal digestible protein (Tamminga et al., 1994).
[6] Net energy lactation calculated using VEM (feed unit lactation) system (Van Es, 1978).
[7] Polyunsaturated fatty acids.

Table 5. Milk production, milk composition and changes in body condition of dairy cows fed maize silages (MS) ensiled at different stages of maturity in combination with a low (LC) and high (HC) degradable carbohydrate concentrate (Con) during week 2 to 15 of lactation.

Parameter	Maize silage[1]					Concentrate			Significance[2]		
	MS30	MS34	MS38	MS42	SEM[3]	LC	HC	SEM	MS	Con	Week
Milk yield											
Milk, kg/d	40.2	40.8	40.8	39.5	1.32	41.3[a]	39.3[b]	0.94	ns	*	***
FPCM[4], kg/d	42.9	43.4	43.8	41.6	1.45	43.2	42.6	1.02	ns	ns	***
Milk composition											
Fat, %	4.25	4.17	4.21	4.05	0.097	4.03[b]	4.30[a]	0.079	ns	***	***
Fat, kg/d	1.66[ab]	1.70[a]	1.70[a]	1.60[b]	0.067	1.67	1.67	0.047	**	ns	***
Protein, %	3.27	3.22	3.28	3.29	0.071	3.25	3.28	0.048	ns	ns	***
Protein, kg/d	1.31	1.33	1.31	1.27	0.060	1.33[a]	1.28[b]	0.058	ns	*	***
Lactose, %	4.66	4.62	4.72	4.64	0.028	4.64[b]	4.68[a]	0.024	ns	*	***
Lactose, kg/d	1.85	1.99	1.90	1.82	0.081	1.91[a]	1.82[b]	0.077	ns	**	***
Body condition											
Body weight, kg	626	640	652	650	16.3	638	646	11.5	ns	ns	ND[5]
BCS[6]	2.7	2.7	2.9	3.0	0.05	2.8	2.8	0.04	ns	ns	ND

[ab] Means within rows with different superscripts differ ($P < 0.05$).
[1] Dry matter contents of 300 (MS30), 340 (MS34), 380 (MS38) and 420 (MS42) g/kg fresh matter.
[2] Ns, not significant; #, $P < 0.1$; *, $P < 0.05$, **, $P < 0.001$;***, $P < 0.001$.
[3] Standard error of the mean.
[4] Fat and protein corrected milk.
[5] Not determined.
[6] Body condition score on a scale of 1 to 5 according to (Edmonson *et al.,* 1989).

Fatty Acid Composition of Milk

The effect of maize silages, concentrate type and lactation stage on milk FA composition of the dairy cows is presented in Table 6. Increasing harvest-maturity of the maize silages from MS30 to MS42, decreased ($P < 0.05$) the content of C18:3n-3, total n-3 and n-6:n-3 ratio in milk fat. Concentrate type significantly altered ($P < 0.05$) the composition of all trans-FAs (Table 6). Inclusion of the HC concentrate in the maize silage based diets

increased the content of all C18:1-trans isomers, C18:2 cis-9, trans-11 and C18:2 trans-10, cis-12. Milk FA composition was strongly influenced by the stage of lactation (Table 6). The content of all even-short and medium chain FAs altered ($P < 0.05$), except for C8:0 and C10:0. The content of C12:0, C14:0 and C16:0 in total fat increased ($P < 0.05$) as a result of advancing lactation with the largest increase (29.1 to 32.4 g/100 g total FAs) observed for C16:0. The content of C4:0 and C6:0 on the other hand decreased ($P < 0.05$) with lactation. Among the pre-formed (not synthesized de novo) saturated FAs, the content of C18:0 showed largest decrease (9.30 to 8.88 g/100 g total FAs) with lactation. Overall, the total content of saturated FAs increased (70.0 to 74.8 g/100 g total FAs) with advancing lactation while the content of C18:1 cis-total and total cis-MUFA decreased with lactation.

Discussion

Research has established that the composition of carbohydrates (starch:NDF ratio) (Kalscheur *et al.,* 1997; Griinari *et al.,* 1998; Shingfield *et al.,* 2005; Nielsen *et al.,* 2006) and content of PUFA (Kelly *et al.,* 1998; Chilliard *et al.,* 2001) in the diets of dairy cows can alter the content and composition of milk fat. Silage maize, next to grass, is a major forage component in rations of dairy cows, under most dietary regimes. The crop is harvested at an advanced ripening stage (for high starch content), but with a wide range in stage of maturation (Phipps *et al.,* 2000; Cone *et al.,* 2008). The variation in maturity at harvest has shown marked influences on the carbohydrate composition (starch:NDF ratio) (Bal *et al.,* 2000; Phipps *et al.,* 2000) and FA content of maize silages (Khan *et al.,* 2011b). The current experiment aimed to provide a comprehensive insight into the effect of maize ensiled at different maturities and supplemented with a high and low degraded carbohydrate concentrate on nutrient and FA intake, milk production, composition of milk and milk fat of dairy cows during early lactation.

The range of maturities (DM content of 300 to 420 g/kg FW) chosen in the present study spanned that normally found in the Netherlands. The increase in starch content with each subsequent harvest is related to the growth of ear and deposition of starch in the grains during maturation (Cone *et al.,* 2008). The substantial increase in starch (grain) content decreased the NDF content in the whole crop DM. The NDF content of the stover increases as maturity advances, however, the NDF content of the whole crop decreases because the

Table 6. Milk fatty acid (FA) content (g/100 g total FAs) of dairy cows fed maize silage (MS) ensiled at different stages of maturity in combination with a low and high degradable carbohydrate concentrate (Con) during week 3, 10 and 15 of lactation.

Fatty acid	Maize silage[1]					Concentrate			Stage of lactation				Significance[2]		
	M30	M340	M38	M42	SEM[3]	HC	LC	SEM	Week 3	Week 5	Week 10	SEM	MS	Con	Week
C4:0	3.37^{AB}	3.52^{AB}	3.53^{A}	3.32^{B}	0.061	3.41	3.45	0.047	3.55^{a}	3.46^{a}	3.29^{b}	0.048	*	ns	***
C6:0	2.35	2.46	2.47	2.36	0.041	2.43	2.38	0.030	2.47^{a}	2.41^{ab}	2.34^{b}	0.034	#	ns	**
C8:0	1.50	1.56	1.57	1.53	0.038	1.58^{a}	1.50^{b}	0.028	1.58	1.55	1.49	0.032	ns	*	ns
C10:0	3.45	3.62	3.70	3.63	0.130	3.71	3.49	0.095	3.53	3.62	3.64	0.106	ns	#	ns
C11:0	0.09	0.09	0.09	0.09	0.007	0.09	0.09	0.005	0.10^{a}	0.10^{a}	0.08^{b}	0.006	ns	ns	**
C12:0	4.54	4.68	4.78	4.83	0.164	4.96^{a}	4.45^{b}	0.123	4.55^{b}	4.66^{ab}	4.92^{a}	0.085	ns	**	*
C13:0	0.10	0.11	0.11	0.11	0.009	0.11	0.1	0.007	0.11	0.10	0.11	0.006	ns	#	ns
Iso-C13:0	0.01^{A}	0.005^{B}	0.009^{AB}	0.011^{AB}	0.002	0.10	0.01	0.001	0.01^{a}	0.01^{a}	0.01^{b}	0.001	*	ns	***
Anteiso-C13:0	0.08	0.08	0.08	0.09	0.005	0.09^{a}	0.08^{b}	0.004	0.08	0.08	0.09	0.004	ns	**	ns
C14:0	11.9	12.1	12.4	12.4	0.24	12.3	12.1	0.18	11.6^{c}	12.2^{b}	12.8^{a}	0.21	ns	ns	***
Iso-C14	0.07	0.06	0.07	0.06	0.004	0.06	0.06	0.003	0.06	0.06	0.06	0.003	#	ns	ns
C14:1 cis-9	0.91	0.85	0.91	0.96	0.041	0.95^{a}	0.87^{b}	0.029	0.85^{c}	0.90^{b}	0.98^{a}	0.025	ns	*	***
C15:0	0.92	0.98	0.89	0.1	0.062	0.95	0.94	0.050	0.09	0.92	0.99	0.050	ns	ns	ns
Iso-C15:0	0.19^{AB}	0.19^{AB}	0.20^{A}	0.17^{B}	0.008	0.19	0.18	0.001	0.18^{b}	0.19^{ab}	0.19^{a}	0.007	*	#	*
Anteiso-C15:0	0.40	0.41	0.40	0.39	0.017	0.41	0.4	0.015	0.39^{ab}	0.40^{b}	0.42^{a}	0.015	ns	ns	*
C16:0	30.4	30.7	30.3	31.0	0.59	29.8^{b}	31.5^{a}	0.05	29.1^{c}	30.3^{b}	32.4^{a}	0.45	ns	**	***
Iso-C16:0	0.16	0.15	0.16	0.15	0.007	0.16	0.16	0.006	0.16	0.16	0.15	0.007	ns	ns	ns
C16:1cis-9	0.47	0.48	0.46	0.46	0.042	0.47	0.46	0.041	1.03^{a}	0.20^{c}	0.16^{b}	0.058	ns	ns	***
C17:0	0.61	0.61	0.61	0.62	0.016	0.61	0.61	0.013	0.62	0.61	0.60	0.012	ns	ns	ns
Iso-C17:0	0.37^{A}	0.36^{B}	0.37^{AB}	0.34^{B}	0.029	0.36	0.36	0.029	0.42	0.34	0.33	0.042	*	ns	ns

Anteiso-C17:0	1.53	1.62	1.66	1.78	0.095	1.59	1.70	0.080	1.11^{c}	1.99^{a}	1.84^{b}	0.075	ns	ns	***
C17:1 cis-9	0.24	0.24	0.22	0.24	0.012	0.23	0.24	0.009	0.27^{a}	0.24^{b}	0.19^{c}	0.009	ns	ns	***
C18:0	9.27	9.12	9.35	8.86	0.251	9.13	9.16	0.19	9.30^{a}	9.26^{a}	8.88^{b}	0.19	ns	ns	*
C18:1 *cis*-9	19.0	17.8	17.7	18.2	0.671	18.3	18.0	0.552	19.3^{a}	18.7^{a}	16.6^{b}	0.665	ns	ns	***
C18:1 *cis*-total[4]	20.1	18.9	18.8	18.2	0.70	19.5	19.1	0.57	20.5 ^{a}	19.8 ^{a}	17.5 ^{b}	0.43	ns	ns	***
C18:1 *trans*-6+8	0.23	0.22	0.22	0.22	0.009	0.24^{a}	0.21^{b}	0.007	0.22	0.22	0.22	0.006	ns	**	ns
C18:1 *trans*-9	0.16	0.16	0.16	0.17	0.004	0.17^{a}	0.15^{b}	0.003	0.16^{b}	0.16^{ab}	0.17^{a}	0.003	ns	***	*
C18:1 *trans*-10,11	1.18	1.12	1.13	1.11	0.052	1.22^{a}	1.05^{b}	0.038	1.18	1.12	1.10	0.030	ns	**	#
C18:1 *trans*-12	0.30	0.31	0.30	0.30	0.009	0.33^{a}	0.28^{b}	0.007	0.30	0.30	0.31	0.007	ns	***	ns
C18:1 *trans*-total[5]	2.16	2.14	2.12	2.09	0.076	2.27^{a}	1.99^{b}	0.054	2.16	2.11	2.12	0.055	ns	***	ns
C18:1 *trans*-16+cis-14	0.31	0.31	0.31	0.29	0.008	0.31^{a}	0.30^{b}	0.007	0.30	0.30	0.31	0.007	#	ns	ns
C18:2 *trans*-9,12	0.15^{B}	0.13^{B}	0.13^{B}	0.16^{A}	0.008	0.14	0.15	0.006	0.16^{a}	0.17^{a}	0.09^{b}	0.005	*	ns	***
C18:2 cis-9,12 (n-6)	1.78	1.71	1.75	1.74	0.038	1.78	1.71	0.027	1.70^{b}	1.76^{a}	1.78^{a}	0.027	ns	ns	**
C18:2 cis-9, trans-11[6]	0.35	0.34	0.31	0.32	0.011	0.34^{a}	0.32^{b}	0.007	0.35	0.33	0.32	0.007	ns	*	*
C18:2 *trans*-10, cis-12[6]	0.01	0.01	0.01	0.01	0.002	0.01^{a}	0.01^{b}	0.002	0.01^{b}	0.00^{c}	0.01^{a}	0.001	ns	**	***
C18:3 n-3	0.42^{A}	0.39^{AB}	0.39^{AB}	0.37^{B}	0.010	0.40	0.39	0.007	0.40	0.40	0.39	0.007	**	ns	ns
C18:3 n-6	0.01^{B}	0.02^{B}	0.02^{AB}	0.02^{A}	0.002	0.02	0.02	0.002	0.01^{b}	0.02^{a}	0.03^{a}	0.001	***	ns	***
C20:0	0.10^{B}	0.10^{B}	0.11^{AB}	0.12^{A}	0.006	0.11	0.11	0.053	0.11	0.10	0.12	0.006	**	ns	#
C20:1 *cis*-11	0.06^{AC}	0.05^{B}	0.06^{BC}	0.07^{A}	0.003	0.06	0.06	0.002	0.06^{b}	0.07^{a}	0.06^{b}	0.002	***	ns	***
C20:2 n-6	0.00^{B}	0.01^{AB}	0.01^{AB}	0.01^{A}	0.001	0.01	0.01	0.001	0.01	0.01	0.01	0.001	*	ns	ns
C20:3 n-6	0.07	0.07	0.07	0.08	0.004	0.08^{a}	0.07^{b}	0.003	0.06^{c}	0.07^{b}	0.08^{a}	0.003	ns	*	***
C20:4 n-6	0. 05^{B}	0.07^{A}	0.07^{A}	0.06^{A}	0.003	0.07^{a}	0.06^{b}	0.003	0.09^{a}	0.02^{b}	0.07^{a}	0.003	***	**	***
C20:5 n-3	0.04	0.04	0.04	0.05	0.002	0.04	0.04	0.002	0.05^{a}	0.04^{ab}	0.04^{b}	0.002	ns	ns	*

Fatty acid	Maize silage[1]					Concentrate			Stage of lactation				Significance[2]		
	M30	M340	M38	M42	SEM[3]	HC	LC	SEM	Week 3	Week 5	Week 10	SEM	MS	Con	Week
C22:0	0.03	0.02	0.03	0.03	0.001	0.03	0.03	0.001	0.03	0.02	0.03	0.001	ns	ns	ns
C22:2 n-3	0.02	0.02	0.01	0.02	0.002	0.02	0.02	0.002	0.01	0.01	0.02	0.001	ns	ns	ns
C22:5 n-3	0.06	0.06	0.06	0.06	0.004	0.06	0.06	0.004	0.06	0.06	0.06	0.004	ns	ns	ns
C24:0	0.02	0.01	0.01	0.02	0.002	0.01	0.01	0.002	0.02	0.01	0.02	0.002	ns	ns	ns
Total saturated FA	71.5	73.0	73.0	72.3	0.73	72.0	72.9	0.61	70.0^{c}	72.6^{b}	74.8^{a}	0.77	ns	#	***
Total *cis*-MUFA[7]	21.87	20.6	20.3	20.9	0.69	21.2	20.7	0.56	22.7^{a}	21.2^{b}	18.9^{c}	0.73	ns	ns	***
Total n-3	0.52^{A}	0.50^{AB}	0.49^{AB}	0.48^{B}	0.012	0.50	0.50	0.054	0.51	0.49	0.49	0.055	*	ns	#
n-6:n-3 ratio	3.99^{A}	4.07^{B}	4.13^{AB}	4.36^{A}	0.101	4.20	4.08	0.090	4.01^{b}	4.14^{a}	4.27^{a}	0.091	**	#	***

Means within rows with different superscripts (^{abc} or ^{AB}) differ ($P < 0.05$).

[1] Dry matter contents of 300 (MS30), 340 (MS34), 380 (MS38) or 420 (MS42) g/kg fresh matter.

[2] Ns, not significant; #, $P < 0.1$; *, $P < 0.05$, **, $P < 0.001$;***, $P < 0.001$.

[3] Standard error of the mean.

[4] Sum of all C18:1 *cis* isomers except cis-14, which was not separated from trans-16.

[5] Sum of all C18:1 *trans* isomers except *trans*-16, which was not separated from cis-14.

[6] Conjugated linoleic acid.

[7] Monounsaturated fatty acids.

proportion of grains in the whole crop DM increases (Bal *et al.,* 2000). The decrease in C18:3n-3 and PUFA content with maturation can be related to the decrease in C18:3n-3 content in the stover (leaves and stems) fraction of maize plants during post-flowering maturation. In maize plants, the membrane glycerolipids are the main pool of C18:3n-3, whereas C18:1 *cis*-9 and C18:2n-6 are the predominant FAs in storage lipids (grains). During grain filling, the content of C18:3n-3 substantially decreases due to a decreasing proportion of the stover in the whole plant DM and the decreasing FA content in the stover (Khan *et al.,* 2011b), due to rapid senescence of leaves (Struik 1983). During leaf senescence, the membrane glycerolipids are oxidized by plant lipoxygenases, causing a rapid decrease in chloroplasts FAs, particularly in C18:3n-3 (Thompson *et al.,* 1998; Mishra*,* Sangwan 2008; Yang*,* Ohlrogge 2009).

The lack of differences in DM intake, milk yield and body condition due to maturity of the maize supports earlier findings (Bal *et al*., 2000; Phipps *et al*., 2000). The increase in starch:NDF ratio resulted in a numeric decrease in milk fat content from 4.25% on MS30 to 4.05% on MS42. Unexpectedly, the combination of the HC concentrate with maize silages resulted in a higher percentage of milk fat compared to the LC concentrate (4.30 *vs.* 4.03). Typically, the combination of the high fermentable carbohydrate concentrate and the low NDF maize silage based diets are associated with a reduction in milk fat (Nielsen *et al.,* 2006). The high milk fat content with the HC concentrate may be due to the large (2 kg/d) decrease in milk yield. Moreover, the fat yield did not differ between the HC and LC concentrates.

The decrease in C18:3n-3, total n-3 and n-6:n-3 PUFA ratio in milk fat content in milk fat with maturity can be related to the parallel decrease in the intake of C18:3n-3 with maturation of maize silages. Although there were small differences in the intake of C18:3n-3, these were reflected in the milk fat composition, which may be due to a lower degradation of the mature leaves of the maize and a high rumen passage rate of maize silages. The changes in carbohydrate composition during maturation, however, did not alter the hydrogenation of dietary PUFA. A plausible explanation for this effect is the relatively small variation in starch:NDF ratio due to maturation from DM content 300 to 420 g/kg FW. A large variation in starch:NDF ratio occurs early during the grain filling period, with the change in crop DM content from 250 to 320 g/kg (Cone *et al.,* 2008). The present study did not include early harvested maize. Concentrate type significantly influenced the composition of milk *trans*-FAs. With the exception of C18:2 *cis*-9, *trans*-11 which can also

be produced via delta-9-stearoyl-CoA desaturase in the mammary gland, the variation in the contents of *trans*-isomers of C18:1 and C18:2 in milk between the two types of concentrates, directly reflect the changes in ruminal biohydrogention of dietary PUFA (Corl *et al.,* 2002; Piperova *et al.,* 2002). High amounts of PUFA or rapidly degradable carbohydrates in the diet can shift rumen biohydrogenation of PUFA towards the production of more *trans* FAs, in particular the *trans*-10 isomer (Griinari *et al.,* 1998; Shingfield *et al.,* 2005b; Nielsen *et al.,* 2006). In the present study, the intake of C18:2n-6 and C18:3n-3 was higher when the LC concentrate was fed, yet the amount of *trans* FAs in milk were higher with the HC concentrate. This indicates that the alteration in milk *trans* FA composition was mainly related to the variation in carbohydrate degradation between the two concentrates. Diets that provide high amounts of readily degradable carbohydrates usually shift the biohydrogenation of dietary PUFA towards *trans*-isomers by changing bacterial population (Griinari *et al.,* 1998; Jurjanz *et al.,* 2004).

Milk fat composition markedly changed with the advancing lactation. Many studies have reported an increase in medium chain saturated FAs (C12-C16:0) during early lactation, whereas C18:0 and C18:1 *cis*-9 follow the reversed pattern (Palmquist *et al.,* 1993; Kay *et al.,* 2005; Garnsworthy *et al.,* 2006; Stoop *et al.,* 2009). In the present study, the changes in FAs content over lactation can be related to the release of FAs from body fat reserves and the consequent shift in *de novo* synthesis of the saturated FAs in the mammary gland (Palmquist *et al.,* 1993). High producing dairy cows are usually in a negative energy balance during early lactation and mobilize considerable amounts of body fat, containing C18:0 and C18:1 *cis*-9 as the predominant FAs (Christie 1981). This can explain the decrease in C18:0 by 4.5 and C18:1*cis*-9 by 14.0%
between week 1 and 10 of lactation. Moreover, a high uptake of long chain FAs inhibits de novo lipogenesis, particularly of the medium chain saturated FAs.

Conclusions

Increasing maize harvest-maturity, crop DM content 300 to 420 g/kg fresh matter, at ensilaging did not affect the DM intake, milk yield and body condition score, but decreased the content of C18:3n-3, total n-3 and n-6:n-3 ratio in milk fat of dairy cows. The combination of maize silage and a high degradable carbohydrate concentrate increased the content of all C18:1 *trans* isomers, C18:2 *cis*-9, *trans*-11, C18:2 *trans*-10, *cis*-12, and total *trans* FA in milk fat compared to a low degradable carbohydrate concentrate. Milk FAs

composition was significantly influenced by stage of lactation. The content of C12:0, C14:0 and C16:0 and total saturate FAs increased, while the content of C18:0, C18:1-*cis* total and total *cis*-monounsaturated FA decreased with advancing lactation.

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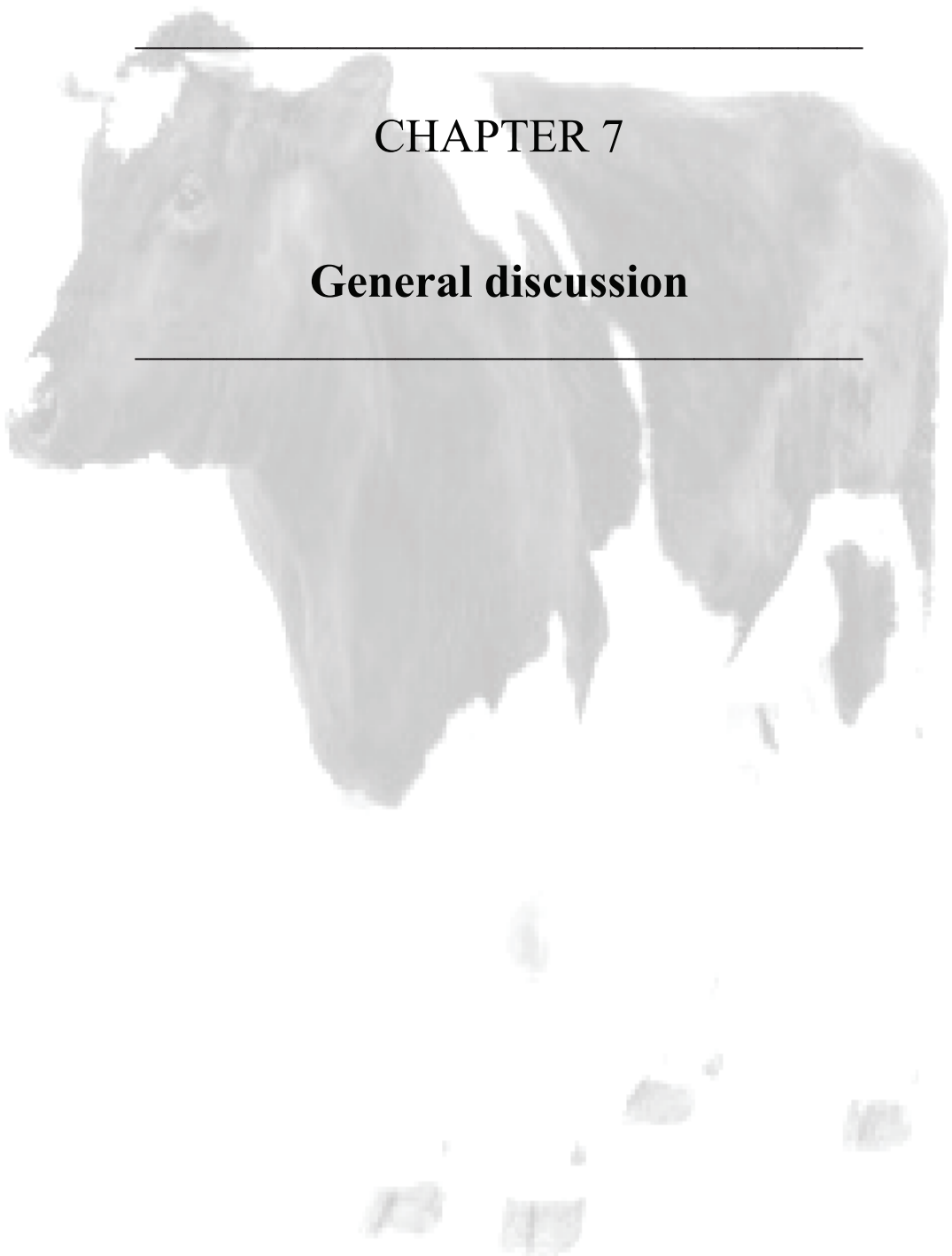

CHAPTER 7

General discussion

The aim of this thesis was to investigate the scope of increasing the content of polyunsaturated fatty acids (PUFA) in grass and maize silages, and to establish relationships between silage quality on the one hand and the fatty acid (FA) content and composition, post-ensiling stability of PUFA and milk FA composition of dairy cows on the other hand. The first focus of this thesis was to quantify the variations in FA content and composition in grass and maize silages and to identify the causes of these variations. The multivariate analysis (Chapter 2) showed that most of the variation in FA content and composition in grass and maize silages originates from differences in maturity at harvest. Therefore, the effect of maturity in variable growing conditions on FA content and composition of silage maize (Chapter 3) and grass (Chapter 1 and 2) were studied. Identifying pre and post-ensiling processes that optimize the stability of PUFA was the next goal. The physical damage and stress caused by mowing and post-harvest field operations activate lipid degrading enzymes, lipases and lipoxygenases, in plant tissues. Plant lipases liberate free FAs from the membrane glycerolipids. The free FAs are dominated by PUFA, which constitute a favourable substrate for enzymatic and non-enzymatic peroxidation. If grass is ensiled directly after cutting, there are no or little oxidative losses as anaerobic conditions establish quickly in well-sealed and compacted silo. However, grass is generally wilted for variable lengths of time to reach a higher DM content at ensiling to improve the silage fermentation process. The wilting of grass prior to ensiling is associated with oxidative losses of FAs. The oxidative losses of FAs mainly depend on the duration of wilting (Chapter 4) with temperature having quantitatively a lesser impact, while light, bruising and tedding of grass does not affect the stability of FAs during wilting (Chapter 2 and 4). The highly esterified lipids of forages are extensively hydrolysed in the silo yielding free FAs which remain stable in well-sealed and compacted silo. The stable environment of the silo dramatically changes after opening the silo, when silages are fed to the animals; because in the presence of oxygen plant lipoxygenases, microbes, light and pro-oxidant metal-ions can oxidise the free FA. The stability of FAs was investigated in grass and maize silages exposed to air for 0, 12 and 24 h (Chapter-5). Maize harvested at different maturities show differences in the content and composition of carbohydrates and FAs that can affect the content and composition of milk fat in dairy cows. The effect of feeding maize silages, ensiled at targeted dry matter (DM) contents of 300, 340, 380 and 420 g/kg fresh matter, on milk FA composition of dairy cows was investigated (Chapter 6).

Variation in Fatty Acid Content and Composition in Grass and Maize Silages

Multivariate analysis was performed on a large number of grass and maize silages, randomly sampled from commercial dairy farms in the Netherlands to quantify the variation in FA contents and to identify key management factors during the entire ensiling process that cause these variations (Chapter 2). The contents of all FAs, in particular the predominant PUFA were highly variable in grass and maize silages. The content of C18:3n-3 showed a large variation (17 g/kg DM) in grass silages, whilst C18:2n-6 showed the largest variation (16 g/kg DM) in maize silages. Moreover, the high intake of grass and maize silages by dairy cows, particularly in winter can further magnify the variation in the intake of PUFA. For example, consumption of 10 kg DM of grass silage produces a difference in intake of C18:3n-3 of 170 g/d and a consumption of 10 kg DM of maize silage produces a difference in intake of C18:2n-6 of 155 g/d. This latter highlights the scope for increasing the intake of PUFA from the grass and maize silages. The variation in FA content of grass silages was predominantly associated with variation in the contents of PUFA, whilst the saturate FAs (SFA) changed marginally (Figure 1, Panel-A). The proportion of C18:3n-3 decreased and the proportion of C16:0 increased with a decrease in total FA.

Quantitatively, grass species and cultivars (Dewhurst *et al.,* 2001; Palladino *et al.,* 2009), N fertilization (Mayland *et al.,* 1976; Elgersma *et al.,* 2005; Witkowska *et al.,* 2008), cutting-date/season (Bauchart *et al.,* 1984; Dewhurst *et al.,* 2001; Boufaïed *et al.,* 2003), environmental conditions at cutting and wilting duration (Dewhurst and King, 1998; Van Ranst *et al.,* 2009a) can all affect the FA contents of grass silages. The multivariate analysis of the data on all these variables and many other potential variables (Chapter 2) showed that quantitatively most of the variation in FA content in grass silages was related to differences in stage of maturity at harvest. Grass silages made from young grass were higher in C18:3n-3 and total FAs. The FA content in grass silages decreased with increasing maturation. During maturation of forages, the content of C18:3n-3 declines at a preferentially faster rate due to the decrease in leaf proportion in the whole plant DM (Bauchart *et al.,* 1984; Boufaïed *et al.,* 2003), maturation of leaves (Hawke, 1973), initiation of flowering (Dewhurst *et al.,* 2006) as well as leaf senescence in more mature grasses (Thompson *et al.,* 1998; Mishra and Sangwan, 2008; Yang and Ohlrogge, 2009). The maturity of grass silages was reflected by their digestibility and the PUFA content of

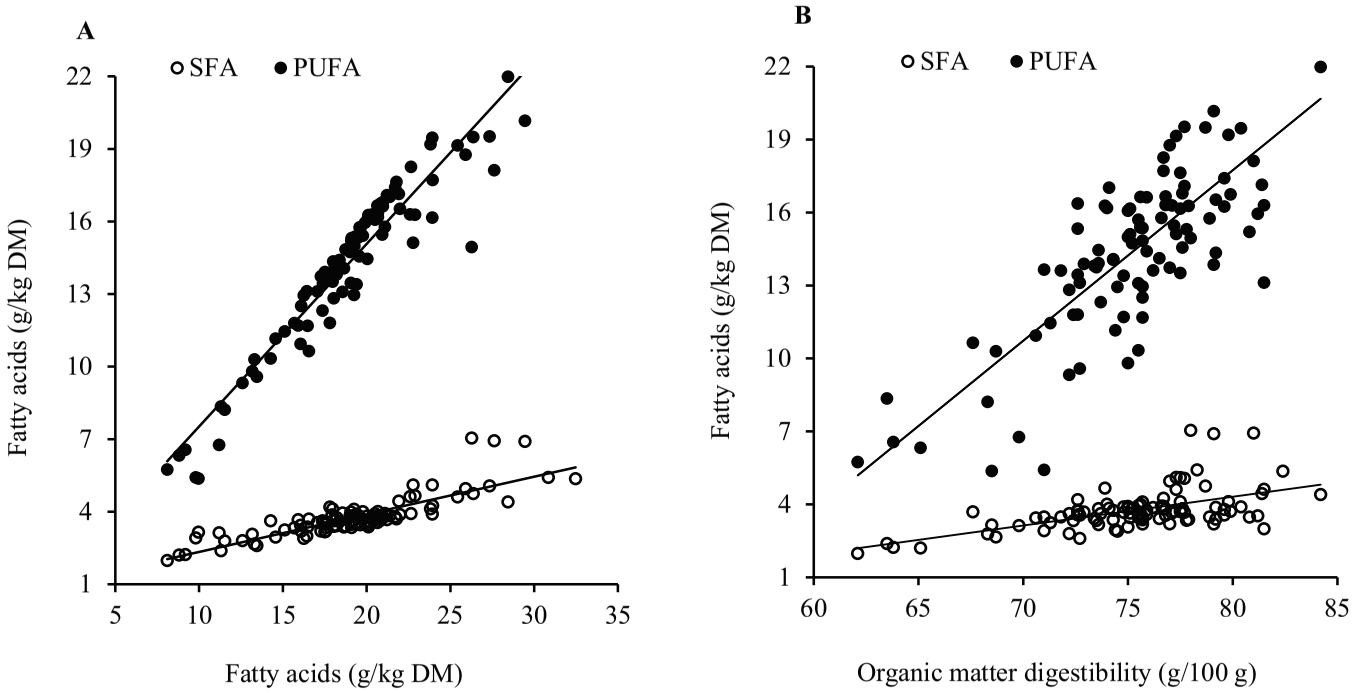

Figure 1. Changes in saturated fatty acids (SFA; C16:0 + C18:0) and polyunsaturated fatty acids (PUFA; C18:3n-3 + C18:2n-6) in relation to total fatty acid content (Panel A) or organic matter digestibility (Panel B) in grass silages (n = 101).

the silages decreased with a decrease in *in vitro* organic matter digestibility, whilst the SFA changed marginally (Figure 1, Panel B).

The large variation in FA content in grass and maize silages highlights the need to determine the quality of individual silages in terms of their FA content and composition. Although the FA contents of silages can be determined using gas chromatograph, which is an accurate method, it requires time consuming extractions, derivitisation and chromatography steps, making this method unfeasible for routine analysis of silages on dairy farms. Alternatively, the multiple regression equations based on chemical composition and feeding values of the silages estimated by near infrared reflectance spectrometry (NIRS) developed here (Chapter 2) can be used. The regression equations had a relatively high coefficient of determination (R^2) for the model fit, predicted R^2 (Table 1; Figure 2 A, B) and low error of cross validation for the content of C18:3n-3 and total FAs in grass silages, and for the content of C18:2n-6 and total FAs in maize silages. The nutrient composition and the feeding value of individual silage clamps in the Netherlands are routinely determined using NIRS analysis in order to optimise the nutrition of dairy cows in practice. Farmers can use information of these predictor variables such as the contents of acid detergent lignin (ADL), neutral detergent fibre (NDF) and crude fat (Cfat); cell walls digestibility (CWD) and organic matter digestibility (OMD), intestinal digestible protein (DVE) and degraded protein balance (OEB) to calculate the FA content of grass silages. Similarly, the content of crude fat, DM, starch and OMD can be used to predict the FA content in maize silage. The predominant PUFA, i.e. C18:3n-3 in grass silages and C18:2n-6 in maize silages may be assessed even more reliably. This can help to design management strategies to increase the content of PUFA in grass and maize silages and enhance the intake of PUFA to favourably modulate milk fat composition of dairy cows.

Table 1. Summary of partial least-squares (PLS) regression analysis.

Silage	PLS model fit			Prediction ability PLS model	
	PLS factors	R^2	SECV[1]	R^2	SEE[2]
Grass					
C18:2n-6	6	0.72	0.69	0.53	0.31
C18:3n-3	4	0.79	0.70	0.75	1.47
Total fatty acids	4	0.74	0.71	0.65	2.48
Maize					
C18:1 n-9	5	0.48	0.90	0.46	0.75
C18:2n-6	4	0.62	0.78	0.64	0.88
Total fatty acids	4	0.57	0.81	0.53	1.20

[1] Standard error of cross validation.
[2] Standard error of estimation.

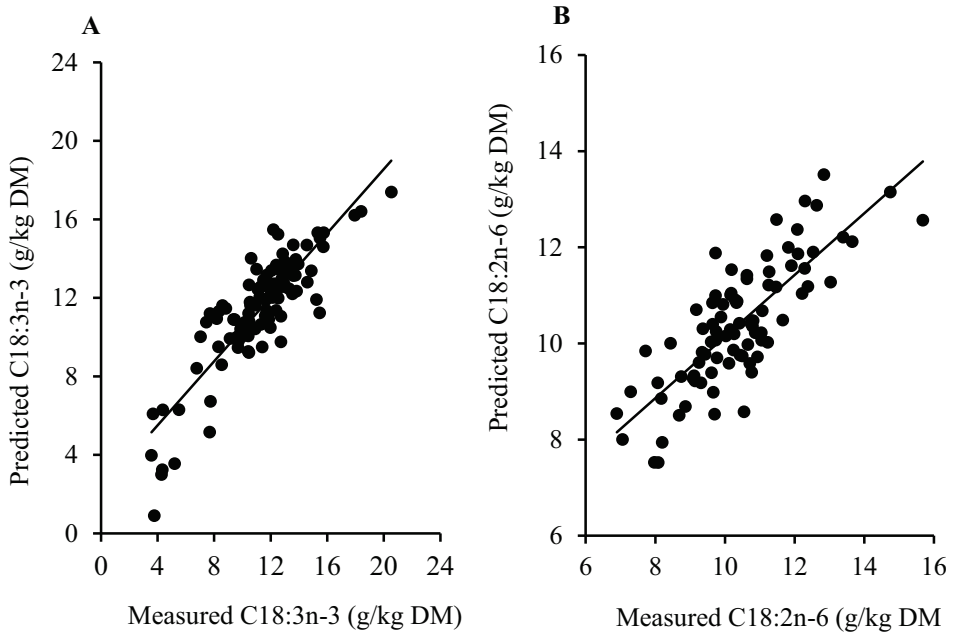

Figure 2. Relationship (1:1) between the contents of C18:3n-3 in grass silages (Panel A) and C18:2n-6 in maize silages (Panel B) as measured by gas chromatography and predicted by PLS model (Table 1).

Optimizing Fatty Acid Content at Harvest in Silage Maize

The multivariate analysis (Chapter 2) showed that there was a large variation in the FA content and composition of maize silages and that this variation primarily originated from the large difference in maturity at harvest. In order to more fully understand this variation the changes in FA content and composition in stover (leaves and stem) and ears (cob, shank and husks) in relation to variables that marked the progress of maturation of the silage maize were studied (Chapter 3). In maize plants, C18:3n-3 is the major FA in the membrane glycerolipids of stover, whilst 18:2n-6 is the major FA in the storage lipids in the ears (grains). During grain filling the FA supply from the membrane lipids of the stover was synergistically decreased by the decreasing proportion of stover in the whole crop and the declining content of FAs in the stover. The stover fraction decreased from 0.90 g/g whole crop DM at 14 d after flowering (DAF) to 0.40 g/g whole crop DM at 84 DAF. During the same period (14–84 DAF) the FA content of stover also declined by 0.59 g/g DM. The decline in C18:3n-3 and total FAs in stover proceeded more or less parallel and showed biphasic rates, with a period of relatively low decrease during 14–56 DAF, where the proportion of C18:3n-3 remained more or less constant (0.60 g/g total FAs). However, in the period from 56 to 84 DAF, the content of C18:3n-3 and total FAs in stover declined rapidly (Chapter 3). The biphasic rate of decline in C18:3n-3 content can partly be explained by a similar pattern of leaf senescence during grain filling. Leaf senescence progresses slowly during early grain filling, however, the rate of leaf senescence increases towards the end of grain filling (Struik, 1983). During leaf senescence, PUFA and in particular C18:3n-3, are oxidized by plant lipoxygenases (Thompson *et al.,* 1998; Mishra and Sangwan, 2008; Yang and Ohlrogge, 2009), which could explain the rapid decrease in the content of C18:3n-3 towards the end of the grain filling period.

The FA supply from the storage lipids of the ears (grains) increased during the grain filling, due to the substantial increase in the proportion (0.10 to 0.60) of the ears in the whole crop and accumulation of FAs in the ears. The maximum accumulation of FAs (g/kg DM) in ears was recorded at 56 DAF (T_{sum} 927 °C.d (Sibma *et al.*, 1987), ear DM content 440 g/ kg) and then the FA content and composition remained more or less constant. Deposition of FAs in the grains occurs only during a brief period early in the development of oil-bearing seeds. Weber (1969) reported a major deposition of lipids in the developing kernels during

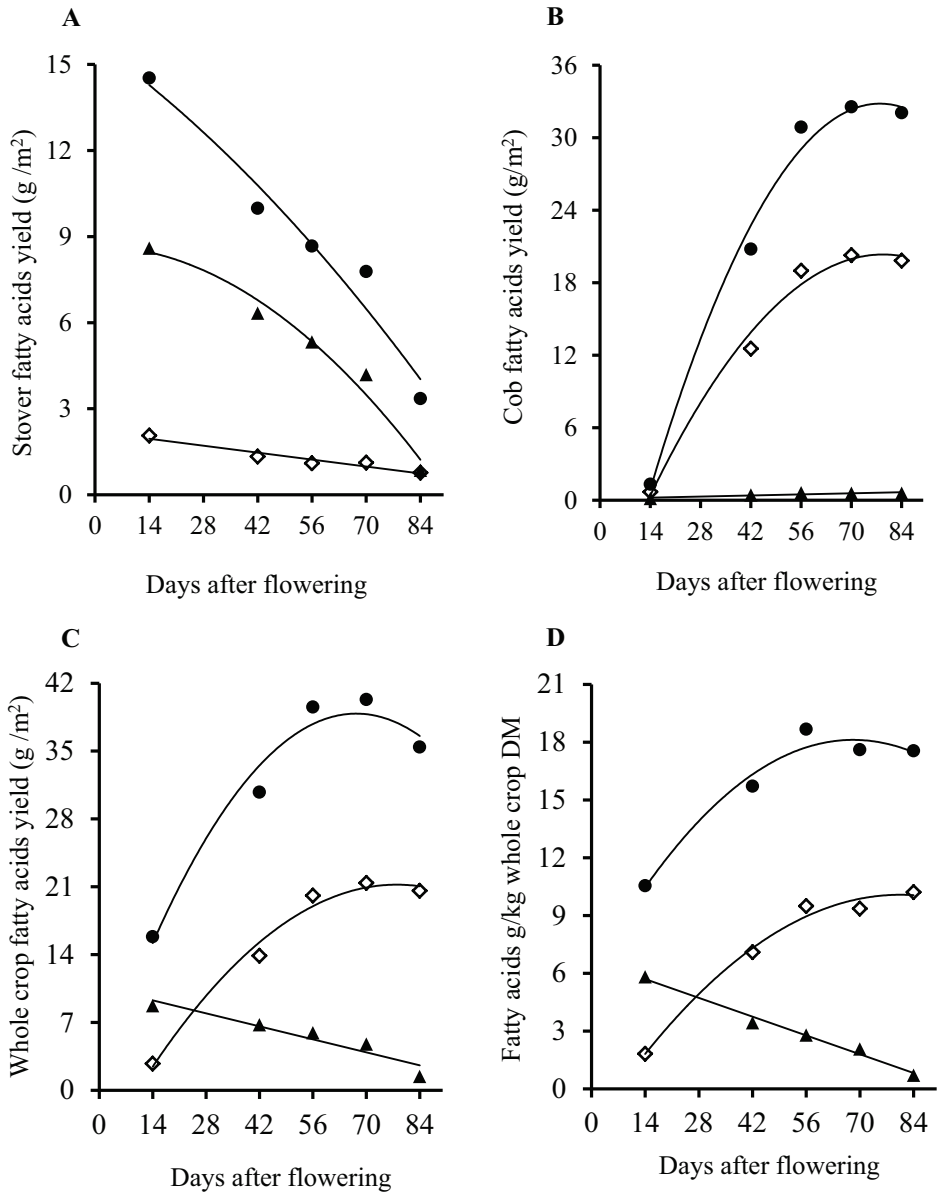

Figure 3. Yield (g fatty acids/m^2) of total fatty acids (•), C18:3n-3 (▲) and C18:2n-6 (◊) in stover DM (Panel A), ear dry matter (DM) (Panel B), whole crop DM (panel C) and, contents (g/kg DM) of total fatty acids (•), C18:3n-3 (▲) and C18:2n-6 (◊) in whole crop DM. Temperature sum (°C.d, with a base temperature of 10 °C) after flowering was 654 (14 DAF), 851 (42 DAF), 927 (56 DAF), 945 (70 DAF) and 968 (84 DAF).

15–45 d after pollination, which coincides with the data here, as nutrients in the ears are mainly stored in the maize kernels.

The yield (g/m^2) of C18:3n-3 in the stover decreased consistently during the grain filling period (Figure 3, Panel A), whereas the yield (g/m^2) yield of C18:2n-6 in the ears increased up to 56 DAF and then remained more or less constant (Figure 3, Panel B). In the whole crop, the content and yield of C18:3n-3 decreased linearly during 14-84 DAF, while the content of C18:2n-6 increased up to 56 DAF and then stayed constant (Figure 3, Panel C, D). Therefore, at a whole plant level the maximum amount of PUFA can be harvested around 56 DAF (T_{sum} 927 °C.d, ear DM content 440 g/kg), which is before the onset of the rapid senescence. These results demonstrate that a delay in harvesting silage maize at the end of the grain-filling period should be avoided in order to minimize the losses of green leaves and with it C18:3n-3 in maize silages. Alternatively, maize hybrids with a high green leaf area index, and which stay greener towards the end of the grain-filling period, can be expected to maintain a higher content of C18:3n-3.

Oxidation of Fatty Acids

Oxidation of FAs during senescence and conservation of forages can lead to substantial losses of PUFA. In forages, lipid peroxidation is initiated by lipases, which liberate free FAs from the membrane glycerolipids. The free FAs are dominated by PUFA, which are highly reactive molecules and are preferred by enzymatic and non-enzymatic oxidation (Mueller, 2004; Liavonchanka and Feussner, 2006). Enzymatic lipid peroxidation is a controlled process and the lipid degrading enzymes, lipases and lipoxygenases, are generally intra-cellularly present in plant tissues. These enzymes degrade membrane lipids only under certain physiological conditions, such as at the onset of leaf senescence and during natural defence responses to physical damage (Thompson *et al*., 1987; Thompson *et al*., 1998). Enzymatic peroxidation is mainly catalysed via the lipoxygenases pathway. Plant lipoxygenases incorporate molecular oxygen into PUFA, leading to different hydroperoxy derivatives (Fall *et al.,* 1999; Feussner and Wasternack, 2002; Hamberg *et al.,* 2005). The hydroperoxy PUFA are highly reactive and these are rapidly catabolised via various lipoxygenase pathways to yield a range of volatile compounds which have a range of biological activities such as defence signalling (jasmonates) and antimicrobial/antifungal activities (leaf aldehydes or divinyl ethers) (Blée, 2002; Liavonchanka and Feussner, 2006).

The majority of lipoxygenases prefer free FAs as substrates (Feussner and Kühn, 2000) and specifically oxygenate PUFA containing *cis* double bonds (Brash, 1999) namely C18:2n-6 and C18:3n-3. Non-enzymatic lipid peroxidation is catalysed by reactive oxygen species (ROS), and proceeds as a free radical-mediated chain reaction involving initiation, propagation and termination. Non-enzymatic peroxidation also prefers PUFA, because they contain multiple double bonds with methylene (CH_2) groups, which possess reactive hydrogen. The most notable ROS in plants are super oxide ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot}$), lipid-oxyl radicals ($LOO^{\cdot}$) and lipid-peroxyl radicals ($OLOO^{\cdot}$). Light energy (ultra violet irradiation, 320-400 nm) can produce singlet oxygen (non-radical ROS) in the presence of sensitizers, such as chlorophyll, to induce photo-oxidation. Membrane degradation, such as during senescence and cutting, can bring the ROS in close contact with PUFA, which can enhance the auto-oxidative effects. In animal tissues, PUFA are predominantly peroxidised non-enzymatically. In plants, however, enzymatic oxidation of PUFA via lipoxygenase predominate (Zhuang *et al*., 2002). Therefore, non-enzymatic oxidation during wilting could be lower, as both ROS and plant lipoxygenase use free PUFA as substrate (Feussner and Wasternack, 2002). However, during advanced senescence the level of non-enzymatic lipid peroxidation increases due to a higher production of ROS and a declining efficacy of various defence mechanisms (Thompson *et al*., 1987).

Oxidation of Fatty Acids During Wilting

After mowing, grass is often wilted for various length of time to reach a higher DM content at ensiling to improve the silage fermentation process (Wright *et al*., 2000). However, wilting of grasses is often associated with extensive losses of PUFA, in particular C18:3n-3. Recent research focusing on the stability of FAs during wilting shows that the losses of FAs are variable with respect to wilting duration and changes in herbage DM contents (Table 2). Moreover, wilting of grass for 20 h at cool temperatures (Arvidsson *et al*., 2009) or 24 h in a hot (50 ºC) air oven (Fievez *et al*., 2004) did not alter the FA composition. The variable effect of wilting on oxidation of FAs can be related to the differences in plant damage at mowing and environmental conditions such as temperature and light during wilting (Van Ranst, 2009). It can be hypothesised that plant damage at mowing and

Table 2. Changes in total fatty acid (FA) content and composition during wilting of grass.

References	Species	Wilting[1]	DMC[2]	g/100g total FAs			Total FA (g/kg DM)
				C16:0	C18:2n-6	C18:3n-3	
Dewhurst and King (1998)	*Lolium perenne L.*	0	151	22.1	12.5	55.4	24.56
		68	230	26.9	12.4	47.6	17.51
Dewhurst *et al.* (2002)	*Lolium perenne L.*	0	NR[3]	18.3	13.3	60.9	29.40
		18	NR	19.3	13.5	60.3	23.10
		48	NR	19.1	13.0	60.3	21.10
Boufaied *et al.* (2003)	*Phleum pratense L.*	0	230	17.7	23.5	48.0	19.29
		few	400	18.7	23.2	48.9	16.60
		3 d	850	18.6	22.3	48.8	16.91
Shingfield *et al.* (2005b)	*Phleum pratense L.*	6	238	20.6	16.6	54.7	18.94
		96	724	33.6	16.5	32.0	8.68
		7 d	858	36.5	14.6	30.3	7.53
Arvidsson *et al.* (2009)	*Phleum pratense*						
	First cut	2	Fresh	16.6	15.9	63.5	30.30[4]
		20	336	17.3	16.0	62.4	27.90
	Second cut	2	Fresh	16.2	16.5	62.1	22.50
		6	350	17.7	17.1	59.9	22.00
Van Ranst *et al*., (2009a)	*Lolium perenne*	0	216	13.7	10.8	66.7	19.21
		48	272	14.8	11.2	64.5	20.01
		72	411	16.1	12.3	62.6	15.32
		120	583	18.5	13.6	58.2	12.83
Van Ranst *et al.* (2009b)	*Lolium perenne*						
	First cut	0	Fresh	13.7	11.9	67.9	26.20
		8	350	14.6	12.1	67.3	24.89
	Second cut	0	Fresh	15.8	12.0	64.5	21.70
		8	350	16.5	13.3	61.8	19.70
	Third cut	0	Fresh	13.1	8.7	70.5	32.90
		8	350	16.7	13.4	60.2	23.29

[1] Duration of wilting in hours unless otherwise stated.
[2] Dry matter content.
[3] Not reported.
[4] Crude fat.

environmental conditions can activity of lipids degrading enzymes and the rate of moisture loss, which can affect the oxidative losses of PUFA during wilting.

A direct comparison of plant damage at cutting, environmental conditions and change in herbage DM content during wilting (Chapter 2, 4) showed that the oxidation of FAs mainly depends on the duration of the wilting period. Temperature has quantitatively a small impact, while light, bruising and tedding of grass did not affect the oxidation of FAs. During 48 h of field wilting, the FA content of the herbage declined consistently at a more or less constant rate, without any influence of the consequent increase in herbage DM content from 197 to 676 g/kg. In the climate chambers, the rate of moisture loss was low, and wilting of grass (for 56 to 62 h) to the initial target DM content of 425 g/kg caused substantial losses of PUFA. However, the FA content did not change with further drying (up to 120 h) to a DM content of 525 and 625 g/kg. In the study of Shingfield *et al.* (2005b), substantial losses of PUFA occurred during drying of grass from 324 to 724 g/kg for 96 h. The losses were, however, much smaller during further drying for 7 d. Similarly, there was a marked decline in the content of total FAs when the red clover was wilted for 5 d to a DM content of 250 g/kg. However, the content of total FAs did not change during drying from 250 to 500 g/kg DM at 35 °C in a hot air oven for 8 h (Van Ranst *et al.,* 2009a). These results demonstrate that PUFA oxidize during the initial period after mowing of grass and then oxidation ceases during further wilting.

In temperate countries, including the Netherlands, grass is often bruised to enhance the rate of moisture loss during wilting. Bruising and subsequent tedding of grass can be hypothesised to disrupt membranes and can bring lipid degrading enzymes, pro-oxidant metal ions, sensitizers and membrane lipids together, which may accelerate the oxidation of PUFA. Chapter 2 and 4 show that compared to untreated grass, bruising did not affect the changes in FA content and composition during wilting. The conditioner-mower used for the bruising in Chapter 4 removes the waxy layers of leaves and stems of the grass and apparently did not sufficiently disrupt the chloroplast membranes to bring the catalyst (enzymes and pro-oxidant ion) and substrate (free PUFA) together in a significant manner. In a recent study by Van Ranst *et al*. (2010) there was also no difference in FA content and composition in undamaged and crushed (squeezed and turned) herbage during 24 h wilting. However, a higher oxidation of C18:3n-3 was observed when the herbage was more severely damaged by freezing and thawing compared to crushed and undamaged herbage (Van Ranst *et al*., 2010). The changes in FA content and composition did not differ when

the grass was wilted in a light or dark environment (Chapter 4). In plants, enzymatic oxidation of PUFA via lipoxygenase is more predominant compared to non-enzymatic oxidation (photo-oxidation) (Feussner and Wasternack, 2002; Zhuang *et al.,* 2002). Therefore, photo-oxidation during wilting can be lower, as both plant lipoxygenase and photo-oxidation use the free PUFA as substrate.

Genetic differences between herbages in the extent of FA oxidation during wilting are not studied extensively. There is some evidence of genetic differences, e.g. the oxidation of C18:3n-3 in perennial ryegrass, red clover and white clover showed large variation despite similar wilting conditions (ventilated oven at 35 °C), duration of wilting (8 h) and DM content (Van Ranst *et al.,* 2009b). This indicates that intrinsic differences among plants such as lipoxygenase activity and antioxidant level can influence FA oxidation during wilting. Plant lipids, particularly C18:3n-3, are localized within the membranes of chloroplasts. Alternatively, production of stay-green varieties that lack one of the enzymes involved in chlorophyll breakdown can reduce the oxidative losses of FAs. Stay-green genotypes retain thylakoid membrane longer during senescence compared to normal grass and show lower losses of FA during senescence (Harwood *et al.,* 1982; Thomas and Smart, 1993) and wilting (Dewhurst *et al.*, 2002).

Stability of Fatty Acids During the Feed-out Period

The highly (> 90%) esterified lipids in forages (Elgersma *et al.,* 2003; Van Ranst *et al.,* 2009b; Van Ranst *et al.,* 2010) are extensively (up to 90%) hydrolysed in the silo with a concomitant increase in the level of free FAs (Lough and Anderson, 1973; Steele and Noble, 1983; Elgersma *et al.,* 2003a; Lourenço *et al.,* 2005; Vanhatalo *et al.,* 2007; Lee *et al.,* 2008; Van Ranst *et al.,* 2009a; Van Ranst *et al.,* 2009b). The free FAs are dominated by C18:3n-3 and C18:2n-6, which are highly reactive molecules and are preferred for enzymatic and non-enzymatic oxidation. In a well-sealed and compacted silo, anaerobic conditions establish quickly after ensiling and the free PUFA remain stable (Dewhurst and King, 1998; Arvidsson *et al.,* 2009; Van Ranst *et al.,* 2009a; Van Ranst *et al.,* 2009b). The stable environment of the silo dramatically changes when the silo is opened to feed the silage to the animals. It can be hypothesized that in the presence of oxygen, plant lipoxygenases, microbes, light and pro-oxidant metal-ions can all oxidize the free FAs.

In Chapter 5, the stability of FAs in grass and maize silages with a wide range of qualities (nutritional and ensiling) was evaluated. Exposure to air for 24 h lead to a quantitatively small, but consistent decline in the contents of the major unsaturated FAs in grass and maize silages, with a concomitant increase in the proportion of C16:0 indicating oxidation of the unsaturated FAs. During the feed out period, the unsaturated FAs can be oxidized enzymatically and non-enzymatically. As discussed earlier, enzymatic lipid peroxidation in plants is mainly catalysed by lipoxygenases, and these enzymes can remain functional after ensiling, even though their activity is generally reduced after wilting and ensiling (Lourenço *et al.,* 2005). Membrane degradation during the ensiling process can bring the free PUFA in contact with ROS (Makoni *et al.,* 1993; Nelson, 1993; Rontani, 2001), which can oxidize the PUFA non-enzymatically. In addition, penetration of air into the silage, promotes the growth of aerobic, acid-tolerant microorganisms which can oxidize the free FAs (Danner *et al.,* 2003). High losses of FAs in very wet maize silages *versus* dry silages can be related to a prolonged fermentation and extensive lipolysis in the high moisture silages. Another plausible explanation might be the high effluent losses in the high moisture silages. The oxidative losses of FAs was lower in maize and grass silages with a higher DM content, which can be related to restricted fermentation and lipolysis in these silages (Van Ranst *et al.,* 2009a). Grass silage pH and NH_3 content did not affect the oxidative stability of FAs during the feed out period. The rate of pH decrease affects the lipolysis and proteolysis (Muck, 1987; Van Ranst, 2009). The pH after 8 weeks does not reflect the differences in the rate of pH decrease and the differences in lipolysis. Moreover, the variation in lipolysis due to silage fermentation characteristics is more distinct at the start (up to 2 weeks) of ensiling and then declines (Van Ranst *et al.,* 2010). The silages were well spread out in the trays and volatile FAs from the silages could have evaporated quickly, thereby marginalizing the effects of pH on the aerobic stability of FA during the feed out period. These results demonstrate that silages should not be exposed to air for longer periods than needed so as to avoid unnecessary oxidative losses of unsaturated FAs.

Effect of Maize Harvest-maturity on Milk Fatty Acid Composition of Dairy Cows

Silage maize, next to grass, is the major forage component in the ration of dairy cows under most dietary regimes. In the Netherlands, but also in other countries in Europe (Phipps *et al.,* 2000), silage maize is harvested during a wide range of maturity, with the whole crop

dry matter (DM) content ranging from 250 to 450 g/kg fresh weight. The variation in maturity at harvest during grain filling produces major changes in the content and composition (starch:NDF ratio) of carbohydrates (Bal *et al.,* 2000; Phipps *et al.,* 2000) and FAs (Chapter 3) of maize silages and these can affect the content and composition of milk fat in dairy cows (Kalscheur *et al.,* 1997; Griinari *et al.,* 1998; Kelly *et al.,* 1998; Shingfield *et al.,* 2005a; Nielsen *et al.,* 2006). The experiment described in Chapter 6 provides a comprehensive insight into the effect of feeding maize silages, ensiled at targeted dry matter (DM) contents of 300 (MS30), 340 (MS34), 380 (MS38) and 420 (MS42) g/kg fresh weight, in combination with high degraded carbohydrates (HC) or low degraded carbohydrate (LC) concentrates on nutrients and FA intake, milk production, composition of milk and milk fat of dairy cows during early lactation. The variation in the content and composition of maize silages and the amount and composition of carbohydrates in the different diets can be hypothesized to affect the milk FA composition of dairy cows.

The content of C18:3n-3, the major FA in stover, decreases with each subsequent harvest. This is related to the declining proportion of stover in whole crop DM and the decrease in FA content in stover due to leaf senescence (Chapter 3). The content of starch in the maize silages increase, and those of neutral detergent fibre (NDF) decrease with increasing maturity, which is related to the substantial growth of the ears and deposition of starch in the grains during maturation (Cone *et al.,* 2008). The NDF content of the stover increases as maturity advances. However, the NDF content of the whole crop decreases because the proportion of grains in whole crop DM increases (Bal *et al.,* 2000). DM intake, milk yield and body condition were not affected by the maturity of the maize silages, supporting earlier findings (Bal *et al.,* 2000; Phipps *et al.,* 2000). However, the intake of starch increased, and those of NDF and C18:3n-3 decreased with increasing maturation. The content of C18:3n-3, total n-3 and n-6:n-3 PUFA ratio in milk fat decreased with maturity of the maize at harvest and used for the silages, which correspond with the parallel decrease in C18:3n-3 intake with maturation of maize. Although there were small differences in the intake of C18:3n-3, these were reflected in the milk fat composition which may be due to a low degradation of the mature leaves of maize and a high rumen passage rate of maize silages. The combination of maize silage and HC concentrate increased the content of all C18:1 *trans* isomers, C18:2 *cis*-9, *trans*-11 , C18:2 *trans*-10, *cis*-12, and total *trans* FA in milk fat, compared to a LC. The alteration in milk *trans* FA composition was mainly related to the variation in carbohydrate degradation between the two concentrates. Diets that provide high amounts of readily degradable carbohydrates usually shift the

biohydrogenation of dietary PUFA towards *trans*-isomers by changing the bacterial population (Griinari *et al.,* 1998; Jurjanz *et al.,* 2004).

Milk fat composition markedly changed with the stage of lactation. The medium chain SFAs (C12-C16:0) increased, whereas C18:0 and C18:1 *cis*-9 followed the reversed pattern with lactation, which is in line with data in the literature (Palmquist *et al.,* 1993; Kay *et al.,* 2005; Garnsworthy *et al.,* 2006; Stoop *et al.,* 2009). The changes in FA content during (early) lactation is related to the release of long chain FAs from body fat reserves and the inhibition of *de novo* synthesis of the SFAs in the mammary gland due to a high uptake of long chain FAs (Palmquist *et al.,* 1993; Chilliard *et al.,* 2000). High producing dairy cows are usually in a negative energy balance during early lactation and mobilize considerable amounts of body fat, containing C18:0 and C18:1 *cis*-9 as the predominant FAs (Christie, 1981). This can explain the decrease in content of C18:0 and C18:1*cis*-9 and increase in the content of the medium chain SFA with advancing lactation (week 3 to week 10).

Conclusions and Implications for Dairy Farmers

The research in this thesis shows that the FA content and composition of grass and maize silages are highly variable, and this variation is primarily caused by differences in the maturity at harvest. Silages made from younger grass and maize have higher contents of C18:3n-3. The variation in total FAs content predominantly originated from the variation in the content of C18:3n-3 in grass silages and C18:2n-6 in maize silages, while SFA varied slightly. Grass silages with higher FA contents had higher proportions (g/g total FAs) of C18:3n-3, whereas any decrease in total FAs content was mainly caused by the losses of C18:3n-3. Chemical components and feeding values that are related to the maturity at harvest can provide relative good estimations for the contents of C18:3n-3 (R^2 = 0.75) and total FAs (R^2 = 0.65) in grass silages, and for the content of C18:2n-6 (R^2 = 0.64) and total FAs (R^2 = 0.53) in maize silages. The nutrient composition and the feeding value parameters of individual silage clamps in the Netherland are routinely measured using NIRS analysis. Farmers can use information on predictor variables provided by this NIRS service to assess the FA content and the predominant PUFA in grass silages and maize silages. During the progression of grain filling, the contents of C18:3n-3 and total FAs in the stover fraction of maize plants declined at a slow rate up to 56 DAF and then decreased rapidly during 56–84 DAF. On the other hand the content of unsaturated FAs (C18:1n-9

and C18:2n-6) in the ears increased up to 56 DAF and thereafter remained more or less constant. The maximum amount of PUFA in silage maize can be harvested around 56 DAF (T_{sum} 927 °C.d; ear DM content 440 g/ kg), which is before the onset of the rapid senescence. Farmers need to avoid a delay in harvesting maize beyond this time, at which a rapid decrease in C18:3n-3 may occur. Alternatively, the use of "stay-green" maize genotypes, which retain thylakoid membrane longer during senescence, might allow the preservation of green leaves and the content of C18:3n-3 towards the end of the grain filling period and combine a high starch content with a high C18:3n-3 content.

The oxidation of FAs during wilting of grass mainly depends on duration of the wilting period and occurs independently of the changes in DM content. The wilting temperature only provokes small differences, whereas mechanical bruising of grass at cutting and light intensity during wilting do not affect the changes in FA content and composition. Stability of FAs in herbage may be increased by minimising the duration of the wilting period. Bruising and tedding increases the rate of moisture losses during wilting which can, in-turn, reduce the duration of the wilting period and hence PUFA losses. Similar effects can be envisaged for the wilting of grass on bright sunny days with higher ambient temperatures. Exposure of grass and maize silages to air during the feed out period results in a quantitatively small (< 0.06 g/g total FAs), but consistent decline in content of major unsaturated FAs with a concomitant increase in the proportion (g/g total FAs) of C16:0. These results demonstrate that during the feed out period, silages should not be exposed to air for longer than is needed in order to avoid oxidation of unsaturated FAs.

The maturity of maize at the time of ensilaging (300 to 420 g/kg fresh matter) does not affect the DM intake, milk yield and body condition score, but linearly decreases the content of C18:3n-3, total n-3 and n-6:n-3 ratio in milk fat of dairy cows. The combination of maize silages and a high degradable carbohydrate concentrate increases the content of all C18:1 *trans* isomers, C18:2 *cis*-9, *trans*-11, C18:2 *trans*-10, *cis*-12, and total *trans* FA in milk fat compared to a low degradable carbohydrate concentrate. At present, farmers in the Netherlands are recommended to harvest silage maize at a DM content of 300 to 420 g/kg fresh matter. The current study indicates that maize harvested at the lower end of this recommendation or the use of stay-green genotypes will result in higher C18:3n-3, total n-3 and n-6:n-3 ratio in the milk fat of dairy cows.

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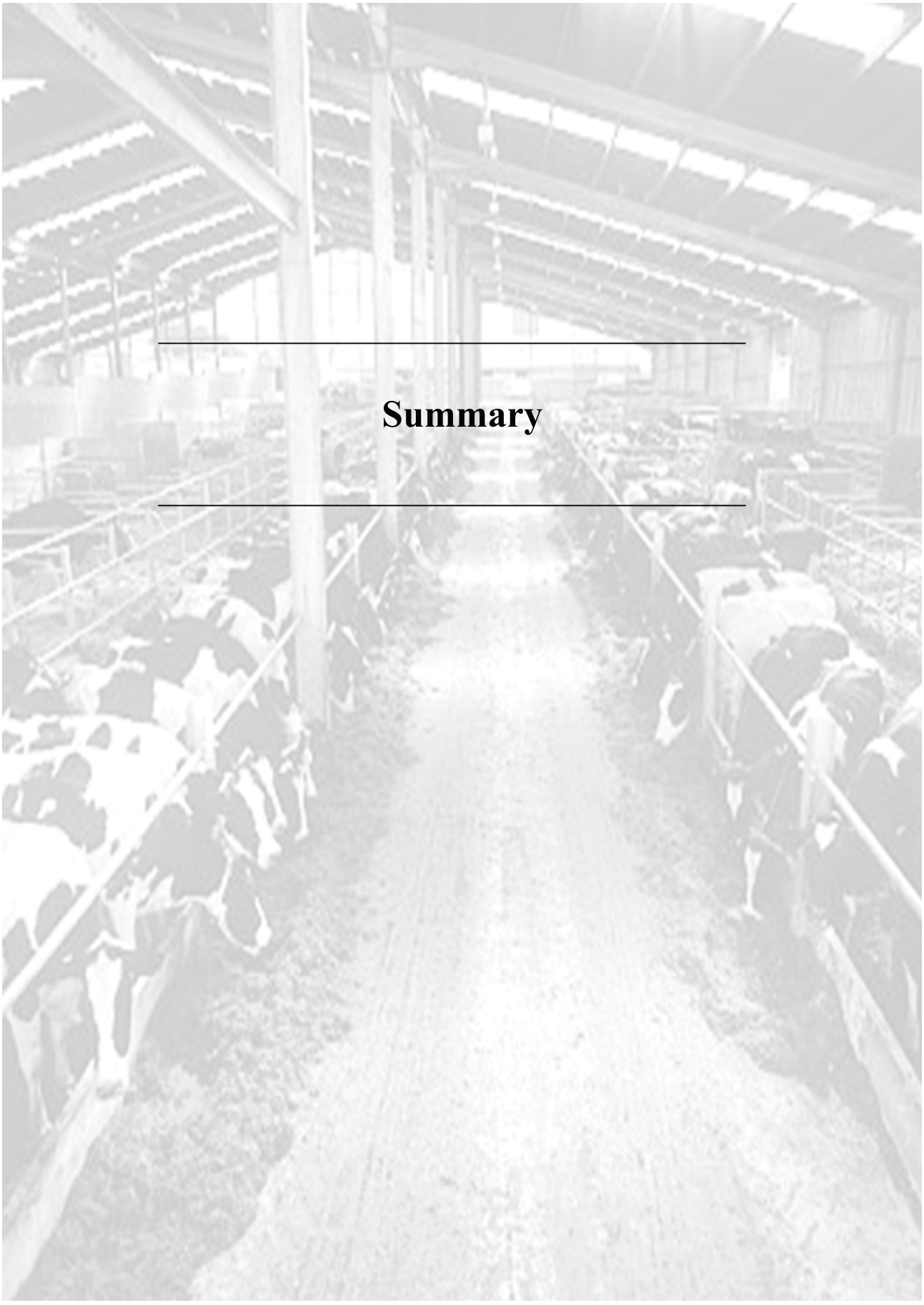

Summary

Summary

Milk and milk products are important components of a human diet in many parts of the world. Raw milk contains fat, high quality protein, lactose, essential minerals and vitamins, and serves as a valuable source of energy and nutrients. Over the past few decades, significant progress has been made in improving milk yield and composition of dairy cows through improved nutrition and breeding. Over this period, however, the fatty acid (FA) composition of milk fat has become less favourable to human health, mainly due to changes in feeding regime, such as the shift from high grazing to more concentrate and silage based diets. The saturated: unsaturated FA ratio has generally increased in milk fat, with a subsequent decrease in the content of essential FAs. Drinking milk with a high proportion of saturated FAs is considered a risk to human health. This makes milk fat a target of growing criticism from dieticians and health-care professionals. However, milk fat also contains a number of FAs such as C18:1 *cis*-9, C18:1 *trans*-11, C18:2 *cis*-9, *trans*-11 conjugated linoleic acid (CLA) and C18:3n-3 that have shown positive health effects. This background provides an impetus for altering the composition of milk fat by decreasing the content of saturated FAs and increasing the content of the beneficial unsaturated FAs.

High polyunsaturated FA-containing forage lipids can be used to favourably modulate milk FA composition of dairy cows at relatively low costs and in an environmentally sustainable manner. Although forages are lower in total FA content (< 45 g/kg dry matter (DM)), the membrane lipids of forages contain a high proportion of polyunsaturated FAs (PUFA; 0.75 ± 0.07 g/g total FA), particularly C18:3n-3 (0.62 ± 0.09 g/g total FA) and dairy cows often consume large quantities of forage lipids. Dairy cows grazing grass ingest large quantities of PUFA and produce milk fat with a lower saturated: unsaturated FA ratio and a high content of beneficial C18:1 *cis*-9, C18:1 *trans*-11, C18:2 *cis*-9, *trans*-11 and C18:3n-3. However, ensiling significantly reduces the positive effects of herbage lipids on the FA composition of milk fat. This unfavourable shift in milk FA composition is partly related to the lower supply of PUFA from ensiled forages due to harvesting of more mature swards and oxidative losses of PUFA during the ensiling process. The aim of this thesis was to investigate the scope of increasing the content of PUFA in grass and maize silages, and to establish relationships between silage qualities on the one hand, and FA content and composition of silages, post-ensiling stability of PUFAs and milk FA composition of dairy cows on the other hand.

In Chapter 2, the variation in the FA content and composition was determined in a large number of grass (n = 101) and maize (n = 96) silages, randomly sampled from commercial dairy farms in the Netherlands. Data related to agronomic practices, sward quality, wilting management, chemical composition, feeding value and ensiling quality of individual silages were subjected to multivariate analysis to search for variables that cause the variation in FA content and composition. The contents of all major individual FAs, in particular the

predominant PUFA, were highly variable in grass and maize silages. The content of C18:3n-3 showed the largest variation (17 g/kg DM) in grass silages and C18:2n-6 showed the largest variation (16 g/kg DM) in maize silages. Multivariate analysis showed that variables related to plants maturity at harvest explained most of the variation in FA content in grass (82%) and in maize (69%) silages, with silages made from young grass and young maize having a high content of C18:3n-3. Grass silages with higher FA contents had a higher proportions (g/g total FA) of C18:3n-3, whereas any decrease in total FA content was mainly caused by the losses of C18:3n-3. Among the nutrient contents and feeding values, variables related to plant maturity were the strongest "predictors" and retained in the regression equations. The regression equations gave relatively good estimations for the content of C18:3n-3 (R^2 = 0.75) and total FAs (R^2 = 0.65) in grass silages, and for the content of C18:2n-6 (R^2 = 0.64) and total FAs (R^2 = 0.53) in maize silages. Farmers and feed manufacturers can use the latter information in conjunction with the routinely provided NIRS derived chemical composition and feeding values to assess the FA content in grass and maize silages, with assessment of the predominant PUFAs being more reliably.

In Chapter 3, the changes in FA content and composition during grain filling were investigated in the stover (leafs and stems) and ear (cob, shank and husk) fractions of maize plants. In maize plants, C18:3n-3 is the major FA in the membrane glycerolipids of stover, whilst C18:1n-9 and 18:2n-6 are the major FAs in the storage lipids of the ears. During the progression of grain filling, 14-84 days after flowering (DAF), the contents of C18:3n-3 and total FAs in the stover DM declined at a slow rate up to 56 DAF (T_{sum} 927 °C.d; ear DM content 440 g/kg) and then decreased rapidly during 56–84 DAF. On the other hand the content of C18:1n-9, C18:2n-6 and total FAs in the ears increased up to 56 DAF and thereafter remained more or less constant. The maximum amount of PUFAs in silage maize can be harvested around 56 DAF, which is before the onset of the rapid senescence.

In Chapter 4, the influence of bruising and environmental conditions on the stability of FAs during the wilting of perennial ryegrass was studied. The untreated and bruised grass was wilted under field conditions for 0, 12, 24, 36 and 48 h, or wilted under controlled climatic conditions at three temperatures (15, 25 or 35 °C) and two light (light and dark) regimes to DM contents of 425, 525 and 625 g/kg fresh weight. The results showed that the oxidation of FAs during wilting of grass mainly depends on the duration of the wilting period and occurs independently of the changes in herbage DM content. The wilting temperature only provoked small differences, whereas mechanical bruising of grass at cutting, and light regimes during wilting did not affect the changes in FA content and composition. Stability of FAs in herbage may be increased by minimising the duration of the wilting period. Bruising and tedding increases the rate of moisture losses during wilting, which can, in-turn, reduce the duration of the wilting period and hence PUFA losses.

The stable environment of the silo dramatically changes, after opening the silo during feed-out period; because in the presence of oxygen, plant lipoxygenases, microbes and light can all oxidize the free PUFAs. In Chapter 5, the stability of FAs was investigated in grass and maize silages, with a wide range of qualities (nutritional and ensiling), exposed to air for 0, 12 and 24 h. Exposure of grass and maize silages to air for 24 h resulted in a quantitatively small (< 0.06 g/g total FA), but consistent decline in the content of the major unsaturated FAs with a concomitant increase in the proportion (g/g total FA) of C16:0. These results demonstrate that during the feed out period, silages should not be exposed to air for longer periods than necessary in order to avoid oxidation of unsaturated FAs.

The large variation in maturity at harvest during grain-filling has a major effect on the carbohydrate composition (starch:NDF ratio) and the FA content of maize silages, which can alter the content and composition of milk fat in dairy cows. The experiment described in Chapter 6 provides a an insight into the effect of feeding maize silages, ensiled at targeted DM contents of 300, 340, 380 and 420 g/kg fresh weight, in combination with a high or low degradable carbohydrate concentrate on nutrient intake, milk yield, milk composition, and milk FA composition in early lactating dairy cows. Sixty-four multiparous Holstein-Friesian dairy cows in their first week of lactation were assigned to the eight dietary treatments according to a randomized complete block design during a 15 weeks experimental period. Maize silage harvest-maturity did not affect the DM intake, milk yield and body condition score, but decreased the content of C18:3n-3, total n-3 and n-6:n-3 ratio in the milk fat of the dairy cows. The combination of maize silage and a high degradable carbohydrate concentrate increased the content of all C18:1 *trans* isomers, C18:2 *cis*-9, *trans*-11, C18:2 *trans*-10, *cis*-12, and total *trans* FA in milk fat compared to a low degradable carbohydrate concentrate.

In Chapter 7, the research results reported in this thesis are discussed and how these findings can be used to increase the PUFA content in grass and maize silages. Maturity at harvest is crucial in achieving a high contents of PUFA and total FAs in grass and maize silages. Avoiding extended maturation of sward and harvesting silage maize around 56 DAF (T_{sum} 927° C.d; ear DM content g/kg), can result in higher PUFA content in grass and maize silages. At present farmers in the Netherlands are recommended to harvest silage maize at a DM content of 300 to 420 g/kg fresh matter. The studies reported here indicate that maize harvested at the lower end of this recommendation result in a higher content of n-3 FA in the milk fat of dairy cows. Alternatively, the use of stay-green genotypes to make silages for dairy cows may further enhance the n-3 FA in milk fat. Management strategies that minimize the wilting period, such as bruising, tedding and harvesting on a warm sunny day, and avoiding a prolonged exposure of silages to oxygen will help to increase the stability of PUFAs during the production-chain.

Samenvatting

Samenvatting

In vele delen van de wereld zijn melk en melk producten belangrijke componenten van het dieet dat door mensen wordt genuttigd. Rauwe melk bevat vetten en ook eiwit van hoge kwaliteit, lactose, essentiële mineralen en vitaminen en dient dus als een zeer belangrijke bron van energie en nutriënten. Gedurende de laatste 20 jaar is er flinke vooruitgang geboekt in melkopbrengst en in melksamenstelling door een verbeterde voeding en door de fokkerij. De samenstelling van het melkvet (vetzuren; FA) is in deze periode niet verbeterd. Integendeel, door veranderingen in de voeding van de koe wordt bijvoorbeeld meer krachtvoer gegeven en ook zijn silages een groter deel van het rantsoen geworden in plaats van vers gras. Daardoor is het melkvet minder goed voor de gezondheid van de mens dan vroeger. De verhouding onverzadigd vet tot verzadigd vet in melkvet is in het algemeen lager geworden met als gevolg een lager gehalte aan essentiele vetzuren. Als er in drinkmelk een groot aandeel verzadigde vetzuren aanwezig is wordt dat beschouwd als een risico voor de gezondheid van de mens. Dit heeft er toe geleid dat de dieet-deskundigen en gezondheidszorg professionals steeds meer kritiek uiten op het gebruik van melkvet in de voeding van de mens. Melkvet bevat echter ook een aantal vetzuren zoals C18:1 *cis*-9, C18:1 *trans*-11, C18:2 *cis*-9, *trans*-11 geconjugeerd linolzuur (CLA) en C18:3n-3 waarvan aangetoond is dat ze positieve effecten hebben op de gezondheid van de consument. Deze feiten over vetzuren in melk zijn de aanleiding en stimulans geweest voor dit onderzoekprogramma naar mogelijkheden tot verandering in de soort vetzuren die in het vet van melk aanwezig is. Er is gezocht naar mogelijkheden om de melkvetsamenstelling te veranderen in de richting van een groter gehalte aan gunstige onverzadigde vetzuren en een lager gehalte aan verzadigde vetzuren.

Als men via het voeren van ruwvoer met een hoger gehalte aan meervoudig onverzadigde vetzuren (PUFAs) de melkvetsamenstelling ook zou kunnen veranderen is dat een vrij goedkope manier die bovendien duurzaam is. Achtergrond is dat hoewel ruwvoer een laag gehalte heeft aan vetzuren (< 45 g/kg droge stof (DS)), membranen van de organellen die voor fotosynthese zorgen een hoog gehalte aan deze PUFAs hebben (wel 0.75 ± 0.07 g/g totaal vetzuren (FA)). Dit is hoofdzakelijk C18:3n-3 (0.62 ± 0.09 g/g totaal FA). Verder kunnen melkkoeien zeer grote hoeveelheden van dit vet via ruwvoer consumeren. Immers tijdens het grazen nemen melkkoeien grote hoeveelheden PUFAs op en produceren in dat geval melk met in het vet een hoge ratio onverzadigde vetzuren ten opzichte van verzadigde vetzuren (FA ratio). Bovendien ie er dan een hoog gehalte aan de gunstige C18:3n-3 en C18:2 *cis*-9, *trans*-11 CLAs. Door het ensileren van gras echter word deze gunstige invloed van gras op melkvetsamenstelling veel minder. Dit effect is tenminste gedeeltelijk gerelateerd aan het lagere gehalte aan PUFA in het gras dat wordt gebruikt voor het maken van silage. Immers dit gras wordt in een meer volgroeid stadium voor ensileren gebruikt dan het groeistadium van gras dat tijdens het grazen door de koe wordt geconsumeerd.

Bovendien verliest het gras tijdens het ensileren PUFAs door oxidatie. Het doel van het onderzoekprogramma was na te gaan welke mogelijkheden en ruimte er zijn om het PUFA gehalte in gras silage en in mais silage te beïnvloeden. Het doel was ook om de relatie vast te stellen tussen silage kwaliteit, FA samenstelling en gehalte van silage , PUFA stabiliteit na het ensileren enerzijds en vetzuursamenstelling van melkvet anderzijds.

In hoofdstuk 2 is de variatie in FA samenstelling van vet in een groot aantal monsters van gras silage (n = 101) en van mais silage (n = 96) bepaald. Monsters werden at random genomen op melkveebedrijven in Nederland. De resultaten werden gerelateerd aan een aantal management maatregelen zoals kwaliteit van het gemaaide gras, mate van voordrogen en chemische samenstelling alsook de voederwaarde en de silage kwaliteit. Via multivariate analyse is onderzocht welke variabelen samenhangen met FA gehalte en met FA samenstelling. De gehaltes aan alle belangrijke FAs en speciaal PUFAs in de verschillende gras silages en in de mais silages blijkt zeer variabel te zijn. De grootste variatie is die in gehalte aan C18:3n-3 (17 g/kg DM) in gras silages en in C18:2n-6 (16 g/kg DM) van maize silages. Multivariate analyse liet zien dat kenmerken die gerelateerd zijn aan groeistadium van de plant bij oogsten het meest verklarend zijn voor de variatie in FA gehalte in gras silage (82%) en in maize silage (69%). Wanneer silage gemaakt is van jong gras of van jonge mais is het gehalte aan C18:3n-3 het hoogste. Gras silages met hoog FA gehalte hadden ook een hoger gehalte aan C18:3n-3(in g/g totaal FA). Ook kwam een verlaging in het totaal FA gehalte hoofdzakelijk door een verlies aan C18:3n-3. Van alle nutriënten die onderzocht werden waren diegene die ook samenhangen met groeistadium het meest voorspellend en deze werden steeds opgenomen in de regressie vergelijkingen. Deze regressie vergelijkingen gaven vrij goede voorspellingen voor de gehalten aan C18:3n-3 (R^2 = 0.75) en totale FAs (R^2 = 0.65) in gras silages, Dit gold ook voor het gehalte aan C18:2n-6 (R^2 = 0.64) en aan totaal FA (R^2 = 0.53) in mais silages. Veehouders en veevoeder leveranciers kunnen deze info gebruiken in combinatie met de NIR bepalingen die routinematig worden gedaan. De met het gebruik van NIRs afgeleide gegevens over de chemische samenstelling en de voederwaarde kan ook gebruikt worden om het FA gehalte te beoordelen in gras en mais silages waarbij de waarde voor PUFA het meest betrouwbaar is .

In Hoofdstuk 3 is het onderzoek beschreven naar de verandering in FA gehalte in verschillende delen van de mais plant en met name in stam, bladeren en in de graan delen van de kolf tijdens het stadium van het vullen van de korrels. In de mais plant is C18:3n-3 de belangrijkste FA in de membranen in de glycerolipiden van de stam, terwijl C18:1n-9 en 18:2n-6 de belangrijkste FAs zijn in de opslagvetten van de mais in de kolf. Tijdens het vullen van de maiskorrel, 14-84 dagen na de bloei (DAF) neemt het gehalte aan C18:3n-3 en aan totaal FAs in de DM van de stam langzaam af tot 56 dagen na bloei. De temperatuursom was T_{sum} 927 °C.d; en het droge stof gehalte was toen 440g per kg..

Daarna daalde het zeer snel van 56–84 dagen na bloei (DAF). Aan de andere kant neemt het gehalte aan C18:1n-9, C18:2n-6 en aan totaal FAs in the kolf toe tot 56 dagen na bloei en daarna blijven deze min of meer constant. Dus wanneer men oogst rond 56 DAF is het gehalte aan PUFAs in de daaruit verkregen silage het hoogste; dat is dus net voor het begin van het afrijpen.

Het onderzoek naar de invloed van kneuzen en omgevingscondities op de stabiliiteit van FAs gedurende het voordrogen van engels raaigras is beschreven in Hoofdstuk 4. Zowel het gekneusde als het onbehandelde gras werd voorgedroogd op het veld gedurende 0, 12, 24, 36 of 48 h ofwel voorgedroogd bij één van 3 gecontroleerde temperaturen 15, 25 of 35 °C en bij twee licht regimes (licht of donker) tot de droge stof gehalten van 425, 525 of 625 g/kg vers gewicht bereikt werden. De resultaten lieten zien dat oxidatie van FAs gedurende het voordrogen van gras vooral wordt bepaald door de duur van het drogen. Bovendien gebeurt dit onafhankelijk van de verandering in droge stof gehalte.. Een verschil in voordroogtemperatuur veroorzaakt slechts kleine verschillen terwijl mechanisch kneuzen en licht of donker geen invloed heeft op verandering in FA gehalte en in samenstelling. Stabiliteit van FAs in ruwvoer kan men verhogen door slechts gedurende korte tijd voor te drogen. Het is dus belangrijk deze tijd te minimaliseren ten einde ook het verlies aan PUFAs te minimaliseren.

De stabiele omgeving in de silo verandert drastisch na het openen van de silo om de silage te voeren. De factoren aanwezigheid van zuurstof, lipoxygenases uit de plant, microben en licht beïnvloeden allemaal de oxidatie van vrije PUFAs. In Hoofdstuk 5 is gerapporteerd over de stabiliteit van FAs in gras en in mais silages. Het onderzoek werd verricht aan silages die een wijde range van kwaliteiten (zowel variatie in nutritionele samenstelling als variatie in ensileren) hadden en die werden blootgesteld aan lucht gedurende 0, 12 of 24 uur. Blootstelling van gras en mais silages aan lucht gedurende 24 uur resulteerde in een kwantitatief kleine (< 0.06 g/g totaal FA), maar wel systematische afname in het gehalte aan de belangrijkste onverzadigde vetzuren met tegelijkertijd een daarmee samenhangende toename in het aandeel (g/g totaal FA) C16:0. Deze resultaten laten zien dat gedurende het voeren uit een geopende silo de silage niet voor langere tijd dan nodig aan lucht mag worden blootgesteld als men tenminste oxidatie van onverzadigde vetzuren zoveel mogelijk wil vermijden.

Als men mais oogst op zeker moment in de periode tijdens de maturatie (gedurende de periode tijdens het vullen van de korrel) leidt dit er toe dat er ook een grote variatie ontstaat in koolhydraatsamenstelling, met name in ratio zetmeel tot NDF, en ook in het FA gehalte van de silage die van die mais gemaakt wordt . Na het voeren van deze mais kan dit het vetgehalte en vetsamenstelling van de melk die door de koe geproduceerd wordt ook beïnvloeden. De resultaten van de proef zoals beschreven in Hoofdstuk 6 geven inzicht in

het effect van het voeren van mais silage die gemaakt is uit mais met verschillende droge stofgehalten (300, 340, 380 of 420 g/kg verse mais. In combinatie met krachtvoer dat een hoog of laag gehalte aan goed of slecht afbreekbare koolhydraten heeft werd de nutriënten opname en melk productie, melksamenstelling en melkvetzuursamenstelling bij nieuwmelkte koeien nagegaan. Vier en zestig Holstein-Frisian melkkoeien met meer dan een pariteit werden in de eerste week van hun lactatie toegewezen aan één van 8 rantsoenbehandelingen. Toewijzing aan behandeling gebeurde via een randomized complete block design en de experimentele periode duurde 15 weken. Mais silage gemaakt van mais die geoogst was op verschillende groeistadia (met betrekking tot vulling van de korrel) had geen invloed op droge stof opname , melkproductie en op conditiescore van de koe. Echter mais silage geoogst tijdens latere stadia van de korrelvulling verlaagde het gehalte aan C18:3n-3, totaal n-3 en n-6:n-3 ratio in het geproduceerde melkvet. De combinatie van mais silage en goed afbreekbare koolhydraten in krachtvoer verhoogde het gehalte aan alle C18:1 *trans* isomeren, C18:2 *cis*-9, *trans*-11, C18:2 *trans*-10, *cis*-12, en totaal *trans* FA in melkvet vergeleken met opname van moeilijk afbreekbare koolhydraten in het krachtvoer .

In Hoofdstuk 7 zijn de resultaten van de onderzoeksresultaten die in deze dissertatie zijn beschreven bediscussieerd. Ook wordt ingegaan op de mogelijkheden om deze resultaten te gebruiken om de PUFA gehalten in gras and mais silages te verhogen. Maturatie stadium bij de oogst van mais en gras voor ensileren is cruciaal om hoge gehaltes aan PUFA en aan totaal FAs in gras silage en in mais silage te bereiken. Men moet vermijden om gras in een laat groeistadium te maaien en bij mais kan men het beste rond dag 56 na bloei oogsten voor het maken van silage (dat is bij een temperatuursom T_{sum} van 927° C.d) Dit kan dan resulteren in een hoog gehalte aan PUFA in de gras silage en in de mais silage. Op het ogenblik wordt de veehouders in Nederland aangeraden mais te oogsten bij een droge stofgehalte van 300 tot 420 g/kg vers materiaal. De studies die hier gerapporteerd zijn laten zien dat mais geoogst bij de lagere droge stof gehalten van deze range na ensilage resulteerde in een hoger gehalte aan n-3 FA in het vet van de koemelk. Anderzijds kan het gebruik van maisgenotypen die langer groen blijven het n-3 FA in melkvet nog verder verhogen. Met betrekking tot management strategie is het belangrijk dat men door minimaliseren van de lengte van de voordroogperiode van gemaaid gras, zoals door kneuzen keren en voordrogen op warme zonnige droge dagen, er voor kan zorgen dat er niet veel PUFAs geoxideerd worden en men dus de stabiliteit van PUFAs gedurende de productieketen waarborgt.

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Curriculum Vitae

About The Author

Nazir Ahmad Khan was born in Swat Valley, Northern Pakistan, in April of 1981. He grew up on a small farm-house of his parents in the valley, where he developed an interest for animal agriculture. In 1997 he qualified Secondary School Certificate (Grade-10) from Deolai High School, Swat, and in 1999 he qualified Higher Secondary School Certificate (Grade-12) from Pak-Turk International College, Islamabad. He graduated with a B.Sc. degree in Animal Husbandry (2000-2004) and an M.Sc. degree in Animal Nutrition (2004-2006) from Kyber-Pukhtunkhwa Agricultural University Peshawar, Pakistan with academic distinctions, (Gold Medals in both B.Sc. and M.Sc.). During M.Sc., he defended a thesis on the nutritional evaluation of local forage tree leaves as a protein supplement to the low quality diets of dairy goats. He worked as livestock production officer from January 2005 to June 2007 in Livestock and Dairy Development Department, Khyber- Pukhtunkhwa-Pakistan. In February 2007, he was awarded with an overseas scholarship by the Higher Education Commission (HEC) Pakistan, to pursue doctorate studies in the Netherlands. In September 2007, he started his PhD at the Animal Nutrition Group of Wageningen University. During his PhD he worked on the changes in fatty acid content and composition during growth and ensiling of grass and maize, in relation with milk fat composition of dairy cows, resulting in this dissertation.

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Training and Supervision Plan		**Graduate School WIAS**	
Name:	Nazir Ahmad Khan		
Group	Animal Nutrition		
Daily supervisors	Dr. John W. Cone and Prof. Dr. Veerle Fievez		
Supervisor	Prof. Dr. Ir. Wouter H. Hendriks		
The Basic Package		**Year**	**Credit***
WIAS introduction course		2008	1.5
Biology underpinning animal sciences		2007	1.5
International conferences			
International Symposium on Ruminant Physiology (ISRP-11), Clermond-Ferrand, France		2009	1.2
International Symposium on Nutrition of Herbivores (ISNH-8), Wales, United Kingdom		2011	1.2
Animal Nutrition Research (ANR) Forum, Ghent and Leuven, Belgium (2x)		2009, 2011	0.6
Seminars and workshops			
WIAS Seminar "Use of biomass: food, feed or fuel: Stakeholder Visions"		2007	0.2
WIAS Seminar "Strategies to improve health and fertility in dairy cows"		2008	0.2
Centre of animal Nutrition (CAN) seminar "Role of plant cell walls in dairy cattle nutrition", Wageningen, The Netherlands		2010	0.2
Dairy solution symposium "Rumen Health: A 360º Analysis", Urecht, The Netherlands		2010	0.6
CAN seminar "Dietary lysine and importance of processing food and feed stuff", Wageningen, The Netherlands		2010	0.2
Animal Welfare Seminar "Scientific research in Animal Welfare", Wageningen, The Netherlands		2011	0.2
WIAS Science Day, Wageningen, The Netherlands (4x)		2008-2011	1.2
ANR Forum, Wageningen and Lelystad, The Netherlands (2x)		2008,2010	1.2
Presentations			
WIAS Science Day (2 posters presentations)		2008, 2009	2.0
Advances in feed evaluation science (oral presentation)		2009	1.0
ANR Forum (2 posters and 1 oral presentations)		2008, 2011	3.0
ISRP-11 (poster presentation)		2009	1.0
ISNH-8 (poster presentations)		2011	1.0
Disciplinary and interdisciplinary courses			
Use of biomass: food, feed or fuel		2007	1.5
Mathematical modelling in boiology		2008	2.0
Nutrition in the omics era		2009	1.0
Nutrient density of milk		2009	0.8
Advances in feed evaluation science		2009	0.3
Statistics courses			
Statistics for life sciences		2008	1.5
Design of animal experiment		2008	1.0
Basic statistics		2009	1.5
MSc level courses			
Working in animal nutrition lab		2009	1.0
Professional Skills Support Courses			
Introduction to Endnote		2008	0.3
Techniques for writing and presenting scientific paper		2010	1.2

Professional Skills Support Courses	**Year**	**Credit***
Scientific publishing	2011	0.3
Interdisciplinary research: crucial knowledge and skills	2011	1.1
Information literacy for PhD	2011	0.6
Research Skills Training		
Preparing PhD research proposal	2008	6.0
Didactic Skills Training		
BSC projects (2x)	2009-2010	2.0
MSC major thesis (1x)	2011	2.0
Tutorship		
Review research master cluster proposals	2010	0.6
Management skills training		
Organizing ANR forum	2008	1.0
Member of WIAS associated PhD Student Council	2008-2009	2.0
Total		**45.7**

*, one ECTS credit equals a study load of approximately 28 hours

Colophon

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