JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Fatty Acids, α-Tocopherol, β-Carotene, and Lutein Contents in Forage Legumes, Forbs, and a Grass–Clover Mixture

Anjo Elgersma,*[,†] Karen Søegaard,[§] and Søren Krogh Jensen[#]

[†]Independent Scientist, P.O. Box 323, 6700 AH Wageningen, The Netherlands

[§]Department of Agroecology and Environment and [#]Department of Animal Sciences, Faculty of Agricultural Science, Aarhus University, Blichers Allé 20, P.O. Box 50, 8830 Tjele, Denmark

ABSTRACT: Fresh forages are an important natural source of vitamins and fatty acids in ruminant diets, and their concentrations in forage species are important for the quality of animal-derived foods such as dairy and meat products. The aims of this study were to obtain novel information on vitamins and fatty acids (FA) in a variety of forage legumes and non-legume forb species compared to a grass–clover mixture and to explore implications for animal-derived products. Seven dicotyledons [four forbs (salad burnet (*Sanguisorba minor*), caraway (*Carum carvi*), chicory (*Cichorium intybus*), and ribwort plantain (*Plantago lanceolata*)) and three legume species (yellow sweet clover (*Melilotus officinalis*), lucerne (*Medicago sativa*), and birdsfoot trefoil (*Lotus corniculatus*))] and a perennial ryegrass–white clover mixture were investigated in a cutting trial with four harvests (May–October) during 2009 and 2010. The experimental design was a randomized complete block, and analyses of variance were performed. In addition, three other forbs were grown: borage (*Borago officinalis*), viper's bugloss (*Echium vulgare*), and chervil (*Anthriscus cerefolium*). Lucerne and yellow sweet clover had the lowest α-tocopherol concentrations (21–23 mg kg^{-1} DM) and salad burnet and ribwort plantain the highest (77–85 mg kg^{-1} DM); β-carotene concentrations were lowest in lucerne, salad burnet, and yellow sweet clover (26–33 mg kg^{-1} DM) and highest in caraway, birdsfoot trefoil, and ribwort plantain (56–61 mg kg^{-1} DM). Total FA concentrations were lowest in lucerne, ribwort plantain, chicory, and yellow sweet clover (15.9–19.3 g kg^{-1} DM) and highest in caraway and birdsfoot trefoil (15.9–19.3 g kg^{-1} DM). Birdsfoot trefoil had the highest (53.6 g 100 g^{-1} FA) and caraway and lucerne the lowest (33.7–35.7 g 100 g^{-1} FA) proportions of n-3 FA. This study demonstrated higher vitamin concentrations in some forbs compared with major forages such as lucerne and grass–clover, more total FA in salad burnet, caraway, and birdsfoot trefoil than in lucerne, and higher n-3 FA concentrations in all forbs than in lucerne. Opportunities are discussed to develop novel biodiverse pastures for particular product quality characteristics.

KEYWORDS: *tocopherols, carotenes, lutein, fatty acids, antioxidants, legumes, herbs, grassland species*

INTRODUCTION

Consumers are increasingly aware of the relationships between their diet, health, and well-being. Retailers and marketers anticipate; this can help to stimulate societal preference for foods that are healthier and more nutritious.[1] Ruminant products have received increased attention due to concern about the environmental impact of ruminant production[2] and health attributes of animal-derived foods due to their high content of saturated FA. Nutritional strategies have been developed to decrease especially the high content of saturated FA and to increase polyunsaturated fatty acids (PUFA) with perceived beneficial effects, for example, conjugated linoleic acid (CLA) (C18:2 *cis*-9,*trans*-11), linoleic acid (C18:2n-6), and α-linolenic acid (C18:3n-3),[3–6] and vitamin content in dairy and meat is of great interest. The amount and fatty acid (FA) profile of fat in milk and meat can be modified by animal diet; that is, the PUFA content increases when feeding ruminants feedstuffs with higher contents of PUFA. In practice, green plants are the main source of PUFA in dairy and meat products. Chloroplast lipid contains high proportions of PUFA, of which α-linolenic acid is usually the predominant fatty acid.[5,6] As α-linolenic acid is the building block of the very long-chain n-3 PUFA (EPA and DHA), feeding forage can increase these beneficial PUFA in milk and meat. Feeding forages represent a low-cost approach to enhance the nutritional quality of milk compared with plant oils or oilseeds and offer the advantage of delivering n-3 FA while minimizing increases in *trans* fatty acids other than C18:1*trans*-11 (vaccenic acid) without negative effects on rumen metabolism. Besides, in forages the lipids are part of a complex matrix and their release in the rumen differs from that of lipids in oils and fats that can impair rumen function when fed in high amounts. The transfer rate of PUFA from forage to milk is high.[3] Forages affect milk fat and protein concentrations and also contribute to nutritive value (vitamins, fatty acids), sensory properties, and physical characteristics of milk and milk products.[7,8] Their impact depends on the species, proportion of forage in the diet, conservation method, and composition of concentrate supplements. After ingestion, lipolysis and biohydrogenation of plant lipids in the rumen play a role.

Milk from grass-fed cows contains higher levels of PUFA such as n-3 FA and CLA than that of silage- and concentrate-fed cows.[4,5] Milk with a high PUFA concentration is more susceptible to oxidation than conventional milk.[6] FA composition may play a role in flavor development over

Received: July 21, 2013
Revised: November 21, 2013
Accepted: November 21, 2013

   dx.doi.org/10.1021/jf403195v | J. Agric. Food Chem. XXXX, XXX, XXX–XXX

time; for example, oxidized flavor in stored milk was positively correlated with levels of linoleic acid, α-linolenic acid, and total PUFA in milk fat.[7] Antioxidants might prevent the development of off-flavors and can increase the shelf life of dairy and meat products. Green forage contains fat-soluble vitamins with antioxidative properties, for example, α-tocopherol and β-carotene (provitamin A),[8] and antioxidants such as lutein. Milk from grass-fed cows contains high levels of antioxidants such as α-tocopherol (vitamin E) and β-carotene.[9] Havemose et al.[10] observed that milk from cows fed grass silage had higher concentrations of the antioxidants β-carotene, lutein, zeaxanthin, and α-tocopherol than milk from cows fed maize silage. Calderon et al.[11] observed a dose−response between β-carotene and α-tocopherol dietary intakes and their secretion in milk. The concentrations of vitamins in forage species are thus important for the vitamin concentration as well as the oxidative stability of animal-derived foods such as dairy[10] and meat products.[12] Lindqvist et al.[13] and Mogensen et al.[14] showed that milk α-tocopherol content is mainly determined by α-tocopherol content in forage and not by the α-tocopherol supplemented with the vitamin−mineral mixture.

Whereas a substantial body of information is available on the differences in composition and sensory properties of products from pasture-based and concentrate-based systems of production,[5,15−18] relatively little information is available on the differences in product quality between species-rich and intensively managed, perennial ryegrass-dominated pastures. Although increased concentrations of polyunsaturated FA in milk from higher altitudes could be related to a higher percentage of forbs (herbs),[19] differences in climate or other yet unknown factors might play a role as well.[20] The concentration and spectrum of antioxidants in milk from cows fed botanically diverse pastures is largely unknown. However, antioxidant properties have been reported for many forb species,[21,22] which if present in botanically diverse pasture may confer added oxidative stability to milk. In forages, various studies on vitamin and fatty acid concentrations were carried out with common grasses[23,24] and some legume species such as white clover and lucerne, but data on non-leguminous forbs are scarce.

Clapham et al.[25] compared traditional and novel forage species grown under greenhouse conditions and observed significant differences in the FA profile of grass and forb species. Few data are available on the FA profile of individual forb species grown under field conditions. Warner et al.[26] found in a cutting trial in The Netherlands that forbs had higher FA concentrations than timothy (*Phleum pratense* L.; 11.5−18.3 g FA kg^{-1} DM), and levels ranged from 18.6 g kg^{-1} DM in yarrow (*Achillea millefolium* L.) to 32.6 g kg^{-1} DM in chicory (*Cichorium intybus* L.). However, this pilot study was carried out during only three harvests in 2007, and no autumn cut was investigated. Wyss and Collomb[27] studied FA in grasses, legumes, and dandelion (*Taraxacum officinale* L.) of two cuts (May and September) during one year. Petersen et al.[28] reported FA concentrations of forb and legume species during a fortnight in late summer. Quantitative data from field experiments carried out during the whole growing season and during multiple years are lacking. As information accumulates on the composition and impact of individual forbs on milk and meat quality, opportunities may arise to develop novel biodiverse pastures for particular product quality characteristics. The aim of this study was to obtain novel data on vitamins and fatty acids in a number of forb and forage legume species compared to a grass/clover mixture, get insight into species differences, and explore implications for animal-derived products.

■ MATERIALS AND METHODS

The experiment was established at the Research Farm Foulumgaard, Aarhus University, Denmark. Swards were established as pure stands of the non-legume forb species salad burnet (*Sanguisorba minor*), caraway (*Carum carvi*), chicory (*Cichorium intybus*), and ribwort plantain (*Plantago lanceolata*) and the legume species yellow sweet clover (*Melilotus officinalis*), lucerne (*Medicago sativa*), and birdsfoot trefoil (*Lotus corniculatus*) and a grass−clover mixture with 15% white clover (*Trifolium repens*) and 85% perennial ryegrass (*Lolium perenne*) (seed weight proportions) in the spring of 2008. Pure stands of chervil (*Anthriscus cerefolium*) were also sown in 2008 and of borage (*Borago officinalis*) and viper's bugloss (*Echium vulgare*) in 2009; the latter two species were not replicated. The experimental setup was a randomized block design with two replications. Net plot size was 1.5 m × 9 m. The plots were harvested four times during 2009 and 2010 to a residual stubble height of 7 cm. Cutting dates were May 29, July 9, August 21, and October 23, 2009, and May 31, July 13, August 19, and October 21, 2010. Agronomic details and herbage yield data were presented earlier.[29]

Sample Processing and Chemical Analyses. After cutting, the herbage was weighed, and subsamples of the harvested herbage were taken.

The botanical composition of the grass−clover mixture was not determined. In the forb plots, unsown species were excluded from the subsamples used for chemical analyses.

A subsample of approximately 0.5 kg of the total herbage was taken from each cut in both years, immediately frozen in a plastic bag at −20 °C, freeze-dried, and subsequently stored in an airtight plastic bag at −20 °C until analysis. Samples were later lyophilized and milled with a 1 mm screen. Of this material, 2 g was saponified in alcohol, and the vitamins were subsequently extracted into heptane and quantified for α-tocopherol, β-carotene (the sum of all isomers), and lutein according to HPLC.[30] In 2009, also γ- and δ-tocopherol were measured. FA was extracted in a mixture of chloroform, methanol, and water according to the method of Bligh and Dyer[31,32] after acidification by boiling in 3 mol L^{-1} HCl for 1 h. Methyl esters was synthesized from alkaline methanol with BF_3 as catalyst and analyzed on gas−liquid chromatography as methyl esters with C17 as internal standard.

Chervil, borage, and viper's bugloss samples were analyzed for FA and vitamins when the amount of herbage was sufficient. These species were excluded from statistical analyses because chervil was present only in the first cut and disappeared thereafter, whereas borage and viper's bugloss were unreplicated.

Statistical Analysis. The experimental design was a randomized complete block with two replications. There were eight species (the seven broad-leaf species plus the mixture) and four harvests per year. Analysis of variance procedures were applied using the MIXED procedures of SAS (version 9).[33] Vitamin concentrations and fatty acid concentration and composition data were evaluated with the following model:

$$Y_{bscy} = \mu + \alpha_s + \beta_c + (\alpha\beta)_{sc} + \delta_y + \lambda_b + (\delta\lambda)_{yb} + A_{bs} + B_{bsc} + C_{sy} + D_{bsy} + E_{bscy}Y_{bscy}$$

where y_{bscy} is the recorded value for species s in cut c of block b in year y, Greek letters denote fixed effects, capital italic Latin letters denote random effects, and lower case italic Latin letters identify the effects and observations.

The following four effects were considered to be random effects: block × species, block × species × cut, species × year, and block × species × year. Because the year × block effect $(\delta\lambda)_{yb}$ was not significant for any of the parameters in a first analysis, this interaction term was deleted and the analysis was repeated.[34] Differences detected among main effects and interactions were assessed using the PDIFF

Table 1. Concentrations of α-Tocopherol, β-Carotene, and Lutein (Milligrams per Kilogram DM) for Four Non-legume Forbs, Three Forage Legume Species and a Perennial Ryegrass–White Clover Mixture, Averaged (n = 16) over Four Cuts in 2009 and 2010[a]

species	salad burnet	caraway	chicory	ribwort plantain	yellow sweet clover	lucerne	birdsfoot trefoil	mixture	SEM species effect	significance of effects			
										spec	cut	S × C	year
α-tocopherol	85 d	58 bcd	55 bc	77 cd	23 a	21 a	65 bcd	39 ab	4.4	**	*	NS	NS
β-carotene	30 ab	61 d	41bc	56 d	33 ab	26 a	59 d	48 cd	4.5	*	***	***	NS
lutein	129 a	174 bc	152 ab	149 ab	131 a	129 a	206 c	195 c	12.6	**	***	**	***

[a]Standard error of the main effect of species (SEM). Significance of main effects of species (Spec) and cut, their interaction (S × C), and year (Y): *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; NS not significant. Within a column, least-squares means without a common letter are significantly different ($P < 0.05$).

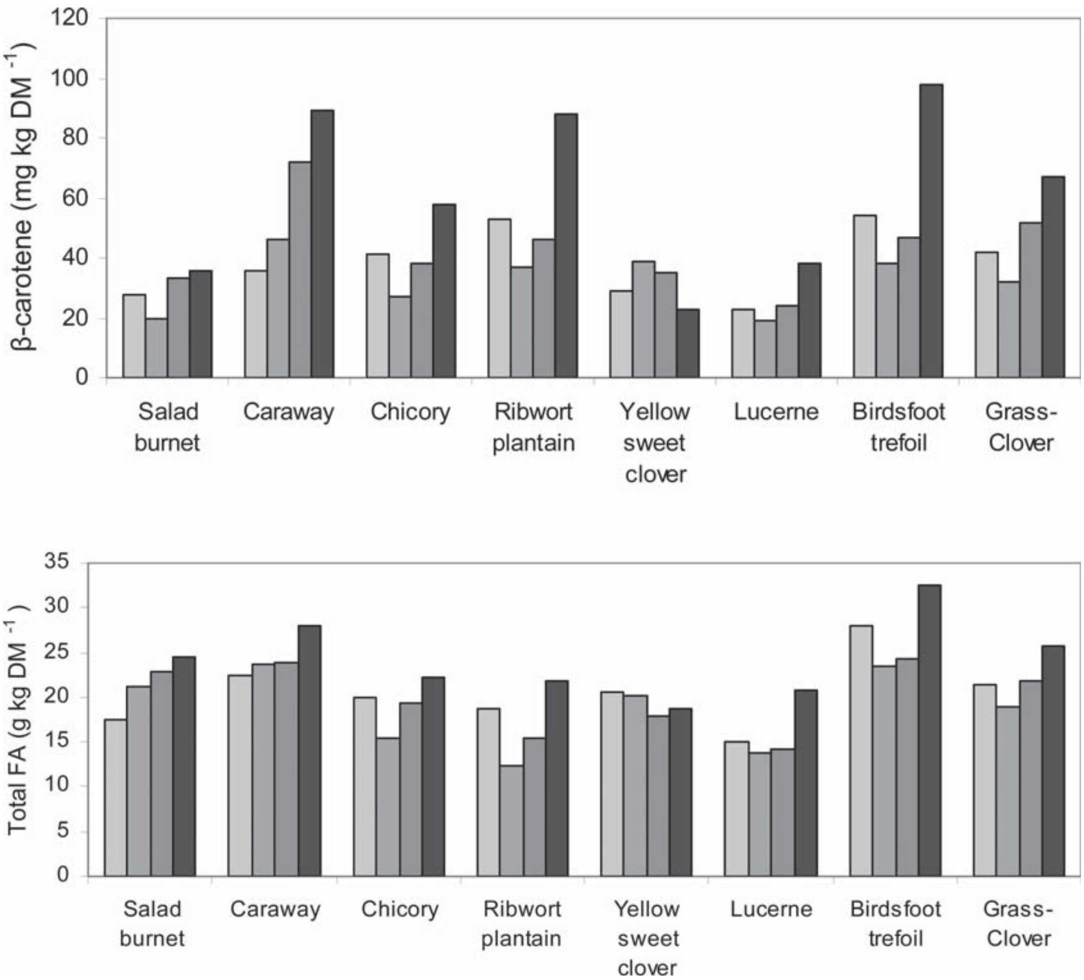

Figure 1. Concentrations of (a, top) β-carotene and (b, bottom) total fatty acids in forage of four non-leguminous forb species, three legumes, and a perennial ryegrass–white clover mixture during four cuts (May, July, August, October), averaged (n = 4) over two years (2009 and 2010).

option in the least-squares means statement. All tests of significance were made at the 0.05 level of probability.

■ RESULTS

Vitamins. The α-tocopherol concentrations were on average lowest ($P < 0.01$) in lucerne and yellow sweet clover and highest in salad burnet and ribwort plantain (ca. 22 versus 80 mg kg^{-1} DM, respectively); the latter two species outperformed the grass–clover mixture ($P < 0.01$, Table 1). The β-carotene concentrations ranged between 26 and 61 mg kg^{-1} DM with salad burnet, lucerne, and yellow sweet clover at the lower end and caraway, birdsfoot trefoil, and ribwort plantain at the top end. Vitamin concentrations differed significantly among harvests (Table 1) and were generally lowest in the second cut and highest in the fourth cut; forage yields were highest in the first and lowest in the fourth harvest.[29] Species × cut interactions that were significant (Table 1) were relatively small compared to main effects (illustrated for β-carotene, Figure 1a).

Birdsfoot trefoil and the grass–clover mixture had the highest lutein concentrations (Table 1).

Table 2. Concentrations of Total Fatty Acids (FA), Individual and Categories of FA (Grams per Kilogram DM) for Four Non-legume Forbs, Three Forage Legume Species, and a Perennial Ryegrass−White Clover Mixture, Averaged (n = 16) over Four Cuts in 2009 and 2010[a]

										signifcance of effects			
species	salad burnet	caraway	chicory	ribwort plantain	yellow sweet clover	lucerne	birdsfoot trefoil	mixture	SEM species effect	S	C	S × C	Y
total FA	22.2 bc	24.5 cd	19.2 ab	17.1 a	19.3 ab	15.9 a	27.0 d	22.0 bc	1.0	**	*	***	**
C10:0	0.1	0.1	0.0	0.1	0.1	0.1	0.1	0.1	0.03	NS	***	NS	***
C12:0	0.1 d	0.0 a	0.0 a	0.0 a	0.1 cd	0.0 b	0.1 c	0.0 b	0.01	***	***	***	NS
C14:0	0.2 a	0.2 a	0.1 a	0.2 a	0.2 a	0.2 a	0.4 b	0.2 a	0.02	*	***	***	NS
C16:0	3.5 b	4.1 cd	3.9 bc	2. a	4.5 de	4.0 cd	4.7 e	4.1 cd	0.12	**	***	NS	**
C16:1n-9	0.3 a	0.4 ab	0.3 a	0.3 a	0.3 ab	0.4 ab	0.4 bc	0.6 c	0.03	*	NS	NS	NS
C18:0	0.8 d	0.3 ab	0.3 a	0.3 a	0.6 cd	0.6 c	0.4 b	0.6 c	0.03	***	*	NS	*
C18:1n-9	1.1	1.3	0.4	0.5	0.6	0.5	0.5	0.9	0.11	NS	***	**	NS
C18:2n-6	4.8 b	6.9 c	4.3 ab	3.6 ab	3.5 ab	3.3 a	4.7 b	3.9 ab	0.27	**	**	**	**
C18:3n-3	10.4 bc	8.3 ab	8.9 b	8.4 b	8.1 ab	5.8 a	14.6 c	10.3 bc	0.65	**	***	*	**
C18:3n-6	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.00	NS	NS	NS	NS
C18:4n-3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.00	NS	NS	NS	NS
C20:0	0.2 c	0.1 a	0.1 a	0.1 ab	0.2 c	0.2 b	0.2 c	0.2 b	0.01	***	***	***	*
C22:0	0.1 a	0.2 b	0.2 b	0.2 b	0.3 c	0.2 b	0.2 b	0.2 b	0.01	**	***	NS	***
C24:0	0.2 ab	0.3 f	0.3 e	0.1 a	0.2 bc	0.3 cd	0.3 de	0.3 de	0.02	***	***	***	***
other FA	0.4 a	2.3 b	0.3 a	0.3 a	0.6 a	0.4 a	0.4 a	0.6 a	0.06	***	***	***	NS
n-3	10.4 b	8.3 b	8.9 b	8.4 b	8.1 ab	5.8 a	14.6 c	10.3 b	0.7	**	***	*	**
n-6	4.8 b	6.9 c	4.4 ab	3.6 ab	3.5 ab	3.3 a	4.8 ab	4.0 ab	0.3	**	**	**	**
n-6:n-3	0.48 bc	0.89 d	0.51 bc	0.48 bc	0.44 bc	0.59 c	0.33 a	0.41 b	0.03	**	***	**	NS
PUFA[b]	15.2 c	15.2 c	13.3 bc	12.0 ab	11.7 ab	9.1 a	19.4 d	14.3 bc	0.8	**	***	**	NS
MUFA[c]	1.3	1.7	0.7	0.7	0.9	0.8	0.9	1.5	0.1	NS	***	**	NS
SFA[d]	5.3 bc	5.3 bc	4.9 b	4.0 a	6.3 de	5.5 bcd	6.3 e	5.6 cd	0.2	**	***	NS	**
MCFA[e]	0.4 de	0.2 ab	0.2 a	0.3 abc	0.4 cde	0.3 abcd	0.5 e	0.3 bcd	0.04	**	***	*	***
ΣC16[f]	3.7 ab	4.5 cd	4.1 bc	3.2 a	4.8 de	4.3 cd	5.1 e	4.6 cde	0.13	**	***	*	**
LCFA[g]	18.1cde	19.7 de	14.9 abc	13.6 ab	14.2 abc	11.3 a	21.4 e	17.1 bcd	0.94	**	***	*	**

[a]Standard error of the main effect of species (SEM). Significance of main effects of species (S) and cut (C), their interaction, and of year (Y): *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; NS not significant. Within a row, least-squares means without a common letter are significantly different ($P < 0.05$). [b]Polyunsaturated fatty acids. [c]Monounsaturated fatty acids. [d]Saturated fatty acids. [e]Medium-chain fatty acids: C10:0 + C12:0 + C14:0. [f]C16:0 + C16:1. [g]Long-chain fatty acids: ≥C18.

The lutein concentration in chervil was 63 mg kg^{-1} DM. Concentrations of α-tocopherol and β-carotene in chervil were very low, that is, 13 and 10 mg kg $^{-1}$ DM in the first harvest of 2009, respectively. However, the γ-tocopherol concentration was 14 mg kg^{-1} DM, whereas in most species γ-tocopherol was not detected. Only the grass−clover mixture and lucerne contained γ-tocopherol, in levels ranging from 0 to 4 mg kg^{-1} DM and from 0 to 2 mg kg^{-1} DM throughout 2009, respectively. δ-Tocopherol was detected only in chicory; concentrations (15 and 67 mg kg^{-1} DM) ranged from 50 to 100% of its α-tocopherol concentrations, for which chicory had an intermediate position compared to other species (Table 1).

Concentrations of α-tocopherol in borage (in replicate 1) ranged from 48 to 73 mg kg^{-1} DM across the four harvests in 2009, whereas 25−38 mg kg^{-1} DM was found in the grass−clover mixture. Concentrations of α-tocopherol in viper's bugloss (in replicate 2) were 69 and 122 mg kg^{-1} DM in harvests 3 and 4 in 2009, whereas 41 and 50 mg kg^{-1} DM were found in the grass−clover mixture.

For β-carotene, concentrations in borage ranged from 11 to 38 mg kg^{-1} DM versus from 32 to 48 mg kg $^{-1}$ DM in grass−clover; values in viper's bugloss were 86 and 101 mg kg^{-1} DM in harvests 3 and 4 versus 56 and 83 mg kg $^{-1}$ DM in grass−clover, respectively.

In 2010, only viper's bugloss samples were analyzed. The concentrations of α-tocopherol were 70, 66, 97, and 98 mg kg^{-1} DM in the four harvests versus 40, 55, 36, and 60 mg kg^{-1} DM in grass−clover; the concentrations of β-carotene were 45, 35, 74, and 104 mg kg DM $^{-1}$ versus 36, 0, 43, and 69 mg kg DM $^{-1}$ in grass−clover, respectively.

Fatty Acids. Total and individual FA concentrations of the seven replicated dicotyledonous species and the grass−clover mixture are shown in Table 2 and their proportions in Table 3. Species differed for absolute amounts as well as proportions of FA. Total FA concentrations were lowest in lucerne, ribwort plantain, chicory, and yellow sweet clover and highest in caraway and birdsfoot trefoil (ca. 17 versus 26 g kg^{-1} DM, respectively) (Table 2). FA concentrations were generally lowest in the second cut in early July and highest in the fourth cut in late October; species × cut interactions that were significant (Tables 2 and 3) were relatively small compared to main effects (Figure 1b).

Generally, α-linolenic acid was the main FA component (Table 2). Concentrations of α-linolenic acid were lowest in lucerne (5.8 g kg^{-1} DM) and highest in birdsfoot trefoil (14.6 g kg^{-1} DM). Birdsfoot trefoil had on average the highest proportion (0.54) of α-linolenic acid, and thus of n-3 FA, and caraway and lucerne the lowest (ca. 0.35 of total FA) (Table 3). Concentrations of linoleic acid were lowest in lucerne (3.3 g kg^{-1} DM) and highest in caraway (6.9 g kg^{-1} DM). The lowest linoleic acid proportions were found in

Table 3. Proportions of Individual and Categories of Fatty Acids (FA) (Grams per 100 g FA) for Four Non-legume Forbs, Three Forage Legume Species, and a Perennial Ryegrass−White Clover Mixture, Averaged over Four Cuts in 2009 and 2010[a]

										significance of effects			
species	salad burnet	caraway	chicory	ribwort plantain	yellow sweet clover	lucerne	birdsfoot trefoil	mixture	SEM species effect	S	C	S × C	Y
C10:0	0.42	0.24	0.24	0.38	0.58	0.43	0.27	0.28	0.13	NS	*	NS	***
C12:0	0.40 d	0.05 a	0.06 ab	0.00 a	0.44 d	0.24 c	0.27 c	0.17 bc	0.03	***	***	***	*
C14:0	1.00 a	0.74 a	0.79 a	1.31 b	1.05 a	1.28 b	1.41 b	0.99 a	0.08	*	**	***	NS
C16:0	15.83 a	17.01 ab	20.60 c	17.77 ab	23.24	25.58 e	17.51 ab	18.75 bc	0.51	***	***	***	*
C16:1n-9	1.21 a	1.52 a	1.49 a	1.54 a	1.62 a	2.42 b	1.72 a	2.61 b	0.16	*	*	NS	*
C18:0	3.61 d	1.30 a	1.42 ab	1.89 b	3.29 cd	3.86 d	1.65 ab	2.81 c	0.15	***	***	NS	NS
C18:1n-9	4.56	5.27	2.13	2.88	3.19	2.85	1.7	4.33	0.46	NS	***	**	NS
C18:2n-6	21.24 c	28.03 d	22.68 c	21.62 c	17.88 ab	20.55 bc	17.47 a	17.92 ab	0.65	**	***	***	NS
C18:3n-3	46.96 bc	33.70 a	45.54 bc	47.51 c	41.52 ab	35.69 a	53.53 d	46.11 bc	1.2	***	***	**	NS
C18:3n-6	0.25 bc	0.12 a	0.32 c	0.21 b	0.23 b	0.23 b	0.17 ab	0.18 ab	0.02	*	NS	NS	*
C18:4n-3	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.00	0.01	NS	NS	NS	NS
C20:0	1.14 ef	0.35 a	0.53 b	0.81 cd	1.27 f	1.04 e	0.90 d	0.72 c	0.04	***	NS	***	NS
C22:0	0.67 a	0.90 bc	1.04 cd	1.30 de	1.42 e	1.44 e	0.79 ab	1.04 cd	0.05	***	*	*	NS
C24:0	0.92 ab	1.39 def	1.58 ef	0.79 a	1.16 bcd	1.6bf	1.03 abc	1.30 cde	0.06	**	NS	**	NS
other FA	1.36 a	9.07 d	1.31 a	1.58 ab	1.76 abc	2.33 c	1.26 a	2.14 bc	0.17	***	***	***	NS
n-3	46.98 cd	33.70 a	45.54 bc	47.51 cd	41.59 b	35.69 a	53.59 d	46.11 bc	0.66	***	***	*	NS
n-6	21.50 c	28.15 d	23.00 cd	21.83 c	18.11 ab	20.79 bc	17.64 a	18.10 ab	1.26	**	***	***	NS
PUFA[b]	68.55 d	61.85 bc	68.54 d	69.34 d	59.67 b	56.48 a	71.23 d	64.21 c	0.97	***	***	NS	**
MUFA[c]	6.19	7.09	3.9	4.83	5.87	5.71	3.7	7.58	0.56	NS	***	**	NS
SFA[d]	24.00 a	21.98 a	26.25 b	24.26 ab	32.29 c	35.49 d	23.76 a	26.06 b	0.80	***	***	***	**
MCFA[e]	1.83 bc	1.03 a	1.09 a	1.69 bc	2.03 c	1.95 c	1.95 c	1.45 ab	0.19	**	**	**	***
ΣC16[f]	17.05 a	18.53 ab	22.09 d	19.31 bc	24.88 e	28.00 f	19.22 abc	21.36 cd	0.59	**	**	**	NS
LCFA[g]	79.76 d	71.37 b	75.51 c	77.42 cd	71.27 b	67.72 a	77.63 cd	75.05 c	0.68	**	**	***	NS

[a]Standard error of the main effect of species (SEM). Significance of main effects of species (S) and cut (C), their interaction, and oyear (Y): *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; NS not significant. Within a row, least-squares means without a common superscript are significantly different ($P < 0.05$). [b]Polyunsaturated fatty acids. [c]Monounsaturated fatty acids. [d]Saturated fatty acids. [e]Medium-chain fatty acids: C10:0 + C12:0 + C14:0. [f]C16:0 + C16:1. [g]Long-chain fatty acids: ≥C18.

birdsfoot trefoil, yellow sweet clover, and grass−clover (ca. 0.18) and the highest proportions in caraway (0.28). The n-6:n-3 ratio was lower in birdsfoot trefoil (0.33) than in all other species; it was highest in caraway (0.89) and higher in lucerne (0.59) than in most other species (Table 2).

Caraway had high amounts and proportions of FA other than the three major FA α-linolenic acid, linoleic acid, and palmitic acid (C16:0); this was also found for borage, chervil, and viper's bugloss. Chervil had a very high total FA concentration (30 g FA kg^{-1} DM in spring) and a distinct FA pattern with linoleic acid (0.45) as main FA, a low proportion of α-linolenic acid (0.19) being similar to that of palmitic acid, and hence a very high n-6:n-3 ratio (2.55). Its oleic acid proportion (0.07) was about 10 times higher than in other species and was an important compound of "other FA" (0.18 of total FA). C18:4n-3 was mainly found in viper's bugloss and borage, ranging from 3.1 to 8.9 g kg^{-1} DM and from 6.2 to 7.2 g kg^{-1} DM, and from 0.02 to 0.04 of total FA and from 0.03 to 0.05 of total FA, respectively.

FA profiles of Boraginaceae were different because "other FA" occurred in very high proportions, stearidonic acid (C18:4n-3) being one of these. Borage had almost equal proportions of α-linolenic, linoleic, and palmitic acid of 0.2, whereas other FA had the largest share (0.4); the n-6:n-3 ratio was 1. Viper's bugloss had a FA composition of 0.39 α-linolenic acid, 0.19 palmitic acid, 0.17 linoleic acid, 0.8 stearidonic acid, and 0.17 other FA and a n-6:n-3 ratio of 0.44. Stearidonic acid proportions were relatively high in viper's bugloss and borage (0.02−0.04 and 0.03−0.05 of total FA, respectively), whereas it was only occasionally present in yellow sweet clover and birdsfoot trefoil at <0.01 (Table 2).

Functional Groups. Whereas in the grass−clover mixture proportions of linoleic and palmitic acid were rather similar (0.18 versus 0.19, Table 3), in the non-leguminous forbs the proportion of linoleic acid was higher than that of palmitic acid, particularly in caraway and salad burnet, whereas the opposite was found in the legumes. At the level of functional groups, forbs had lower concentrations of saturated FA and C16 FA, a higher proportion of linoleic acid, and a higher n-6:n-3 ratio than legumes but, generally, within each functional group large differences were found among individual species. For example, lucerne and yellow sweet clover were similar in having low FA concentrations, low proportions of PUFA and long-chain FA, and high proportions of C22:0, saturated FA, medium-chain FA, and C16 FA (Table 3), but birdsfoot trefoil was quite different from the other legumes. In the forbs, for example, ribwort plantain had a lower FA concentration than caraway, whereas salad burnet and ribwort plantain had higher proportions of n-3 FA, PUFA, medium-chain FA, long-chain FA, and C18:0 and lower proportions of n-6 FA, C20:0, C24:0, and other FA and a lower n-6:n-3 ratio than caraway.

Vitamin contents differed largely among legume species, as birdsfoot trefoil had a significantly higher α-tocopherol concentration than lucerne and yellow sweet clover, which was also the case for concentrations of β-carotene and lutein. The grass−clover mixture had a numerically intermediate content (Table 1), but no information is available on the grass and clover vitamin contents and yields, hampering a direct

dx.doi.org/10.1021/jf403195v | J. Agric. Food Chem. XXXX, XXX, XXX−XXX

comparison with grass. No common feature was found among the four non-leguminous forb species.

■ DISCUSSION

Vitamins and Fatty Acids. Samples were frozen within 2 h after harvest, and wilting in this period was avoided as much as possible by storage in plastic bags that were kept out of the sun, so no effect of wilting was expected. Mean concentrations of α-tocopherol and β-carotene in the grass−clover mixture (39 and 48 mg kg^{-1} DM, respectively) were comparable to those in mixtures in the study of Lindqvist et al.[13] Legumes usually contain less α-tocopherol than grasses,[35] but in Norwegian alpine grazing plants,[36] grasses had a lower content (28 ± 11 mg kg^{-1} DM) of α-tocopherol than forbs (215 ± 94 mg kg^{-1} DM). In the latter study, a different analytical method was used. Small-sized and fine-leaved grass species had very low levels of α-tocopherol (2−6 μg g^{-1} DM), whereas the contents for large and broad-leaved grasses were significantly higher (48−82 μg g^{-1} DM). As in our study species differences within each of the functional groups of forbs and legumes were large, and no contrast for forage yield[29] or vitamin contents was found between these functional groups. Chicory was the only species containing δ-tocopherol.

In line with our findings, high total FA concentration levels in forbs were also found by Warner et al.:[26] on May 14, chicory contained most ($P < 0.01$) total FA (32.6 g kg^{-1} DM) followed by yarrow (25.9 g kg^{-1} DM), parsnip (*Pastinaca sativa* L.) (25.0 g kg^{-1} DM), and ribwort plantain (20.8 g kg^{-1} DM), whereas timothy had the lowest FA content (18.3 g kg^{-1} DM); the same ranking order was found in June and August. Clapham et al.[25] also reported that chicory contained the most total FA.

There are several fatty acids in green plants, and α-linolenic acid is often the main FA, followed by linoleic acid and palmitic acid.[37] Proportions of ca. 0.5−0.6, 0.2, and 0.2 of total FA, respectively, were reported in forbs and legumes[25,26,28] and in perennial ryegrass.[4,38,39] In young leafy plants, the proportion of α-linolenic acid can be >0.6.[27,39]

In this study in all species α-linolenic acid was the main component, although the FA profile differed among species. Petersen et al.[28] found α-linolenic acid proportions of 0.38 in white clover, 0.45 in lucerne, chicory, and ribwort plantain, 0.50 in yellow sweet clover and salad burnet, and 0.58 in birdsfoot trefoil and perennial ryegrass; concentrations ranged from 3.4 g kg^{-1} DM in white clover to 10.2 g kg^{-1} DM in perennial ryegrass. Their linoleic acid concentrations ranged from 1.8 to 2.9 g kg^{-1} DM and were higher in caraway (4.0 g kg^{-1} DM); proportions ranged from 0.14 in birdsfoot trefoil and perennial ryegrass to 26 in caraway and ribwort plantain, which is in line with our findings.

In our study chervil had a high oleic acid content. Petersen et al.[28] even found oleic acid to be the main FA (0.47) in chervil, with linoleic acid as second (0.36) and α-linolenic acid only 0.03. Caraway and chervil belong to the Apiaceae, as does parsnip, where also a high proportion of linoleic acid (0.39) and a low proportion of α-linolenic acid (0.27) were found.[26]

In summary, reported (fragmented) data suggest that chervil and dandelion[27] had the highest FA concentrations, chicory sometimes had high and sometimes rather low contents compared with other forb and legume species, and grasses often had the lowest contents, although not always. The n-6:n-3 ratio was highest in caraway and lowest in birdsfoot trefoil. Forb species belonging to the Boraginacea and Apiaceae had high amounts and proportions of FA other than the three major FA. This study demonstrated higher vitamin concentrations in some forbs compared with major forages such as lucerne and grass−clover, more total FA in salad burnet, caraway, and birdsfoot trefoil than in lucerne, and higher n-3 FA concentrations in all forbs than in lucerne.

Effects on Animals and Animal-Derived Products. Van Ranst et al.[40] postulated that PUFA could be protected against biohydrogenation through encapsulation in protein−phenol complexes. Specific secondary plant metabolites, such as condensed tannins or saponins, may inhibit lipase activity. Salad burnet contains a.o. phenols; Loges[41] found 18.6% of DM in the first cut in 2010, whereas chicory and ribwort plantain contained around 6.5% phenols. The higher PUFA content in meat and milk of animals grazing species-rich relative to improved grass swards may relate to inhibited or modified fatty acid metabolism in the rumen. The changes may be caused by plant secondary compounds, which are associated with the numerous dicotyledonous species common in species-rich grassland. However, the presence of specific metabolites in forbs of botanically diverse forage, which might modify rumen fatty acid metabolism or transfer efficiency of α-linolenic acid from the duodenum to the mammary gland, still needs to be tested in vivo. Few studies have been carried out to examine the effect on milk composition of forage plants (chicory and birdsfoot trefoil, respectively) containing such metabolites;[42,43] beneficial effects of these species on animal performance due to reduction of parasites have been reported.[44,45]

Increased knowledge of the fate of the lipid-rich chloroplast in the rumen represents an opportunity to deliver more beneficial n-3 PUFA from rumen through to the small intestine and hence to milk and meat lipids.[46]

The high vitamin concentrations of some forbs as found in this study offer perspectives for naturally improved milk vitamin composition. In a pilot study comparing the transfer efficiency and content in milk of n-3 and n-6 FA and vitamins of cows fed a TMR, fresh grass−clover forage, or a mixture of forb species in Denmark, the FA increased with forbs due to an increased transfer rate from feed to milk, but apart from a higher retinol content with forbs, no significant differences were observed in the vitamin content of the various milks;[3] there were, however, increased contents of n-3 and n-6 fatty acids (FA) in milk of forb-fed cows that could be due to an increase in transfer efficiency from feed to milk for n-3 FA compared to both grass−clover and TMR diets. Further studies are underway.

■ AUTHOR INFORMATION

Corresponding Author

*(A.E.) E-mail: anjo.elgersma@hotmail.com.

Funding

The project was funded by the Danish Fund for Organic Farming. Financial support of A.E. (EU-COST FA0802 Feed for Health grants for short-term scientific missions to Foulum) is gratefully acknowledged.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank M. Würtz Reeh and E. Lyng Pedersen for performing the laboratory work and Dr. K. Kristensen for statistical advice.

■ REFERENCES

(1) Elgersma, A. New developments in The Netherlands: dairies reward grazing because of public perception. *Grassl. Sci. Eur.* **2012**, *17*, 420−422.

(2) Steinfeld, H.; Gerber, P.; Wassenaar, T.; Castel, V.; Rosales, M.; De Haan, C. Livestock's long shadow. *Environmental Issues and Options*; FAO: Rome, Italy, 2006.

(3) Petersen, M. B.; Søegaard, K.; Jensen, S. K. Herb feeding increased n-3 and n-6 fatty acids in cow milk. *Livest. Sci.* **2011**, *14*, 90−94.

(4) Dewhurst, R. J.; Shingfield, K. J.; Lee, M. R. F.; Scollan, N. D. Increasing the concentrations of beneficial polyunsaturated fatty acids in milk produced by dairy cows in high-forage systems. *Anim. Feed Sci. Technol.* **2006**, *131*, 168−206.

(5) Elgersma, A.; Tamminga, S.; Ellen, G. Modifying milk composition through forage − a review. *Anim. Feed Sci. Technol.* **2006**, *131*, 207−225.

(6) Murphy, J. J. Synthesis of milk fat and opportunities for nutritional manipulation. *Milk Composition*; Occ. Publ. 25; British Society Animal Science: Midlothian, UK, 2000; pp 201−222.

(7) Timmons, J. S.; Weiss, W. P.; Palmquist, D. L.; Harper, W. J. Relationship among roasted soybeans, milk components, and spontaneous oxidized flavour of milk. *J. Dairy Sci.* **2001**, *84*, 2440−2449.

(8) Ballet, N.; Robert, J. D.; Williams, P. E. V. *Vitamins in Forages*; CABI Publishing: Wallingford, UK, 2000.

(9) Agabriel, C.; Cornu, A.; Journal, C.; Sibra, C.; Grolier, P.; Martin, B. Tanker milk variability according to farm feeding practices: vitamins A and E, carotenoids, color, and terpenoids. *J. Dairy Sci.* **2007**, *90*, 4774−4896.

(10) Havemose, M. S.; Weisbjerg, M. R.; Bredie, W. L. P.; Nielsen, J. H. Influence of feeding different types of roughage on the oxidative stability of milk. *Int. Dairy J.* **2004**, *14*, 563−570.

(11) Calderón, F.; Chauveau-Duriot, B.; Pradel, P.; Martin, B.; Graulet, B.; Doreau, M.; Nozière, P. Variations in carotenoids, vitamins A and E, and color in cow's plasma and milk following a shift from hay diet to diets containing increasing levels of carotenoids and vitamin E. *J. Dairy Sci* **2007**, *90*, 5651−5664.

(12) Mercier, Y.; Gatellier, P.; Renerre, M. Lipid and protein oxidation in vitro, and antioxidant potential in meat from Charolais cows finished on pasture or mixed diet. *Meat Sci.* **2004**, *66*, 467−473.

(13) Lindqvist, H.; Nadeau, E.; Jensen, S. K. α-Tocopherol and β-carotene in legume-grass mixtures as influenced by wilting, ensiling and type of silage additive. *Grass Forage Sci.* **2012**, *67*, 119−128.

(14) Mogensen, L.; Kristensen, T.; Søegaard, K.; Jensen, S. K.; Sehested, J. α-Tocopherol and β-carotene in roughages and milk in organic dairy herds. *Livest. Sci.* **2012**, *145*, 44−54.

(15) Coulon, J. B.; Priolo, A. Influence of forage feeding on the composition and organoleptic properties of meat and dairy products, bases for a 'terroir' effect. *Grassl. Sci. Eur.* **2002**, *7*, 513−524.

(16) Scollan, N. D.; Dewhurst, R. J. ; Moloney, A. P.; Murphy. J. J. Improving the quality of products from grassland. In *Grassland: A Global Resource*; McGilloway, D. A., Ed.; Wageningen Academic Publishers: Wageningen, The Netherlands, 2005; pp 41−56.

(17) Scollan, N. D.; Hocquette, J.-F.; Nuernberg, K.; Dannenberger, D.; Richardson, I.; Moloney, A. Innovations in beef production systems that enhance the nutritional and health value of beef lipids and their relationship with meat quality. *Meat Sci.* **2006**, *74*, 17−33.

(18) Doreau, M.; Bauchart, D.; Chilliard, Y. Enhancing fatty acid composition of milk and meat through animal feeding. *Anim. Prod. Sci.* **2010**, *51*, 19−29.

(19) Collomb, M.; Bütikofer, U.; Sieber, R.; Jeangros, B.; Bosset, J. O. Correlation between fatty acids in cow's milk fat produced in the lowlands, mountains and highlands of Switzerland and botanical composition of the fodder. *Int. Dairy J.* **2002**, *12*, 661−666.

(20) Leiber, F.; Kreuzer, M.; Nigg, D.; Wettstein, H. R.; Scheeder, M. R. L. A study on the cause for the elevated n-3 fatty acids in cows' milk of alpine origin. *Lipids* **2005**, *40*, 191−202.

(21) Al-Mamum, M.; Yamaki, K.; Masumizu, T.; Nakai, Y.; Saito, K.; Sano, H.; Tamura, Y. Superoxide anion radical scavenging activities of herbs and pastures in northern Japan determined using electron spin resonance spectrometry. *Int. J. Biol. Sci.* **2007**, *3*, 349−355.

(22) Xu, B. J.; Chang, S. K. C. A comparative study on phenolic profiles and antioxidant activities of legumes as affected by extraction solvents. *J. Food Sci.* **2007**, *72*, S159−S166.

(23) Dewhurst, R. J.; Scollan, N. D.; Youell, S. J.; Tweed, J. K. S.; Humphreys, M. O. Influence of species, cutting date and cutting interval on the fatty acid composition of grass. *Grass Forage Sci.* **2001**, *56*, 68−74.

(24) Elgersma, A.; Ellen, G.; van der Horst, H.; Muuse, B. G.; Boer, H.; Tamminga, S. Influence of cultivar and cutting date on the fatty acid composition of perennial ryegrass (*Lolium perenne* L.). *Grass Forage Sci.* **2003**, *58*, 323−331.

(25) Clapham, W. M.; Foster, J. G.; Neel, J. P. S.; Fedders, J. M. Fatty acid composition of traditional and novel forage species. *J. Agric. Food Chem.* **2005**, *53*, 10068−10073.

(26) Warner, D.; Jensen, S. K.; Cone, J. W.; Elgersma, A. Fatty acid composition of forage herb species. *Grassl. Sci. Eur.* **2010**, *15*, 491−493.

(27) Wyss, U.; Collomb, M. Fatty acid composition of different grassland species. *Grassl. Sci. Eur.* **2010**, *15*, 631−633.

(28) Petersen, M. B.; Søegaard, K.; Jensen, S. K. Fatty acid content and lipid fractions in herbs. *Grassl. Sci. Eur.* **2012**, *17*, 314−316.

(29) Elgersma A.; Søegaard, K.; Jensen, S. K. Herbage dry matter production and forage quality of three legumes and four non-leguminous forbs in single-species stands. *Grass Forage Sci.* **2014**, *69*, DOI: 10.1111/gfs.12104.

(30) Jensen, S. K.; Jensen, C.; Jakobsen, K.; Engberg, R. M.; Andersen, J. O.; Lauridsen, C.; Sørensen, P.; Henckel, P.; Skibsted, L. H.; Bertelsen, G. Supplementation of broiler diets with retinol acetate, β-carotene or canthaxanthin: effect on vitamin and oxidative status of broilers *in vivo* and meat stability. *Acta Agric. Scand. Sect. A: Anim. Sci.* **1998**, *48*, 28−37.

(31) Bligh, E. G.; Dyer, W. J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Phyisol.* **1959**, *37*, 911−917.

(32) Jensen, S. K. Improved Bligh and Dyer extraction procedure. *Lipid Technol.* **2008**, *20*, 280−281.

(33) Littell, R. C.; Milliken, G. A.; Stroup, W. W.; Wolfinger, R. D. *SAS Systems for Mixed Models*; SAS Institute: Cary, NC, USA, 2006

(34) Cochran, W. G.; Cox, G. M. *Experimental Designs*; Wiley: New York, 1957.

(35) Danielsson, H.; Nadeau, D.; Gustavsson, A.-M.; Jensen, S. K.; Søegaard, K.; Nilsdotter-Linde, N. Contents of α-tocopherol and β-carotene in grasses and legumes harvested at different maturities. *Grassl. Sci. Eur.* **2008**, *13*, 432−434.

(36) Sickel, H.; Bilger, W.; Ohlson, M. High levels of αptocopherol in Norwegian alpine grazing plants. *J. Agric. Food Chem.* **2012**, *60*, 7573−7580.

(37) Bauchart, D.; Vérité, R.; Rémond, B. Long-chain fatty acid digestion in lactating cows fed fresh grass from spring to autumn. *Can. J. Anim. Sci.* **1984**, *64* (Suppl.), 330−331.

(38) Elgersma, A.; Maudet, P.; Witkowska, I. M.; Wever, A. C. Effects of N fertilization and regrowth period on fatty acid concentrations in perennial ryegrass (*Lolium perenne* L.). *Ann. Appl. Biol.* **2005**, *147*, 145−152.

(39) Witkowska, I. M.; Wever, A. C.; Gort, G.; Elgersma, A. Effects of nitrogen rate and regrowth interval on perennial ryegrass fatty acid content during the growing season. *Agron. J.* **2008**, *100*, 1371−1379.

(40) Van Ranst, G.; Fievez, V.; Vandewalle, M.; De Riek, J.; Van Bockstaele, E. Influence of herbage species, cultivar and cutting date on fatty acid composition of herbage and lipid metabolism during ensiling. *Grass Forage Sci.* **2009**, *64*, 169−207.

(41) Loges, R. Urter og tanninrige planter − den nyeste tyske forskning. *Plantekongres 2012 − Produktion, Plan og miljø*, Herning, Denmark; Videncentret for Lanbrug: Aarhus, Denmark, 2012; pp 363−365

(42) Kälber, T.; Meier, J. S.; Kreuzer, M.; Leiber, F. Flowering catch crops used as forage plants for dairy cows: influence on fatty acids and tocopherols in milk. *J. Dairy Sci.* **2011**, *94*, 1477−1489.

(43) Höjer, A.; Adler, S.; Martinsson, K.; Jensen, S. K.; Steinshamn, H.; Thuen, E.; Gustavsson, A.-M. Effect of legume−grass silages and α-tocopherol supplementation on fatty acid composition and α-tocopherol, β-carotene and retinol concentrations in organically produced bovine milk. *Livest. Sci.* **2012**, *148*, 268−281.

(44) Marley, C. L.; Cook, R.; Keatinge, R.; Barrett, J.; Lampkin, N. H. The effect of birdsfoot trefoil (*Lotus corniculatus*) and chicory (*Cichorium intybus*) on parasite intensities and performance of lambs naturally infected with helminth parasites. *Vet. Parasitol.* **2003**, *112*, 147−155.

(45) Moss, R. A.; Vlassoff, A. Effect of herbage species on gastro-intestinal roundworm populations and their distribution. *N.Z. J. Agric. Res.* **1993**, *36*, 371−375.

(46) Morgan, S.; Huws, S. A.; Scollan, N. D. Progress in forage-based strategies to improve the fatty acid composition of beef. *Grassl. Sci. Eur.* **2012**, *17*, 295−307.