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

F. Calderón,* B. Chauveau-Duriot,* P. Pradel,† B. Martin,* B. Graulet,* M. Doreau,* and P. Nozière^{1*}

*INRA, UR1213 Unité de Recherche sur les Herbivores, F-63122 St Genès Champanelle, France

†INRA, UE0373 Domaine de la Borie, Marcenat, F-15190 Marcenat, France

ABSTRACT

This experiment was conducted to determine the variations in carotenoid, vitamins A and E concentrations, and color in the plasma and milk of dairy cows following a shift from a hay diet to diets containing increasing levels of carotenoids and vitamin E. This study was performed on 32 multiparous Montbéliarde dairy cows in midlactation. After a 6-wk preexperimental period on a diet based on hay and concentrates, the cows were allocated to 4 homogeneous groups, and thereafter fed for 6 wk on isoenergetic experimental diets where the hay was replaced by an experimental feed rich in carotenoids and vitamin E, consisting in 75% grass silage and 25% alfalfa protein concentrate (PX Agro Super Desialis, Châlons en Champagne, France). The hay-to-experimental feed ratios were 100/0 in group 1, 67/33 in group 2, 33/67 in group 3, and 0/100 in group 4, providing 1.6, 3.6, 5.4, and 7.4 g/d of total carotenoids, respectively. Variations in carotenoid, vitamins A and E concentrations as well as variations in color index (CI) were monitored from d –7 through to d 42 on the experimental diets. Zeaxanthin, lutein, 13-*cis*- β -carotene, and all-*trans*- β -carotene accounted for an average 3, 10, 9, and 78%, respectively, of total carotenoids in plasma and 0, 17, 12, and 71%, respectively, of total carotenoids in milk. The switch from preexperimental to experimental diets only slightly affected zeaxanthin, lutein, and vitamin A concentrations in plasma and milk. A rapid increase in vitamin E and β -carotene (BC) was observed during the first week in both plasma and milk. For vitamin E, the time to reach a plateau was from 8 d (group 2) to 28 d (group 4) in plasma, and 5 d (groups 2–4) in milk. Plasma concentrations of BC had stabilized after 28 d in group 2 but were not stabilized after 42 d in groups

3 and 4, whereas milk concentrations of BC plateaued from d 21 in group 2 and d 28 in groups 3 and 4. At the end of the experimental period, BC and vitamin E concentrations in plasma and vitamin E concentrations in milk fat were linearly related to the proportion of experimental feed in the diet. In contrast, BC concentrations in milk fat did not differ between groups 2, 3, and 4, reflecting saturation at high levels of carotenoid intake (i.e., when plasma BC exceeded 5 μ g/mL). These results suggested that under high-carotenoid diets, milk secretion of BC is not limited by the amount of plasma BC arriving to the mammary gland but by mechanisms involved in the BC transfer from plasma to milk. These mechanisms will need to be investigated. The BC concentrations were responsible for more than 80% of CI variations in plasma and 56% of CI variations in milk, where there was wide variability among individuals. Plasma CI appeared to be a more promising tool than milk CI as an indicator of the carotene content of the diets ingested by dairy cows.

Key words: carotenoid, cow, dairy product, plasma

INTRODUCTION

The nutrient composition of dairy products is strongly influenced by the nature of the diet ingested by the cows, which is becoming an increasing important criterion in consumer perception of product quality. In certain countries in particular, dairy products derived from animals fed grass-based diets are perceived positively as having a “clean and green image”, and have been shown to be related to specific compounds of nutritional or sensorial interest (Martin et al., 2004), that is, certain fatty acids and fat-soluble micronutrients. Thus, there has been increasing interest in recent years in using feed management to control the composition of the fat-soluble fraction in milk and dairy products. Among these fat-soluble micronutrients, carotenoids and vitamin E in their role as antioxidants or carotenoids in their role as vitamin A precursors directly influence the nutritional quality of the end products. Carotenoids are also able to influence the sensorial

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¹Corresponding author: noziere@clermont.inra.fr

characteristics of products, not only directly by conferring a yellow color but also indirectly via their antioxidant properties (Barrefors et al., 1995). Furthermore, the assessment of the yellow color in milk has been considered as a potential biomarker indicating feed management quality in dairy cows, and a simple method has been proposed based on the quantification of carotenoids in end products (or animal fluids) using a color index (CI; Prache et al., 2002).

The respective influence of dietary and nondietary factors on β -carotene (BC) concentrations in the plasma and milk of dairy cows have been determined (see Nozière et al., 2006b, for review). Type of forage is the main factor of variation in BC in both plasma and milk. The BC concentrations in plasma and milk are highly dependent on the BC concentration in the diet, but very high dietary levels of carotenoids have not yet been studied in dairy cows. Furthermore, the majority of related results have been obtained after animals have been given several weeks of adaptation to the diet. Moreover, unlike in humans (Reboul et al., 2007) or cattle (Knight et al., 1994, 1996; Mora et al., 2001), there have been few studies on the kinetics of carotenoid concentrations (but also of other fat-soluble micronutrients and CI) following a change in diet in dairy cows, despite the fact that this information is essential for the development of traceability tools. Recently, Nozière et al. (2006a) demonstrated that the switch from a high-carotenoid (grass silage) to a low-carotenoid (hay) diet led to a rapid decrease in BC and vitamin E concentrations as well as CI in plasma and milk during the first 2 wk following the diet shift; however, to our knowledge, the switch from a low- to a high-carotenoid diet and the subsequent variations in plasma and milk concentrations of fat-soluble micronutrients have not yet been studied. The aim of the present study was therefore to determine the kinetics of carotenoids, vitamin A and vitamin E concentrations, and CI in the plasma and milk of dairy cows shifted from a low-carotenoid diet to diets containing increasing levels of carotenoids.

MATERIALS AND METHODS

This research was conducted on a French National Agronomic Research Institute (INRA, Marcenat) experimental farm located in the French Massif Central (1,100 m above sea level). The study was carried out in accordance with French Ministry of Agriculture guidelines on the use of experimental animals.

Animals and Diets

During a preexperimental period (6 wk, from February 7 to March 20), 32 multiparous Montbéliarde breed

dairy cows in midlactation were fed a low-carotenoid diet based on hay and concentrates. They were then allocated to 4 homogeneous groups according to parity (2.4 ± 1.9), DIM (94 ± 10 d), BW (625 ± 58 kg), body condition score (1.6 ± 0.4), milk yield (19.2 ± 3.8 kg/d), and milk fat content (36.8 ± 4.4 g/kg), and to the CI of plasma (31.9 ± 6.8) and milk (453 ± 70) as determined after at least 10 d on a high-carotenoid diet based on grass silage distributed before the beginning of the pre-experimental period.

The experimental period began on March 21 (d 1). Throughout the 6-wk experimental period, the cows were fed isoenergetic experimental diets differing in carotenoid and vitamin E contents. These diets were based on hay and on an experimental feed consisting in 75% (wt/wt) grass silage and 25% (wt/wt) alfalfa protein concentrate (APC), on a DM basis. The hay/experimental feed ratios were 100/0 in group 1, 67/33 in group 2, 33/67 in group 3, and 0/100 in group 4. Within each group, the daily amount of forage (hay, experimental feed, or both) was kept limited, similar for all animals, and constant over the experimental period to provide a constant carotenoid contents. The dietary carotenoid contents for each group (1–4, respectively) were 1.6, 3.6, 5.4, and 7.4 g/d (i.e., 0.15, 0.65, 1.13, and 1.63 g of β -carotene/d, respectively). The diets were individually supplemented with concentrates according to the cow's NE_L and nitrogen requirements for lactation, according to INRA guidelines (1989). In addition, each animal also received 200 g/d of a mineral-vitamin mix (6% P, 218 Ca, 5% Mg, 5% Na, 1,300 mg/kg of Cu, 6,000 mg/kg of Zn, 3,500 mg/kg of Mn, 80 mg/kg of I, 32 mg/kg of Co, 20 mg/kg of Se, 600,000 IU/kg of vitamin A, 120,000 IU/kg of vitamin D₃, and 1,300 IU/kg of vitamin E) to meet individual requirements. The diet was distributed via 2 equal meals per day, after milking.

The same hay was used in both the preexperimental and experimental periods. It was prepared from orchardgrass (*Dactylis glomerata*) grazed until June 1 and cut on July 26 then field-dried and harvested on July 28, under sunny and windy conditions. Grass silage was prepared from perennial ryegrass (*Lolium perenne*) on June 16 and directly ensiled with formic acid as preservative. Only one silo was used throughout the experiment. Feed composition, nutritive value, and carotenoids concentrations are given in Table 1.

Sampling and Measurements

Intake was recorded individually on 4 d/wk. The DM content (103°C for 24 h) was determined 4 times a week for silage and hay and once a week for soybean meal and concentrates. A representative sample of each feed

Table 1. Composition, nutritive values, and average intake of feeds

Item ¹	Hay	Grass silage	APC ²	Concentrate ³
DM (g/kg)	859	217	918	896
OM (g/kg of DM)	927	870	877	950
CP (g/kg of DM)	89	125	547	150
Crude fiber (g/kg of DM)	373	363	28	73
OM digestibility (%)	50	55	90	88
Net energy (MJ/kg of DM)	4.27	4.91	8.96	7.76
PDIN (g/kg of DM)	59	77	462	110
PDIE (g/kg of DM)	68	81	437	110
Carotenoids (µg/g of DM)	165	441	1,119	0
Lutein	106	223	697	0
Zeaxanthin	13	45	18	0
Violaxanthin	13	33	69	0
Antheraxanthin	9	17	46	0
Unknown xanthophyll	8	26	42	0
all- <i>trans</i> -β-carotene	15	98	247	0
13- <i>cis</i> -β-carotene	0	0	0	0
Vitamin A (µg/g of DM)	0	0	0	1.9
Vitamin E (µg/g of DM)	ND	ND	500	10
Intake (kg of DM/d)				
Group 1	9.79	0	0	7.39
Group 2	6.86	3.26	0.89	6.52
Group 3	3.43	6.45	1.79	4.76
Group 4	0	9.78	2.71	2.99

¹Dry matter was determined by drying at 103°C for 24 h. Organic matter was determined by drying at 550°C for 6 h. Crude protein was determined by the Kjeldahl method (AOAC, 1990). Crude fiber was determined by the Wende method (AOAC, 1990). The OM digestibility was measured on sheep for hay and grass silage, and calculated according to Sauvant et al. (2004) for APC and concentrate. Net energy and nitrogen values were calculated according to INRA guidelines (1989). PDIN = proteins digestible in the intestine, when degraded N is a limiting factor. PDIE = proteins digestible in the intestine, when energy is a limiting factor. Carotenoids were determined according to Cardinault et al. (2006). Unknown xanthophyll quantified according to an epilutein standard which presents the same retention time and molecular mass. Vitamin E was not determined (ND) in hay and grass silage; the value in APC was provided by Desialis (Châlons en Champagne, France); the value in concentrate was provided by UCL Centraliment (Aurillac, France).

²APC = alfalfa protein concentrate (PX Agro Super, Desialis, Châlons en Champagne, France).

³Concentrate contained 6,400 IU/kg of vitamin A, 1,250 IU/kg of vitamin D₃, and 10 mg/kg of vitamin E.

was kept under vacuum in dark conditions at -15°C for silage or at +4°C for hay and concentrates, until carotenoid determination. Vitamin E was not determined in feeds.

Individual plasma samples (from caudal blood withdrawn before the morning meal using L-heparin as anticoagulant) were collected 7 d before (d -7) and at 5, 8, 14, 21, 28, and 42 d after (d 5 to 42) the beginning of the experimental period. The cows were milked twice daily at 0600 and 1600 h, and individual milk yields were recorded. Individual milk samples were collected 2 d per week on morning and evening milkings to determine milk fat and protein contents (AOAC, 1990) by the infrared method (Milkoscan4000, Foss System, Hillerød, Denmark). Additionally, individual milk samples

(morning) were collected 7 d before (d -7) and at 5, 8, 14, 21, 28, and 42 d after (d 5 to 42) the beginning of the experimental period.

Plasma and milk samples were either used immediately after sampling for CI determination or were stored at -15°C away from light until carotenoids and vitamin (A and E) were determined. Briefly, the spectrum between 450 and 530 nm was translated to make the absorbance (plasma) or reflectance (milk) value at 530 nm equal to zero, and the CI corresponded to the absolute value of the integral of the translated spectrum between 450 and 530 nm. The CI was determined by absorbance for plasma using a Hitachi U-2000 spectrophotometer (Hitachi Ltd., Tokyo, Japan), and by reflectance for morning milk using a Minolta CM 2002 spectrophotometer (Minolta France S.A., Carrières-sur-Seine, France), as previously described (Prache et al., 2002; Nozière et al., 2006a). Carotenoids (epilutein, lutein, zeaxanthin, violaxanthin, antheraxanthin, all-*trans*-β-carotene, and 13-*cis*-β-carotene), vitamin A (retinol), and vitamin E (α-tocopherol) were determined by HPLC using the technique described by Lyan et al. (2001) for plasma, and the same technique but modified according to Nozière et al. (2006a) for milk and according to Cardinault et al. (2006) for feedstuffs. All the extractions were performed under yellow light at room temperature, using echinenone as internal standard. The HPLC apparatus consisted of a Waters Alliance 2996 HPLC system with photodiode array detector monitoring between 280 and 600 nm. Carotenoids, vitamin A, and vitamin E were simultaneously separated on a 150 × 4.6 mm, RP C18, 3-µm Nucleosil column coupled with a 250 × 4.6 mm RP C18, 5-µm Vydac TPS4 column (Interchim, Montluçon, France), as previously described (Calderón et al., 2006). Millennium 32 software (version 3.05.01) published by Waters SA (Saint Quentin-en-Yvelines, France) was used for instrument control, data acquisition, and data processing. The detection wavelengths for carotenoids, vitamin A, and vitamin E were 450, 325, and 292 nm, respectively, and the compounds were identified by comparing retention times and spectral analyses with those of pure standards (>95%). Compound concentrations were calculated using an external standard curve and were then adjusted by percentage recovery of the added internal standard.

Statistical Analysis

Milk concentrations of fat-soluble micronutrients were related to the milk fat content determined in the corresponding sample and expressed as micrograms per gram of fat. Data were analyzed statistically as repeated measurements using the MIXED procedure of

Table 2. Intake, milk production and composition, and energy and protein balance in cows fed diets differing in levels of carotenoids for 6 wk¹

	Group ²				SE	Effect ³		
	1	2	3	4		G	T	G × T
Intake								
Net energy, MJ/d	100	103	99	96	3	0.392	<0.001	<0.001
Protein, g of PDI ⁴ /d	1,403	1,778	2,048	2,308	24	<0.001	<0.001	<0.001
Total carotenoids, mg/d	1,619	3,574	5,412	7,352	40	<0.001	<0.001	<0.001
β-carotene, mg/d	150	646	1,129	1,633	17	<0.001	<0.001	<0.001
Production								
Milk yield, kg/d	18.5	19.2	19.0	19.3	0.8	0.853	<0.001	<0.001
Milk yield 4%, kg/d	17.0	17.7	17.2	17.5	0.6	0.836	<0.001	0.009
Milk fat, g/kg	35.6	33.9	34.2	33.7	0.7	0.152	<0.001	<0.001
Fat yield, g/d	641	669	642	654	25	0.816	<0.001	0.083
Milk protein, g/kg	32.3	30.9	30.3	30.0	0.4	<0.001	<0.001	0.083
Protein yield, g/d	592	590	576	579	23	0.935	<0.001	<0.001
Balance								
Net energy, MJ/d	2.11	2.66	-1.71	-3.98	1.33	<0.001	<0.001	<0.001
Protein, g of PDI ³ /d	214	530	781	1,052	22	<0.001	<0.001	<0.001

¹Values are means for 8 cows per group over 6 wk.

²Groups were fed various proportions of hay:experimental feed = 100:0 (group 1), 67:33 (group 2), 33:67 (group 3), or 0:100 (group 4); experimental feed consisted in grass silage and alfalfa protein concentrate (75:25).

³Effects: G = group, T = time.

⁴PDI = protein digestible in the intestine.

the SAS software package (2000), with group (1 to 4), time (d 5 to 42), and their interactions as fixed effects, and animal as random effect. For each cow and each variable, the data obtained at the end of the preexperimental period (d -7 or wk -1) was included in the model as a covariate. A *P*-value < 0.05 was considered significant, and a *P*-value < 0.10 was considered a trend. Means separation was performed using the PDIF option of the LSMEANS procedure. The experimental unit used for intake, milk yield, and milk fat and protein contents was mean per animal per week. The experimental unit used for micronutrient concentrations and percentages, CI in plasma and milk, and milk micronutrient yield was the data per animal per day.

The relationships between BC or total carotenoid concentrations (X) and CI (Y) in both plasma and milk were assessed by linear regression: $Y = \alpha + \beta X + e$ (where *e* = error). Within-animal relationships were assessed by variance-covariance models with animal as fixed effect: $Y = \alpha + \beta X + \text{animal} + (\text{animal} \times X) + e$ (where *e* = error). For both global and within-animal relationships, fat-soluble micronutrients in milk were expressed in micrograms per milliliter.

RESULTS

Intake, Milk Yield and Composition, and Energy and Protein Balance

The average values for intake, milk production and composition, and energy and protein balance are given in Table 2. As expected, there were no between-group

differences in intake of NE_L, which averaged 100 MJ/d throughout the experiment, whereas digestible protein and carotenoids intakes increased with the proportion of experimental feed in the diet (groups 4 > 3 > 2 > 1). Transitory refusals of experimental feed (grass silage + APC) were observed during wk 1 (0.46, 1.14, and 2.97 kg of DM for groups 2, 3, and 4, respectively), inducing group × time interactions for the intakes of NE_L, digestible protein, and carotenoids. No significant changes in intake of NE_L, digestible protein, or carotenoids were observed between wk 2 and 6 (Figure 1).

On average, throughout the experiment as a whole, there were no between-group differences in milk yield, milk fat content, and protein yield, which averaged 19.0 kg/d, 34.4 g/kg, and 651 g/d, respectively (Table 2). However, after a transitory increase between wk 1 and 2 related to the concomitant increase in NE_L intake (Figure 1), milk yield decreased in group 1 (from 19.7 to 17.7 kg/d between wk 2 and 6, respectively), whereas it remained stable in the other 3 groups (averaging 19.4 kg/d). In parallel, milk fat content remained stable throughout the experiment in group 1, whereas it decreased in the other groups (+0.3, -1.3, -2.3, and -4.1 g/kg from wk 1 to 6 for groups 1-4, respectively). Milk protein content was higher in group 1 (32.2 g/kg) than in the other 3 groups (30.4 g/kg on average), but there were no between-group differences in protein yield, which averaged 584 g/d. Excluding a transitory and moderate increase in wk 4, milk protein content remained stable throughout the experiment. From wk 2 to the end of the experimental period, the daily secretion of

Figure 1. Evolution in net energy and carotenoid intakes, milk yield and milk fat, in dairy cows fed diets differing in hay:experimental feed ratios: 100:0 (group 1 ○), 67:33 (group 2 △), 33:67 (group 3 ▲), or 0:100 (group 4 ●). Experimental feed consisted in grass silage and lucerne protein concentrate (75:25). All groups were fed hay during a preexperimental period of 6 wk. Means $\pm$ SEM for 8 cows per group are presented.

fat and protein in milk decreased, this decrease being more marked for group 1 (–82 g of fat and –50 g of protein) than for the other 3 groups (–42 g of fat and –10 g of protein, on average).

On average, throughout the experiment as a whole, NE_L balance was positive for groups 1 and 2 and negative for groups 3 and 4. The between-group differences, which reached 6.6 MJ/d between groups 2 and 4, were mostly due to differences in NE_L intake (reaching 7 MJ/d) rather than differences in milk yield. Protein balance increased between groups 1 and 4, consistently with the between-group differences in PDI intake (mainly from APC). The NE_L and protein balances increased between wk 2 and 6 by an average +4.9 MJ/d and +87 g of PDI/d for the 4 groups.

Plasma Carotenoid, Vitamin A and Vitamin E Concentrations, and Color Index

Average plasma carotenoid, vitamin A and vitamin E concentrations, and CI are shown in Table 3. Figure 2 shows variations with time during the experiments. Among the 5 xanthophylls identified in the diet, only 2 (lutein and zeaxanthin) were found in plasma, accounting on average for 9.9% (se = 3.1) and 2.7% (se = 1.5) of total carotenoids, respectively (Table 3). Only

the all-*trans* isomer of BC was identified in the diets, whereas 2 isomers (all-*trans* BC and 13-*cis* BC) were found in plasma, accounting on average for 78.9% (se = 4.1) and 8.6% (se = 1.4) of total carotenoids, respectively. Vitamin A concentrations were similar in the 4 groups, averaging 0.44 μ g/mL. In contrast, plasma concentrations of carotenoids (mainly BC) and vitamin E as well as color index all significantly increased with the ratio of experimental feed in the diet.

Plasma lutein, zeaxanthin, and vitamin A concentrations did not significantly vary over time in the experiment. Over the experimental period (Figure 2), plasma concentrations of all-*trans*-BC, 13-*cis*-BC and vitamin E, as well as CI, remained constant in group 1 and increased in the other groups. There were significant between-group differences (1 vs. 4) from d 5 for all-*trans*-BC and CI, and from d 8 for 13-*cis*-BC and vitamin E. Assuming that covariable measurements (d –7) reflected concentrations at d 1 (beginning of the experimental period), plasma concentrations of BC and vitamin E increased linearly over the first week of the experiment, with an average rate of 0.09, 0.12, and 0.20 μ g of BC/mL/d and 0.12, 0.15, and 0.25 μ g of vitamin E mL/d for groups 2, 3, and 4, respectively. All-*trans*-BC and 13-*cis*-BC concentrations plateaued from d 28 in group 2 but were not stabilized at d 42 in groups 3 and

Table 3. Plasma concentration of carotenoids, vitamins A and E, and color index in cows fed diets differing in levels of carotenoids for 6 wk¹

Item	Group ²				SE	Effect ³		
	1	2	3	4		G	T	G × T
Zeaxanthin, µg/mL	0.11	0.12	0.14	0.16	0.02	0.031	0.571	0.305
Lutein, µg/mL	0.47	0.49	0.48	0.55	0.03	0.024	0.484	0.064
All- <i>trans</i> -β-carotene, µg/mL	2.58	4.28	5.00	5.73	0.33	<0.001	<0.001	<0.001
13- <i>cis</i> -β-carotene, µg/mL	0.31	0.47	0.54	0.59	0.04	<0.001	<0.001	<0.001
Total xanthophylls, µg/mL	0.58	0.61	0.64	0.70	0.06	0.253	0.386	0.160
Total-β-carotene, µg/mL	2.90	4.75	5.55	6.32	0.36	<0.001	<0.001	<0.001
Total carotenoids, µg/mL	3.49	5.38	6.17	6.99	0.38	<0.001	<0.001	<0.001
Vitamin A, µg/mL	0.42	0.44	0.45	0.44	0.02	0.469	0.055	0.124
Vitamin E, µg/mL	3.33	4.13	4.55	5.14	0.37	<0.001	<0.001	0.056
Color index	32.0	41.5	46.0	50.7	3.2	<0.001	<0.001	<0.001

¹Values are means for 8 cows per group over 6 wk.²Groups were fed various proportions of hay:experimental feed = 100:0 (group 1), 67:33 (group 2), 33:67 (group 3), or 0:100 (group 4); experimental feed consisted in grass silage and alfalfa protein concentrate (75:25).³Effects: G = group, T = time. **Figure 2.** Evolution in lutein, all-*trans*-β-carotene, 13-*cis*-β-carotene, vitamin A, vitamin E, and color index in plasma of dairy cows fed diets differing in hay:experimental feed ratios: 100:0 (group 1 ○), 67:33 (group 2 △), 33:67 (group 3 ▲), or 0:100 (group 4 ●). Experimental feed consisted in grass silage and lucerne protein concentrate (75:25). All groups were fed hay during a preexperimental period of 6 wk. Means ± SEM for 8 cows per group are presented.

4. Vitamin E concentrations were stabilized from d 8 in groups 2 and 3 and from d 28 in group 4. The CI was stabilized from d 17 in group 2, from d 21 in group 3, and from d 28 in group 4.

At the end of the experimental period, plasma concentrations in groups 1, 2, 3, and 4 reached 0.44, 0.48, 0.49, and 0.60 $\mu\text{g/mL}$ for lutein; 0.10, 0.14, 0.15, and 0.18 $\mu\text{g/mL}$ for zeaxanthin; 2.70, 5.29, 6.47, and 7.79 $\mu\text{g/mL}$ for all-*trans*-BC; 0.32, 0.65, 0.80, and 0.94 $\mu\text{g/mL}$ for 13-*cis*-BC; 3.17, 4.47, 4.80, and 6.07 $\mu\text{g/mL}$ for vitamin E; and 29.9, 50.9, 58.3, and 66.8 for IC, respectively. There were linear relationships between the amount of experimental feed in the diet and the plasma concentrations of lutein ($P < 0.01$, $\text{Syx} = 0.13$, $R^2 = 0.20$), zeaxanthin ($P < 0.01$, $\text{Syx} = 0.05$, $R^2 = 0.25$), all-*trans*-BC ($P < 0.001$, $\text{Syx} = 1.15$, $R^2 = 0.72$), 13-*cis*-BC ($P < 0.001$, $\text{Syx} = 0.18$, $R^2 = 0.60$) and vitamin E ($P < 0.001$, $\text{Syx} = 1.06$, $R^2 = 0.47$). The relationship was quadratic for IC ($P < 0.001$, $\text{Syx} = 8.0$, $R^2 = 0.74$) and nonsignificant for vitamin A.

Milk Carotenoid, Vitamin A and Vitamin E Concentrations, and Color Index

Average CI, concentrations (expressed in $\mu\text{g/mL}$ and $\mu\text{g/g}$ of fat), and yield (in mg/d) values for carotenoids, vitamin A, and vitamin E in milk are reported in Table 4. Figure 3 illustrates the variations with time in experiment. Lutein, 13-*cis*-BC, and all-*trans*-BC were identified in milk, accounting on average for 16.9% (SE = 5.3), 11.8% (SE = 5.6), and 71.3% (SE = 7.5), respectively, of total carotenoid content (Table 4). Although present in plasma, zeaxanthin was not present in milk. There were no between-treatment differences in lutein concentrations, yield, and CI, which averaged 0.73 $\mu\text{g/g}$ of fat, 0.45 mg/d , and 433 units, respectively. There was a significant increase in milk 13-*cis*-BC and vitamin E concentrations and yield between groups 1 and 4, with intermediate figures recorded for the other groups. Milk all-*trans*-BC and vitamin A concentrations and yield were lower in group 1 but remained similar in the other 3 groups.

The concentration and yield of 13-*cis*-BC in milk did not vary significantly according to time during the experiment, but there were significant between-group differences after d 5. Milk concentrations of lutein, vitamin A, and vitamin E did not vary significantly between d 5 and 42, but were transitorily lower at d 21. The daily yield of lutein slightly decreased between d 5 and 42, from 0.49 to 0.42 mg/d on average for the 4 groups. A similar trend was observed for vitamin A (from 3.83 to 3.46 mg/d , $P = 0.07$), but not for vitamin E (from 6.45 to 5.91 mg/d , $P = 0.28$).

Milk concentrations and yield of all-*trans*-BC remained constant over the experimental period in group 1, whereas they increased in the other groups. There were significant differences between group 1 and the other 3 groups from d 5 (group 4) or d 8 (groups 2 and 3). A plateau was reached from d 21 (group 2) or d 28 (groups 3 and 4). Except at d 5, milk concentrations and yield of all-*trans*-BC did not vary significantly between groups 2, 3, and 4. There was no significant difference in CI between d 1 and 42, but there was a drastic decrease between d 1 and 5 (from 462 to 350 units, on average for the 4 groups), followed by an increase that plateaued as from d 10.

At the end of the experimental period (d 42), milk concentrations in groups 1, 2, 3, and 4 reached 0.70, 0.69, 0.77, and 0.73 $\mu\text{g/g}$ of fat for lutein; 0.53, 0.67, 0.73, and 0.67 $\mu\text{g/g}$ of fat for 13-*cis*-BC; 2.14, 3.93, 4.13, and 4.15 $\mu\text{g/g}$ of fat for all-*trans*-BC; 5.03, 6.72, 6.82, and 5.39 $\mu\text{g/g}$ of fat for vitamin A; 8.51, 10.08, 10.17, and 13.62 $\mu\text{g/g}$ of fat for vitamin E; and 465, 410, 463, and 459 for IC, respectively. There were positive relationships (Figure 4) between the amount of experimental feed in the diet and the concentrations of all-*trans*-BC (quadratic, $P < 0.001$, $\text{Syx} = 0.76$, $R^2 = 0.53$) and vitamin E (linear, $P < 0.01$, $\text{Syx} = 3.05$, $R^2 = 0.22$) in milk fat. The relationships were not significant for lutein, 13-*cis*-BC, vitamin A, or CI.

Relationship Between Carotenoids and CI in Plasma and Milk

Global regression analyses revealed a significant positive and linear relationship between BC and CI in plasma but not in milk:

$$\text{Plasma: CI} = 14.0 \text{ (SE 1.1; } P < 0.001)$$

$$+ 6.54 \text{ (SE 0.21; } P < 0.001) \times \text{BC}$$

$$(n = 191; \text{RMSE} = 5.8; R^2 = 0.84);$$

$$\text{Milk: CI} = 373 \text{ (SE 31; } P < 0.001)$$

$$+ 294 \text{ (SE 246; } P = 0.23) \times \text{BC}$$

$$(n = 124; \text{RMSE} = 91; R^2 = 0.03).$$

The within-animal models revealed a significant relationship between BC and CI in both plasma and milk.

$$\text{Plasma: CI} = 20.1 \text{ (SE 1.5; } P < 0.001)$$

$$+ 5.30 \text{ (SE 0.30; } P < 0.001) \times \text{BC}$$

$$(\text{Nanim} = 32; n = 191; \text{RMSE} = 5.1; R^2 = 0.87);$$

Table 4. Milk concentration and secretion of carotenoids, vitamins A and E, and color index in cows fed diets differing in levels of carotenoids for 6 wk¹

Item	Group ²				SE	Effect ³		
	1	2	3	4		G	T	G × T
Lutein								
μg/mL	0.024	0.024	0.023	0.024	0.002	0.785	<0.001	0.333
μg/g of fat	0.72	0.73	0.71	0.77	0.08	0.742	0.001	0.776
secreted, mg/d	0.43	0.46	0.44	0.47	0.06	0.773	<0.001	0.737
all- <i>trans</i> -β-carotene								
μg/mL	0.079	0.114	0.115	0.112	0.008	0.001	<0.001	0.001
μg/g of fat	2.31	3.54	3.57	3.55	0.25	<0.001	<0.001	<0.001
secreted, mg/d	1.39	2.28	2.21	2.27	0.226	0.004	<0.001	<0.001
13- <i>cis</i> -β-carotene								
μg/mL	0.017	0.020	0.017	0.021	0.003	0.158	0.377	0.077
μg/g of fat	0.44	0.56	0.56	0.67	0.11	0.010	0.323	0.335
secreted, mg/d	0.26	0.35	0.37	0.41	0.07	0.008	0.532	0.507
Total-β-carotene								
μg/mL	0.094	0.132	0.133	0.132	0.010	<0.001	<0.001	0.066
μg/g of fat	2.74	4.10	4.13	4.21	0.31	<0.001	<0.001	0.001
secreted, mg/d	1.62	2.64	2.56	2.68	0.261	0.003	<0.001	0.015
Total carotenoids								
μg/mL	0.119	0.155	0.157	0.155	0.012	0.002	0.002	0.254
μg/g of fat	3.47	4.82	4.85	4.97	0.447	<0.001	<0.001	0.041
secreted, mg/d	2.09	3.11	3.00	3.14	0.301	0.006	0.005	0.105
Vitamin A								
μg/mL	0.171	0.193	0.193	0.181	0.014	0.059	0.001	0.106
μg/g of fat	5.02	6.00	5.93	5.79	0.441	0.004	0.026	0.350
Secreted, mg/d	3.01	3.73	3.73	3.57	0.303	0.008	0.033	0.261
Vitamin E								
μg/mL	0.266	0.299	0.322	0.351	0.038	0.030	<0.001	0.468
μg/g of fat	7.80	9.25	9.76	11.31	1.186	0.005	<0.001	0.795
Secreted, mg/d	4.40	5.75	6.25	6.68	0.802	0.003	<0.001	0.623
Color index	436	428	430	437	20	0.965	<0.001	0.219

¹Values are means for 8 cows per group over 6 wk.

²Groups were fed various proportions of hay:experimental feed = 100:0 (group 1), 67:33 (group 2), 33:67 (group 3), or 0:100 (group 4); experimental feed consisted in grass silage and alfalfa protein concentrate (75:25).

³Effects: G = group, T = time.

Milk: CI = 319 (SE 34; $P < 0.001$)

+ 735 (SE 274; $P < 0.010$) × BC

(Nanim = 32; n = 124; RMSE = 13; $R^2 = 0.54$).

With these models, the individual intercepts varied significantly among animals, but the individual slopes were not significantly different between animals.

DISCUSSION

In the present work, we choose providing carotenoids by a natural source (grass silage and alfalfa protein concentrate), so that the increase in carotenoid intake was accompanied by a parallel increase in vitamin E and digestible protein intake. Possible interactions between digestible protein intake and absorption of carotenoids may not be excluded, but knowledge on interaction between protein and absorption of carotenoids or other lipophilic compounds remains scarce. In contrast, clear evidence of an interaction between dietary vita-

min E (α-tocopherol) and plasma BC has been reported in ruminants (Yang et al., 2002). This may be related to interactions for intestinal absorption, competition for transport in plasma in lipoproteins, or both. It must be underlined that our experimental design did not allow to assess the extent of these interactions and that observed effects are global effects of the experimental feed.

Intake and Milk Production

Refusals of experimental feed (grass silage + APC) were observed only transitorily during wk 1, reflecting an adaptation period to this new regimen. The inclusion of 1.5 kg of APC/d in the diet of dairy cows has already been shown to lead to a slight decrease (−0.3 kg/d) in the intake of grass silage over 4 wk (Dewhurst and Coulmier, 2004). It should be underlined that the dietary level of carotenoids reached 475 μg/kg of DM in with group 4. Although these were high levels compared with winter rations (Chauveau-Duriot et al., 2005) and

Figure 3. Evolution in lutein, all-*trans*- β -carotene, 13-*cis*- β -carotene, vitamin A, vitamin E, and color index in milk fat of dairy cows fed diets differing in hay:experimental feed ratios: 100:0 (group 1 $\circ$), 67:33 (group 2 $\triangle$), 33:67 (group 3 $\blacktriangle$), or 0:100 (group 4 $\bullet$). Experimental feed consisted in grass silage and lucerne protein concentrate (75:25). All groups were fed hay during a preexperimental period of 6 wk. Means $\pm$ SEM for 8 cows per group are presented.

some grasslands (Calderón et al., 2006), they remained lower than most pasture-based diets (Prache et al., 2003; Graulet et al., 2006) as quantified by a similar methods.

There was a 2-fold stronger decrease in milk production over the experimental period in group 1 (-14% /month) than in the other 3 groups (averaging -7% /month). Given the protein and energy balance, this decrease was not due to the lower protein supply in group 1 but to the higher efficiency of grass silage compared with hay for milk production, as reported by Coulon and Pradel (1997) with isoenergetic and isoproteic diets. Nevertheless, for the 4 groups, the evolution of milk yield remained consistent with what is commonly observed during this lactation period for multiparous animals with similar potential (Faverdin et al., 1987), and the between-group differences in milk yield over the experimental period were not statistically significant.

Carotenoid Concentrations and Diversity in Forage, Plasma, and Milk

Total carotenoid concentrations in ryegrass silage ($441 \mu\text{g/g}$ of DM) and the respective proportions of carotene and xanthophylls (22/78) were consistent with previous reports on direct grass silages (350 to $520 \mu\text{g/g}$ of DM, Chauveau-Duriot et al., 2005; Calderón et al., 2007). In contrast, the total carotenoid concentrations recorded in the hay ($165 \mu\text{g/g}$ of DM) were much higher than previously reported for sun-dried hay (70 to $100 \mu\text{g/g}$ of DM, Chauveau-Duriot et al., 2005), suggesting that maximal carotenoid degradation had not been achieved after 2 d of sun-drying in the present study. Also, the proportion of carotene in total carotenoids was lower in hay (9%) than in grass silage (22%), which is consistent with Chauveau-Duriot et al. (2005) who reported that the carotenoid loss between direct-cut

Figure 4. Relationships between the proportions of experimental feed (75% grass silage + 25% alfalfa protein concentrate) in the basal diet, and the concentrations of total β -carotene (all-*trans* + 13-*cis*- β -carotene) and vitamin E in plasma (A) and milk fat (B) after 42 d on experiment. Means $\pm$ RMSE for 8 cows per group are presented.

silage and sun-dried hay is more marked for carotene than for xanthophylls, particularly lutein. The 5 xanthophylls quantified in the forages and in APC in the present work have previously been identified in a range of grasslands including fresh grass (Calderón et al., 2006), grass silage (Calderón et al., 2007), or hay (Cardinault et al., 2004), but to our knowledge, this is the first time they have been reported in cultivated monospecific swards. As already reported with preserved forages from cultivated monospecific swards (Chauveau-Duriot et al., 2005), no *cis*-BC isomers were found in our feedstuffs, whereas the same analytical technique revealed significant amounts of 13-*cis*-BC (Prache et al., 2003; Cardinault et al., 2004; Calderón et al., 2006; Graulet et al., 2006) and 9-*cis*-BC (Graulet et al., 2006) together with all-*trans*-BC in diverse natural grassland.

Although xanthophylls account for the majority of dietary carotenoids, it has been clearly demonstrated that BC is the main circulating carotenoid in bovines (Yang et al., 1992). However, the mechanisms that could explain the low apparent transfer of xanthophylls from forages to plasma in bovines have not been clearly described. In humans, specific facilitative epithelial transporters implied in the intestinal absorption of ca-

rotenoids have been evidenced (During and Harrison, 2005) and may be responsible of the more efficient absorption of polar xanthophylls compared with less polar carotenes. A greater apparent intestinal digestibility in sheep (Cardinault et al., 2006) or appearance rate in the plasma of preruminant calves (Bierer et al., 1995) has also been observed for lutein compared with β -carotene. In cows, preliminary results were unable to identify specificity in ruminal metabolism or efficiency of intestinal absorption among carotenoids from pasture (Graulet et al., 2006). Taken together, these results would suggest that metabolic rather than digestive processes explain the very low concentrations of xanthophylls in plasma of ruminants.

In the present work, xanthophylls (lutein + zeaxanthin) accounted for 10 to 17% of total plasma carotenoids, which is greater than previously reported in other experiments using the same analytical method (3 to 4%, Graulet et al., 2006; Nozière et al., 2006a; Calderón et al., 2007). This may be related to the greater proportion of dietary xanthophylls in the present study than the above mentioned reports (78 to 91% vs. 76 to 79% of total carotenoids, respectively). To our knowledge, lutein has been the only xanthophyll thus far identified in bovine plasma, although other xantho-

phylls have been identified in bovine specific tissues (e.g., zeaxanthin in retina, Dachtler et al., 1998). To our knowledge, zeaxanthin was detected for the first time in bovine plasma in the present work, although it was not recovered in the milk. Although the underlying mechanisms are still unclear, it has been shown that there is a regulated uptake of polar carotenoids into milk in humans (Lietz et al., 2006). Nevertheless, significant amounts of zeaxanthin have already been quantified together with lutein in cow's milk (Havemose et al., 2004; Hulshof et al., 2006). In the present work, the only xanthophyll recovered in milk was lutein, accounting for 18 to 26% of total carotenoids. This is consistent with previous results reported using the same analytic method in milk from cows fed a wide variety of forages, including hays, grass and corn silages, and pasture (12 to 25% Martin et al., 2004; Calderón et al., 2006, 2007), whereas lower proportions were reported by other groups (1 to 3% by Havemose et al., 2004; 10% by Hulshof et al., 2006).

Although the only ingested BC was the all-*trans* isomer, we also detected 13-*cis*-BC isomer in both plasma (9 to 11% of total plasma BC) and milk (14 to 16% of total milk BC). Significant BC proportions of 13-*cis* isomer have rarely been reported, although recent studies have cited up to 30 and 21% of total BC in plasma and milk, respectively (Nozière et al., 2006a; Calderón et al., 2007). As previously reported and discussed (Calderón et al., 2007), the exact site of BC isomerization remains unknown, although the rumen, enterocytes, liver, plasma, or peripheral tissues, including adipose tissue and mammary gland, are all good candidates.

Variations in Plasma Following Change in Diet

Plasma concentrations of zeaxanthin, lutein, and vitamin A were only slightly affected following changes in diet, which is in good agreement with the majority of published studies reporting plasma concentrations of xanthophylls (Nozière et al., 2006a; Calderón et al., 2007) and vitamin A (see Nozière et al., 2006b for review) in dairy cows. In contrast, plasma concentrations of all-*trans* and 13-*cis*-BC as well as vitamin E all increased very rapidly following the changes in diet, and the amount of experimental feed in the diet appeared to positively affect i) the rate of increase of plasma BC and vitamin E concentrations, ii) the time elapsed before reaching a plateau, and iii) the peak value at plateau.

The rates of increase in plasma BC and vitamin E concentrations over the first week were comparable, in absolute terms, with those reported for dairy cows in midlactation shifted from a grass silage to a hay diet [i.e., $-0.21 \mu\text{g/mL}$ of BC/d and $-0.12 \mu\text{g/mL}$ of vitamin

E/d over the first 10 d following change in diet (Nozière et al., 2006a)]. These comparable rates between studies may reflect the turnover rate of the plasma HDL and LDL, through which carotenoids and vitamin E are carried in blood.

The time elapsed before plasma concentrations reached a plateau increased from 8 to 28 d for vitamin E, and from 28 to more than 42 d for BC between groups 2 and 4, respectively. This delay is comparable for vitamin E but much higher for BC than the duration of persistence (both 12 d) observed following shift from grass silage to hay (Nozière et al., 2006a). This may be related to the much higher BC supply in the present experiment. In steers supplemented with 352 mg of β -carotene/kg of DM (that is more than 3 times higher than in the present work for group 4), the concentrations in serum did not reach plateau 30 d after beginning of supplementation, but were 2 times lower than in the present work on dairy cows (Mora et al., 2001).

The peak concentrations obtained at the end of the experiment were linearly related with the amount of experimental feed, reaching $8.7 \mu\text{g/mL}$ of BC and $6.1 \mu\text{g}$ of vitamin E/mL, respectively, in group 4. This suggests that the BC and vitamin E absorption and transport capacities of the lipoproteins had not yet been saturated, even in group 4. In agreement with these results, plasma BC and vitamin E have already been reported to reach 8.4 and $10.1 \mu\text{g/mL}$, respectively, in Montbéliarde cows at pasture (J. B. Coulon, B. Martin, and P. Grolier, INRA, Theix, France, unpublished results, cited by Nozière et al., 2006b).

Variations in Milk Following Change in Diet

The transitory decrease in milk concentrations of lutein, vitamin A, and vitamin E at d 21 may be related to a concomitant decrease in milk fat content, but there were no significant variations in concentrations between d 5 and 42 for these micronutrients or for 13-*cis*-BC. Nevertheless, milk mirrored plasma in that the vitamin E concentrations were linearly related to the amount of experimental feed in the diet. However, unlike in plasma, although the milk BC concentrations rapidly increased following change in diet, both the rates of increase and the peak concentrations reached (plateau) remained similar between groups 2, 3, and 4, averaging $0.10 \mu\text{g/g}$ of fat/d over the first week, and $4.8 \mu\text{g/g}$ of fat, respectively. Also, the peak concentrations were reached more rapidly in milk than in plasma (d 28 in milk vs. more than 42 d in plasma for group 4). These results clearly indicate that despite the linear increase in plasma BC in response to dietary carotenoid levels, the milk secretion of BC did not increase when plasma BC exceeded $5 \mu\text{g/mL}$. The very few published

results on plasma, milk BC, or both in Montbéliarde cows nevertheless highlighted only slight differences with Holstein cows, and a review of the data, including on Holsteins, clearly suggests linearity between plasma and milk BC concentrations when the plasma concentration is under 5 $\mu\text{g/mL}$ (see Nozière et al., 2006b, for review). This is in strong agreement with the variations between groups 1 and 2 observed in the present work. In contrast, plasma concentrations higher than 5 $\mu\text{g/mL}$ of BC have been only rarely reported. With a very high BC plasma concentration (12.4 $\mu\text{g/mL}$) in Holstein cows, Morris et al. (2002) demonstrated a comparable milk BC concentration (5.2 $\mu\text{g/g}$ fat) as in the present work. Furthermore, the majority of BC values reported for the milk of both Montbéliarde or Holstein cows, even in pasture, are below 6 $\mu\text{g/g}$ of fat (see Nozière et al., 2006b for review; Hulshof et al., 2006). Our results clearly show that when plasma BC exceeds 5 $\mu\text{g/mL}$, the mechanisms involved in the transfer of BC from plasma to milk are limiting in terms of BC secretion. A previous report by Jensen et al. (1999) suggested that daily secretion of BC in dairy cows is quantitatively limited by genetic factors. In the present work, there was no increase in milk vitamin A concentrations between groups 2 and 4. This indicates that the limitation of BC secretion may not be due to a higher cleavage of BC in the mammary gland, but rather to a limited uptake by the mammary gland or limited transport by binding β -lactoglobulin (Dufour and Haertlé, 1991) and/or to saturation of milk fat globules.

Transfer of Carotenoids and Fat-Soluble Vitamins from Plasma to Milk

Given a flow through the mammary gland of 400 L of plasma/kg of milk produced (Delamaire and Guinard-Flament, 2006), it is possible to estimate the efficiency of micronutrient transfer from plasma to milk. The rate of efficiency in the present study averaged 0.07 and 1.06 mg/g for BC and vitamin A, respectively, which is similar to the previous figures reported for dairy cows in midlactation (0.08 and 0.95 mg/g, respectively, Nozière et al., 2006a), whereas higher values were reported in early lactation (0.20 and 1.50 mg/g, respectively, Calderón et al., 2007). This may suggest that the efficiency of BC transfer from plasma to milk decreases through stage of lactation. However, the mechanisms involved in the uptake of carotenoids from plasma lipoproteins (mainly HDL in bovines, Schweigert et al., 1987; Yang et al., 1992) to the mammary gland and in the following release into milk remain unknown, and further studies are needed to clarify the issue. In the present study, the efficiency of lutein and vitamin E transfer averaged 0.12 and 0.18 mg/g, respectively,

which is comparable to results previously obtained in early lactation (0.08 and 0.20 mg/g; Calderón et al., 2007), whereas a higher efficiency of vitamin E transfer was reported in midlactation using another analytical method (0.64 and 0.48 mg/g, Jensen et al., 1999; Nozière et al., 2006a). Compared with the other fat-soluble micronutrients, the higher efficiency of vitamin A transfer was consistent with these reports, which may reflect a more efficient uptake of vitamin A from plasma by the mammary gland, and/or the ability of the mammary gland to cleave BC into vitamin A, as demonstrated by Schweigert and Eisele (1990). Lastly, it should be highlighted that there was no variation in the efficiency of transfer of lutein, BC, vitamin A or vitamin E between-group or according to time during the experiment.

Relationship Between BC and Color Index

The CI in plasma and milk have been considered promising tools for feeding management traceability in dairy cows (Martin et al., 2005a; Nozière et al., 2006a). In the present work, BC explained more than 80% of CI variations in plasma, a much higher figure than in previous studies conducted in either midlactation (58%, Nozière et al., 2006a) or early lactation (51%, Calderón et al., 2007) over a similar range of BC concentrations. In contrast, the CI variations in milk, and particularly the decrease during the first week in experiment, were unexpected. Also, with the global model, BC explained only 0.3% of CI variations in milk, whereas an R^2 of 37-40% was achieved in previous works. Indeed, in the present study, unlike BC, CI showed stronger between-individual variation ($P < 0.001$) than between-treatment variation ($P = 0.97$). Genetic studies have shown that milk color (Winkelman et al., 1999) is highly variable among individuals. Nevertheless, it should be underlined that the 56% variability explained with the within-animal model in the present work was comparable to the 60% value reported previously by Calderón et al. (2007). Taken together, these results emphasize the likely presence of variable amounts of undetected compounds absorbing between 450 and 530 nm (i.e., riboflavin?) mainly in milk.

CONCLUSIONS

The present study demonstrated that replacing 33, 67, or 100% of the hay-based diet component by an experimental feed (consisting in a 75/25 mixture of grass silage and lucerne protein concentrate) induced a rapid increase in plasma concentrations of BC and vitamin E. The time to plateau varied according to the amount of experimental feed in the diet, i.e., from 8 to

28 d for vitamin E and from 28 to more than 42 d for BC. Plasma concentrations of BC and vitamin E were linearly related to the amount of experimental feed in the diet. Similar responses were observed in milk for vitamin E, whereas there was an apparent saturation in milk concentrations of BC at high levels of carotenoid intake, i.e., when plasma BC exceeded 5 µg/mL. These results suggest that milk secretion of BC under high-carotenoid diets is not limited by the amount of BC arriving at the mammary gland but by mechanisms involved in BC transfer from plasma to milk. Further studies are required to elucidate these mechanisms. Lastly, plasma CI appeared to be a more promising indicator than milk CI of the dietary carotene content ingested by dairy cows.

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