Ultraviolet-absorbing compounds in milk are related to forage polyphenols

J. M. Besle,^{*1} D. Viala,^{*} B. Martin,^{*} P. Pradel,[†] B. Meunier,^{*} J. L. Berdagué,[‡] D. Fraisse,[§]

J. L. Lamaison,[§] and J. B. Coulon^{*}

^{*}INRA, UR1213 Herbivores, F-63122 Saint Genès Champanelle, France

[†]Domaine de la Borie, INRA, UE1296 des Monts d'Auvergne, F63210 Orcival, France

[‡]INRA, UR370 Qualité des Produits animaux, F-63122 Saint Genès Champanelle, France

[§]Faculté de Pharmacie, Laboratoire de Pharmacognosie, BP 38, F-63000 Clermont-Ferrand, France

ABSTRACT

The aim of this work was to characterize UV-absorbing compounds (UAC) in milk in relation to diet. In winter, 4 groups of cows each received a different diet: concentrate rich containing 35% cocksfoot hay (CCH), maize silage (MS), rye grass silage (RS), or rye grass hay (RH). In summer, 2 additional diets were given: mountain grassland hay (GH) and mountain grassland pasture (GP). Polyphenols were analyzed by HPLC and Folin reaction on forages and UAC were extracted from milks and analyzed by HPLC. In forages, the number of polyphenols was lowest in MS (57) and greatest in GP (85). Twenty-four peaks were identified, accounting for 28 to 47% peak area at 280 nm. Caffeoyl compounds and flavonoid glycosides were mainly found in RH, GH, and GP. Hydrolyzed compounds such as hydroxycinnamic acids and aglycones were found in MS and RS. Estimated amounts of polyphenols were lowest for MS (3.7 g/kg), roughly similar for CH, RS, and RH (about 15 g/kg), and greatest for GH and GP (21.6 and 35.3 g/kg, respectively). About 230 different peaks were separated in milks. Milks from RH and GP contained the lowest (87) and the greatest (127) numbers of peaks, respectively. Only 10 peaks were identified, accounting for 21 to 54% of the total spectra area. In addition to the major compound hippuric acid, phenylacetic acid, benzoic acid, 4-hydroxybenzoic acid benzaldehyde, catechol, and small amounts of ferulic acid were found in varying amounts depending on the diet. Flavonoids such as quercetin, luteolin, and apigenin were also present. Hippuric acid was clearly related to the presence of caffeoylquinic compounds in forages. Other identified UAC may originate essentially from forage simple polyphenols or from cell wall aromatics. Some of the several unknown compounds may also originate from the transformation of other nutrients. Estimated amounts of UAC were widely variable within each animal group.

They were surprisingly high in CCH and roughly similar in all milks from preserved forages (about 3.6 mg/L), with generally greater values for GH milks, whereas the greatest amount was found in GP milks (13.3 mg/L). Hierarchical clustering clearly discriminated the 6 diets, showing that there were major differences in GP milks. Some UAC were specific to one or a group of diets. Ultraviolet-absorbing compounds are therefore a potential tool to distinguish between milks according to diet. In addition, they may have a bioactive effect on milk component conservation or on human health.

Key words: traceability, transfer to milk, polyphenol, phenolic compound

INTRODUCTION

Forages contain large amounts of aromatic compounds both in the insoluble cell wall (Besle et al., 1994) and in the cellular content in the form of water and ethanol-soluble polyphenols (Fraisse et al., 2007) specific to each plant taxon (Harborne, 1999). These compounds are partially degraded in the rumen to form several aromatic compounds (Scheline, 1991). Some other components of the diet are also fermented to form nitrogen- and sulfur-containing aromatic or heterocyclic products (Martin, 1973), and some aromatic hydrocarbons may occasionally be present (Bakke and Scheline, 1970). All these compounds are partly absorbed, transformed in the animal tissues, and then excreted (Scheline, 1991) in a pool of UV-absorbing compounds (UAC), which can be recovered in milk.

The metabolism of phenols has been known since the end of 19th century in monogastrics (Bauman, 1876). A first fraction of volatile UAC was identified in the 1970s as contributing to the flavor of dairy products. It contained mostly monophenols, heterocycles, phenyl ketones, and alkylbenzoic compounds (Urbach et al., 1972). Slightly later, other volatile or more complex compounds resistant to vacuum drying were identified (Brewington et al., 1974; Lopez and Lindsay, 1993). Their effect on the flavor of milk was assessed (Schutte, 1999). In addition, isoflavones and the products of their

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¹Corresponding author: besle@clermont.inra.fr

fermentation in the rumen were isolated in the milk of cows receiving commercial diets (Hoikkala et al., 2007).

In several other reports, other micronutrients were studied in milk in relation to diet. The knowledge of the laws governing the transfer of micronutrients into milk was used to assess the nutritional properties of milk (Lucas et al., 2005; Fedele, 2008) and for traceability studies (Prache et al., 2007). The major compounds studied in this respect were terpenoids (Fernandez et al., 2003), polyunsaturated fatty acids (Ferlay et al., 2006), and carotenoids (Nozière et al., 2006).

Very few studies have assessed the relationships between diet and UAC. It has been shown that red clover is the source of isoflavones in milk (King et al., 1998). Scehovic et al. (1998) observed a significant difference in total phenolic between cheeses from lowland and highland pastures. With a diet rich in *Borago* and *Crataegus*, De Feo et al. (2006) identified rutin and quercetin in cow's milk. Ultraviolet-absorbing compounds are therefore of potential interest for traceability studies because they represent a hydrophilic fraction (O'Connell and Fox, 2001) complementary to other volatile or lipophile tracers. In addition, they are partly involved in milk flavor (Schutte, 1999) and are probably of nutritional interest, with possible effects on health (Clifford, 2000).

The aim of this work was to study the transfer of polyphenols from forages to milks in the case of the most common cow diets and to verify their potential interest as a tool for the authentication of animal diet.

MATERIALS AND METHODS

Experimental Scheme

Thirty multiparous lactating cows from the Institut National de la Recherche Agronomique experimental farm of Marcenat (Cantal, France) were divided into 6 groups of 5 animals at a similar lactation stage with comparable milk yield, milk fat and protein contents, and parity. Each group included 3 Montbéliarde and 2 Tarentaise cows. The cows produced on average 13.2 (SE = 0.6) kg of milk/cow per day and the mean lactation stage was 94 (SE = 3.9) d post-calving at the first day of the experimental period. The experiment was divided into 2 periods, preexperimental and experimental, of 3 and 6 wk, respectively. During the preexperimental period, the cows were fed a diet containing on average 10.9 kg of maize silage and 1.8 kg of concentrate (57% barley, 26% soybean meal, and 17% urea). The first week of the experimental period was used for a progressive adjustment to diets, and wk 5 and 6 were used for data and sample collection.

In winter, 4 of these groups were then fed for the duration of the experimental period 1 of 4 diets composed of the following forages: concentrate rich (66% DMI) plus cocksfoot hay (**CH**; 34% DMI), forming the **CCH** diet; maize silage (**MS**; 86% DMI); perennial rye grass silage (**RS**; 85% DMI); and rye grass hay (**RH**; 90% DMI). In the CCH diet, the concentrate contained 90% barley, 5% soybean meal, 3% CaCO₂, and 2% urea. In the MS diet, the concentrate contained 43% barley, 28% soybean meal, 18% CaCO₂, and 5% urea. In the other diets, the concentrate contained only barley and soybean meal (70 and 30% in the RS and RH diets, respectively).

In spring, the 2 other groups of cows were either fed diversified grassland hay (**GH**; 87% DMI) and concentrate (68% barley and 32% soybean meal) or grazed a diversified mountain (1,100 m altitude) grassland pasture (**GP**) with 0.5 kg/d of soybean meal. In addition, the 6 groups received 0.2 kg/d of the same mineral and vitamin premix throughout the experiment.

A first cutting of rye grass (June 6, 1999) was used for silage making with formic acid as additive (3 L/t), and a second cutting of the same plot was carried out 6 wk later to make hay. Grassland hay was prepared from a first cut (May 5, 1999) at the same altitude on different, less sloping ground than the grassland pasture, which was grazed from May 30 to June 26, 2000. All hays were barn dried. In both groups, the quantities of concentrates were adjusted to the individual requirement so as to cover at least 105% of the INRA (1989) energy and protein allowances.

The intake and refused fractions were measured for all the preserved diets. An aliquot of the ingested preserved fodders was sampled twice a week and frozen (−18°C). A milk aliquot was collected from the morning and evening milkings 2 d/wk, mixed according to the respective amount produced, and kept at −18°C.

All experimental procedures were conducted according to the French recommendations for the use of experimental animals, including animal welfare and appropriate conditions (Guidelines April 18 1988, cited by Veissier, 1999).

Sample Preparation and Extraction of UAC

Forages. Samples of GP were collected and botanical composition was determined according to Fraisse et al. (2007). The preserved forages were only roughly examined for their botanical composition. The rye grass and cocksfoot fields were contaminated by about 20% (wet weight) other plants and biodiversity was lower in grassland hay than in the grazed grassland. Ingested forage aliquots from 2 consecutive weeks of the experimental period were analyzed and were freeze-dried and ground

to pass a 1-mm sieve. The polyphenols were extracted according to Fraisse et al. (2007). Briefly, the samples (200 mg) were extracted with 12 mL of ethanol/water (50:50 vol/vol) under reflux for 30 min, then filtered through a sintered crucible porosity P2 (41–100 μm). This treatment was repeated once on the residue. After washings, the filtrates were placed together and the final volume was adjusted to 25 mL. Of this volume, 10 mL was vacuum dried, solubilized into 1 mL of ethanol/water (50:50 vol/vol), and filtered through a 0.2- μm cellulose acetate filter (Sartorius, Goettingen, Germany). The filter was washed with ethanol/water (50:50 vol/vol) to recover 1.5-mL samples. In these conditions we checked that the recovery of several polyphenolic compounds added to a control forage was total. Because the polyphenol content of concentrates was negligible, only the data regarding forages are presented.

Milk. For each cow, the samples from the 2-d collections of wk 5 and 6 were thawed and mixed. Thus, for each cow, 2 samples (1 for each week) were analyzed in duplicate. Ultraviolet-absorbing compounds were extracted according to a modified procedure of King et al. (1998). Briefly, 19 mL of acetonitrile was added to 8 mL of whole milk. After mixing, the samples were left overnight at 4°C and mixed again. The mixture was then warmed to reach ambient temperature, thoroughly mixed, and centrifuged at $1,000 \times g$ at ambient temperature. The supernatant was evaporated under nitrogen or vacuum dried in a SpeedVac (Jouan, St-Herbain, France) at 55°C. For deconjugation, the samples were incubated overnight at 37°C with 800 μL of acetate buffer (0.2 M, pH 5) and 10 μL of glucuronidase/sulfatase (*Helix pomatia*) (G-0876, Sigma, St. Louis, MO). The pH was then adjusted to 2.5 with 6 M HCl (about 40 μL) and extraction was performed 3 times with 2 mL of ethyl acetate. The mixed solvent phases were evaporated to dryness under nitrogen at 50°C and 45 μL of acetate buffer plus 105 μL of methanol was added to solubilize the compounds with a vortex.

As the samples were vacuum dried, the volatile UAC were not recovered. Thus, it was checked that after spiking a control milk with standard compounds, phenol, guaiacol, *p*-cresol, 3,4-dimethylpropylphenol, 2-isopropylphenol, indole, or skatole were not recovered whereas only $16.6 \pm 1.6\%$ of carvacrol was recovered. However, several other compounds tested in triplicate were recovered partially or totally with a reliable accuracy (CV < 6%). For all the compounds identified in this work, the recovery of triplicates added in 3 different assays varied from $68.7 \pm 5.7\%$ for quercetin to $100 \pm 4.4\%$ for apigenin. This recovery factor was used to calculate the amount of each type of compound.

Because milk contains arylsulfatase (Kilic and Lindsay, 2005) and glucuronidase, we checked with 4-methy-

lumbelliferone, 4-methylumbelliferyl- β -O-glucuronide, 4-methylumbelliferyl- β -O-sulfate, *p*-nitrocatechol, *p*-nitrocatechol sulfate, β -estradiol, β -estradiol-17- β -D-glucuronide, and control milk that an amount of enzyme 10 times less (i.e., 1 μL of glucuronidase/sulfatase per sample) than that used in this method was sufficient for a total hydrolysis of the conjugates. However, for security, a larger amount was preserved. Because cow milk is devoid of phosphate conjugates (Lopez and Lindsay, 1993), it was not necessary to add any aryl phosphatase to achieve total deconjugation. It was also checked that skimming of milk did not change the final composition of the milk extracts nor the yields of any of the aromatic compounds added.

Chromatographic Separation and Identification

Each sample was analyzed on a chromatograph equipped with a photodiode array detector (200–400 nm, Kontron, Serlabo Technologie, Entraigues, France). Elution was carried out through a Superspher column 60 RP8 (12.5 cm $\times$ 0.4 cm, 5 μm ; Merck, Whitehouse Station, NJ) with the solvents A ($\text{H}_2\text{O}/\text{H}_3\text{PO}_4$; 85%; 100/0.3 vol/vol) and B ($\text{MeCN}/\text{H}_2\text{O}/\text{H}_3\text{PO}_4$; 85%; 94/6/0.3 vol/vol/vol) forming a gradient from 0 to 70% B. The identification of the compounds was based on the comparison of retention time and spectra with those of appropriate commercial standards and by coelution with added standard compounds.

In forages, identified compounds were quantified using the peak area at 280 nm. An estimation of unknown compounds was made on the assumption that the overall relationship between peak area and concentration was the same for unknown and identified compounds. In milk samples, because the maxima of the spectra varied from 200 to 360 nm, there was no common wavelength able to detect or quantify all the compounds. Thus, to facilitate identification, quantification, and classification, the spectra of UAC peaks at apex time were used. They were digitized using a screen snapshot of the chromatograph software (Chromasystem 2000, Kontron, Serlabo Technologie). These images were automatically analyzed using a homemade visual basic program developed under Visilog 6.0 (Noesis, Gif sur Yvette, France) to retrieve the 200 to 400 nm spectra data. These data were recorded and processed under Excel (Microsoft Corp., Redmond, WA). Thus, the area under the spectra at the optimal retention time was used to calculate the amounts of identified compounds. It was verified, with pure standard compounds, that the area under the spectra and concentration were linked by a close linear relationship ($r > 0.999$ for all compounds tested). Unknown compounds were estimated either 1) on the assumption that the overall relationship between

peak spectra area and concentration was roughly similar in unknown fraction to that of identified compounds or 2) using catechol as standard. Peaks found in only 1 cow were eliminated.

Colorimetric Measurements

Total phenolics were determined according to the Folin-Ciocalteu procedure (Singleton and Rossi, 1965). To 150 μL of forage extract, 750 μL of diluted (1:10 vol/vol in water) Folin reagent and 600 μL of aqueous Na_2CO_3 (75 g/L) were added. The mixture was kept in a water bath at 50°C for 5 min and was then chilled on ice before reading of absorbance at 760 nm versus catechin solutions.

Data Analysis

The effect of diet on the UAC compounds was studied by ANOVA according to the general linear model procedure $X_{ij} = \mu + D_i + \varepsilon_j$, where X_{ij} = area under the spectra between 200 and 400 nm at apex time of a given peak, μ = mean effect, D_i = diet effect, and ε_j = residual variation. A principal component analysis was performed to compress the data (more than 99% of the total variance was contained into the first 10 axes of the principal component analysis) in order to perform a hierarchical clustering analysis (computations on Euclidian metric and Ward method for aggregation of samples), which was then carried out to illustrate the closeness of the type of feeding (Statistica Software, 2009).

RESULTS

Polyphenolic Composition of Forages

In forages, the UAC were essentially polyphenols. Their amounts estimated by HPLC were in rough agreement with those determined by the Folin method ($R^2 = 0.90$), but MS was significantly greater with the latter method (Table 1). Of the preserved forages, MS had the lowest amount of polyphenols (3.7 g/kg of DM measured by HPLC) and GH had the greatest. Grassland pasture had by far the greatest polyphenolic content (35.3 g/kg of DM). The number of compounds separated by HPLC was lowest in MS (57) and greatest in GP (85). According to their spectra, these compounds were essentially hydroxycinnamic or o-diphenolic acids and flavonoids with a very different composition for each forage. The fraction of area identified varied from one-quarter to about one-half of the total area measured in MS and GP, respectively.

Protocatechuic, neochlorogenic, and chlorogenic acids were the major acids found in hays. In addition, GP contained verbascoside and 3,5- and 1,5-dicaffeoylquinic acids. Among the flavonoids, apigenin was ubiquitous, homoorientin and luteolin were found in all hays and green pasture, and schaftoside was found in all samples except RH. Several luteolin and apigenin glycosides were found in all hays and GP but only a few were identified. Grassland hay was particularly rich in luteolin 7-O-glucoside and in a luteolin glycoside, and CH was characterized by apigenin 7-O-rutinoside, apigenin 7-O-glucoside, and an apigenin glycoside that was also observed in the 2 other hays. Quercitrin and quercetin 3-glucuronide were found in GH and RH, respectively.

Rye grass silage and RH had only 28 peaks (out of a total of 100 different peaks) with similar spectra and retention time. The silages contained several compounds resulting from the hydrolysis of cell wall or cellular polyphenols. Phenolic acids such as 4-hydroxybenzoic, *p*-coumaric, and ferulic acids were found in both silages, although in different amounts. In addition, RS contained protocatechuic and gallic acids and unhydrolyzed remaining chlorogenic acid. Both silages lacked O-glycosides but contained the highest amounts of aglycones such as luteolin and apigenin. Homoorientin, a flavonoid C-glucoside, quercetin, and kaempferol were also identified in RS.

The amounts of polyphenols in the diet were quite low for CCH and MS diets (4.7 and 3.2 g/kg of DMI, respectively), owing either to the low proportion of CH in the diet or to the small amount of polyphenols in MS. For the other preserved forages, the proportion of polyphenols ingested was considerably higher, with the greatest ingested proportion (35.3 g/kg of DMI) being for GP.

Milk UAC Composition

Several compounds were not present in all the animals receiving a diet. Thus, taking into account only the peaks present in at least 2 cows/diet, a total of 230 different peaks were separated in the different milk samples, some of them representing coeluted compounds. For each diet, the total number of different compounds separated in at least 2 cows receiving preserved forages was lowest for RH (87) but surprisingly high for CCH (118; Table 2). It was also high for the biodiverse GH and reached 127 for GP diets. Of the total number of peaks, 17 were the same (on spectra and retention time basis) for all diets but in varying amounts. We observed peaks specific to 1 diet. Their number, considering those found in the milk of at least 4 cows, were 1 for RS, 2 for CCH, RH, and GH, 5 for

Table 1. High performance liquid chromatography and colorimetric analysis of polyphenolic compounds in ingested forages

Item	Forage						RSD ¹
	Cocksfoot hay	Maize silage	Ryegrass silage	Ryegrass hay	Grassland hay	Grassland pasture	
Nb peaks	69	57	66	75	76	85	
Identified compounds (g/kg of DM)							
Gallic acid	— ^a	— ^a	0.83 ^b	— ^a	— ^a	— ^a	0.38
Protocatechuic acid	0.15 ^a	— ^a	0.89 ^b	0.30 ^a	0.34 ^a	— ^a	0.20
Neochlorogenic acid	0.21 ^b	— ^a	— ^a	0.86 ^c	0.64 ^d	0.65 ^d	0.09
Hydroxybenzoic acid	— ^a	0.34 ^{ac}	0.69 ^{bc}	— ^a	— ^a	— ^a	0.23
Chlorogenic acid	1.13 ^b	— ^a	1.32 ^b	3.89 ^c	1.51 ^b	4.90 ^d	0.35
Luteolin glycoside	— ^a	— ^a	— ^a	— ^a	1.30 ^b	— ^a	0.24
<i>p</i> -Coumaric acid	— ^a	0.30 ^b	0.26 ^b	— ^a	— ^a	— ^a	0.06
Ferulic acid	— ^a	0.06 ^a	0.48 ^b	— ^a	— ^a	— ^a	0.10
Schaftoside	0.38 ^b	— ^a	— ^a	— ^a	0.32 ^b	0.60 ^c	0.07
Homoorientin	1.00 ^b	— ^a	0.28	0.20	1.14 ^b	0.84 ^b	0.22
Quercitrin	— ^a	— ^a	— ^a	— ^a	0.58 ^b	— ^a	0.18
Quercetin 3-glucoside	— ^a	— ^a	— ^a	0.24 ^b	— ^a	— ^a	0.05
Luteolin 7-O-glucoside	— ^a	— ^a	— ^a	— ^a	0.67 ^b	0.55 ^b	0.15
Luteolin 7-rutinoside	— ^a	— ^a	— ^a	— ^a	— ^a	0.80 ^b	0.02
Verbascoside	— ^a	— ^a	— ^a	— ^a	— ^a	0.45 ^b	0.02
3,5-dicaffeoylquinic acid	— ^a	— ^a	— ^a	— ^a	— ^a	5.70 ^b	0.05
1,5-dicaffeoylquinic acid	— ^a	— ^a	— ^a	— ^a	— ^a	1.90 ^b	0.02
Apigenin 7-O-rutinoside	0.17 ^b	— ^a	— ^a	— ^a	— ^a	— ^a	0.03
Apigenin glycoside	0.09 ^b	— ^{ac}	— ^{ac}	0.04 ^{bc}	0.12 ^b	— ^a	0.03
Quercetin	— ^a	— ^a	0.68 ^b	— ^a	— ^a	— ^a	0.11
Apigenin 7-O-glucoside	0.45 ^b	— ^a	— ^a	— ^a	— ^a	— ^a	0.17
Luteolin	— ^a	0.13 ^a	0.35 ^b	0.12 ^{ab}	— ^a	— ^a	0.12
Kaempferol	— ^a	— ^a	0.36 ^b	— ^a	— ^a	— ^a	0.03
Apigenin	0.06 ^{ac}	0.19 ^{bd}	0.22 ^b	0.15 ^{abd}	0.01 ^c	0.06 ^{acd}	0.06
Total identified (g/kg of DM)	3.63 ^a	1.03 ^b	5.55 ^c	5.80 ^c	6.63 ^c	16.45 ^d	1.02
Identified area (% total area)	27.6	28.4	37.9	40.2	30.7	46.6	5.02
Total estimated ² (g/kg of DM)							
HPLC	13.4 ^a	3.7 ^b	16.8 ^a	14.5 ^a	21.6 ^c	35.3 ^d	1.95
Folin	15.5 ^{ad}	6.5 ^b	20.3 ^c	13.9 ^a	17.5 ^d	31.6 ^e	1.49
Ingested amounts (g/kg of DMI)	4.7 ^a	3.2 ^a	14.4 ^b	13.0 ^b	18.8 ^c	35.3 ^d	1.61

^{a–e}Means within a row with different superscripts differ ($P < 0.05$).

¹RSD = residual standard deviation.

²Unknown compounds of preserved forages were estimated assuming that the global relationship peak area/concentration was similar for unknown compounds (UC) and for identified compounds (IC) (i.e., $UC = IC/IA \times 100$, with $IA = \% \text{ identified area}$). In grassland pasture, they were estimated according to Fraisse et al. (2007).

MS, and 20 for GP. Other peaks were present in several milks but in different amounts.

Only 10 peaks, accounting for 21 to 54% of the total spectra area, were identified according to their spectra and retention times versus standard compounds. As indicated by the high residual standard deviation values, large animal variability was observed for all compounds (Table 2).

Hippuric acid (**HA**) was the major compound and was found particularly in milks from MS and RH, with highest amounts from GP diets. Phenylacetic acid (**PA**) and benzoic acid (**BA**) were found in milks from all diets but in different amounts, being particularly high for the GP diet. 4-Hydroxybenzoic acid was not found with the CCH and MS diets but was observed particularly with the RH diet and, above all, the GP diet. Ferulic acid (**FA**) in small amounts was observed only for RH and GP diets. Catechol was found in low

amounts for hay-rich diets (RH and GH) and in high quantities for GP diets. Benzaldehyde was observed in only 2 or 3 cows/group receiving the silages and biodiverse forages (GH and GP). Milks from GP had the highest amount of these last 2 compounds. The flavonoid aglycones quercetin, luteolin, and apigenin were observed in low amounts in milks from all diets; GP, however, was devoid of quercetin, and MS contained the lowest amount of apigenin.

The lowest total amount of identified compounds was found in CCH milks (0.8 mg/L). The other preserved forage diets were the source of intermediary amounts (1–1.8 mg/L), and the highest amount was found in GP milks (6.9 mg/L). The amount of unknown compounds estimated by method 1 (similar mean absorption for unknown and identified compounds) was the lowest for milks from CCH and RS (3.1 and 3.2 mg/L, respectively). In the other preserved forage diets, the milks from

Table 2. High performance liquid chromatography analysis of UV-absorbing compounds (UAC) in milk according to the diets

Item	Diet ¹						RSD ²
	CCH	MS	RS	RH	GH	GP	
Nb peaks	118	94	94	87	109	127	
Identified UAC (µg/L)							
4-Hydroxybenzoic acid	— ^a	— ^a	4.7 ^a	52.5 ^b	4.4 ^a	245 ^c	30.9
Phenylacetic acid	238 ^a	311 ^{ac}	215 ^a	414 ^{ac}	525 ^{bc}	1,298 ^d	201.3
Catechol	— ^a	— ^a	— ^a	9.9 ^a	10.1 ^a	590 ^b	94.4
Hippuric acid	466 ^a	1,352 ^b	839 ^{ab}	1,214 ^b	424 ^a	4,629 ^c	542
Benzoic acid	43.8 ^{ab}	59.0 ^a	43.0 ^{ab}	51.8 ^a	60.3 ^a	30.2 ^b	16.5
Ferulic acid	— ^a	— ^a	— ^a	3.9 ^a	0.9 ^a	14.7 ^b	3.4
Benzaldehyde	— ^a	1.2 ^{ab}	0.7 ^{ab}	— ^a	0.9 ^{ab}	2.4 ^b	1.4
Quercetin	12.4 ^a	7.0 ^a	14.3 ^a	16.0 ^a	10.4 ^a	— ^a	12.7
Luteolin	4.7 ^a	3.5 ^{ab}	3.6 ^{ab}	6.5 ^a	0.7 ^b	5.9 ^a	3.1
Apigenin	48.5 ^{ab}	25.3 ^a	75.0 ^b	71.5 ^b	56.5 ^{ab}	60.5 ^{ab}	29.0
Total identified UAC (µg/L)	813 ^a	1,759 ^b	1,196 ^{ab}	1,841 ^b	1,094 ^{ab}	6,877 ^c	682
Identified area (% total area)	26.9 ^a	44.6 ^b	37.3 ^{ab}	47.7 ^b	21.0 ^a	54.4 ^b	13.3
Total estimated UAC (µg/L)							
UC vs. mean IC absorption ³	3,154 ^a	3,640 ^{ab}	3,206 ^{ab}	3,657 ^{ab}	5,688 ^b	13,311 ^c	1,816
UC vs. catechol ⁴	2,439 ^a	3,290 ^a	2,893 ^a	3,295 ^a	3,365 ^a	12,223 ^b	1,475

^{a-d}Means within a row with different superscripts differ ($P < 0.05$).

¹CCH = concentrate rich + cocksfoot hay; MS = maize silage; RS = ryegrass silage; RH = ryegrass hay; GH = grassland hay, GP = grassland pasture.

²RSD = residual standard deviation.

³Estimation of unknown compounds (UC) assuming that the global relationship peak area/concentration was similar for UC and for identified compounds (IC) (i.e., $UC = IC/IA \times 100$, with $IA = \% \text{ identified area}$).

⁴Quantification of unknown compounds (UC) versus catechol.

MS and RH had greater values overall whereas GH had significantly higher values. Grassland pasture diets had milks with considerably higher amounts of UAC, reaching 13 mg/L. The second method of calculation (vs. catechol) yielded lower figures than did method 1 but the pattern was similar ($R^2 = 0.97$), except a particularly low figure for GH. The GP milks reached a UAC content of 12 mg/L.

Discrimination of the Diets by Milk UAC

The hierarchical clustering achieved with the peaks that were the most affected by the diets ($F > 10$) showed that the 6 diets were clearly distinct (Figure 1). The clustering segregated 3 groups below $25 < Dlink/Dmax < 35\%$: 1) GP milks at a great distance from the preserved forages; 2) GH milks from the more biodiverse forage, followed by RH and RS milks; and 3) MS and CCH milks derived each from very different diets.

The presence and amounts of different compounds in milks defined a pattern that was specific to each diet, thus providing a tool to recognize the diet consumed. Some examples of compounds present in all milks for a diet are shown in Figure 2. Catechol clearly differentiates GP milks from GH and RH milks (containing very little catechol) and from other milks (devoid of catechol). Unknown peak 120 differentiated between CCH and MS but was not found in other milks. Unknown

peak 53 was specific to rye grass (RS and RH) milks and was also present in higher amounts in GP milks. Unknown peak 34 was present only in RS milks.

DISCUSSION

Polyphenols in Forages

Several compounds identified in this study were also observed in other studies, namely caffeoyl compounds and apigenin glycosides (Jay and Lumaret, 1995), schaftoside and homoorientin (Fiasson et al., 1987) in *Dactylis glomerata*, and chlorogenic acid in *Lolium perenne* (Takahashi et al., 1992). However, in this study the number of compounds (about 70) separated in monospecific hays was much greater than those separated in earlier studies. Because the polyphenols were analyzed in whole ingested forage containing about 20% contaminating plants, some of the additional separated compounds (identified or not) may originate from the other taxons. In RS, *p*-coumaric acid, ferulic acid, quercetin, and kaempferol are formed from the hydrolysis of esterified hydroxycinnamic acids (Hartley, 1972) and of the respective flavonoid glycosides (Wilson, 1985), which were all found in *Lolium perenne*. In MS, the number of peaks separated is also higher than those in several parts of *Zea mays* in other studies (20 compounds according to Harborne, 1999). Several com-

Figure 1. Hierarchical ascendant clustering of UV-absorbing compounds in milk according to the 6 diets. It was carried out using the 5 most significant axes of the principal component analysis, the Ward method, and the Euclidian distance for computation of the data. GP = grassland pasture; GH = grassland hay; RH = ryegrass hay; RS = ryegrass silage; MS = maize silage; CCH = concentrate rich + cocksfoot hay.

pounds identified by Pedreschi and Cisneros-Zevallos (2007) in whole fresh maize were not present in the silage but, like in RS, cell wall-hydrolyzed compounds such as hydroxycinnamic acids (Leclerc et al., 2007) and a flavonoid aglycone (luteolin) were isolated. Because grassland pasture was grazed in a field with a higher biodiversity (Fraisie et al., 2007) than grassland hay, the number of peaks was greater in pasture, although some were similar. In addition, the presence of flavonoids mainly in the form of glycosides in all the hays argue in favor of the suitability of barn drying in darkness for preserving polyphenols.

In contrast, it has been shown with red clover that silage fermentation and oven drying are both suitable methods for preserving polyphenol aglycones in forages (Sakakibara et al., 2004a). Thus, the preservation method is probably not the explanation for the large differences in composition and the higher amounts observed in RS than in RH. Very likely the major causes of these discrepancies are differences in the cycle and stage of collection of silage and hay (first cut, young stage for silage versus regrowth and late mowing for hay), in agreement with similar observations by Jeangros et al. (2001). The total amounts of phenols (Folin

method) in CH and in RH were consistent with those observed by Jeangros et al. (2001), who used the same method for similar plants and stages. The slight differences (amounts and pattern) between HPLC and Folin methods are attributable to a different reactivity of several compounds, or the presence in MS of polymerized compounds that were not detected by HPLC, or both.

Origin of the Milk UAC

Most of the compounds isolated arose from the ruminal degradation of soluble polyphenols and of cell wall aromatics. Several reactions of reduction, demethylation, dehydroxylation, and decarboxylation with a slow or rapid rate resulted in several aromatic compounds that are able to be absorbed (Besle et al., 1995). The compounds are absorbed through the rumen and intestinal mucosa and immediately conjugated before they can be transformed in the liver and excreted in urine or milk (Scheline, 1991). The main phenolic acids observed in the rumen of sheep receiving forages were 3-phenylpropionic acid (PPA), PA, cinnamic acid (CA), and BA (Martin, 1973; Chesson et al., 1982). In the blood,

Figure 2. Examples of UV-absorbing compounds (areas of the spectra under the peak at apex time) present in milks secreted by all animals receiving the 6 diets. CCH = concentrate rich + cocksfoot hay; MS = maize silage; RS = ryegrass silage; RH = ryegrass hay; GH = grassland hay; GP = grassland pasture.

the major compounds were PPA and BA (Cremin et al., 1995). 3-Phenylpropionic acid is transformed into BA in the liver by beta oxidation (Scheline, 1991). The pool of BA is then conjugated with glycine to form HA (Gatley and Sherratt, 1977), or with glucuronic acid or sulfate. Chlorogenic acid is rapidly cleaved in the rumen into caffeic and quinic acids. Caffeic acid is partly absorbed or transformed in the rumen into cinnamate and then into PPA; quinic acid aromatization is not very efficient (Martin, 1982a) but it gives rise to BA. Parts of all these compounds are then directly absorbed. The pool of BA in blood is excreted in milk (and in urine) as hippuric acid and some BA conjugates. Thus, each chlorogenic acid molecule may give roughly 2 HA equivalents (Gonthier et al., 2003) and the diets with a high amount of chlorogenic acid, neochlorogenic acid (namely RH and GP), or caffeoyl compounds (GP) will give milks with corresponding high HA content. However, MS also gives milks with a high amount of HA, which cannot be explained by its content in identified compounds. In addition to the ferulic fraction released by the ensiling process in MS, some unhydrolyzed fraction of esterified plus ether-linked ferulic acid in the cell wall was expected to be released in the rumen, thereby providing a large amount of ferulate (Ostrander et al.,

1999) that may then have been transformed into HA; BA, however, remained at a low level in milk and did not follow the same pattern as HA. This may be attributable to either a limitation in blood of the fraction of BA conjugated with glucuronide and sulfate or a specific absorption of HA through the udder mucosa.

The presence of an overwhelming amount of HA in milk was first reported by Karabinos and Dittiner (1943). Although the amount of HA was lower in the present study than that reported by Svensen (1974), its variation was similar (i.e., a greater amount in milk from grazed pasture than from indoor forages). Benzoic acid was identified in whole milk by Brewington et al. (1974).

The absorbed fraction of caffeic acid may be methylated into FA in the ruminant's liver as in the rat (Gonthier et al., 2003). When cereal cell wall was fed to rats, 5% of linked FA was recovered in urine (Adam et al., 2002). It was therefore not surprising to find FA in milk from cows receiving the caffeoyl-rich RH and especially GP diets. To our knowledge, it is the first time that FA has been identified in milk.

Benzaldehyde, which was observed in few animals and in only 4 diets, was commonly found in whole milk by several authors (Lopez and Lindsay, 1993; Moio

et al., 1993). 4-Hydroxybenzoic acid, an intermediary compound present only in milk from forage-rich diets, was found earlier by Walker and Manning (1976).

It is very unlikely that phenylacetic acid and catechol are obtained from the degradation of cell wall aromatics because the first is produced very slowly and the second is found at the end of several reactions (Besle et al., 1995). They might both be formed from polyphenol degradation. Catechol has been observed in sheep urine after infusion of protocatechuic acid in the rumen (Martin, 1982b). Rutin, quercetin (Sharma et al., 1981), and a quercetin 3-glycoside (Schneider et al., 1999) may give rise to PA after rumen fermentation. In the present study, PA was present in all milks but was particularly abundant, like catechol, in milks corresponding to hays or GP rich in protocatechuic acid and other o-dihydroxypolyphenols. Phenylacetic acid has been frequently identified in milk (Lopez and Lindsay, 1993; Bendall, 2001), whereas catechol was found only by Brewington et al. (1974).

Untransformed aglycones quercetin, luteolin, and apigenin recovered in milk were formed after hydrolysis of their glycosides, absorption, and conjugation. Amounts were small and greatest in the flavonoid-rich GP. Isoflavone aglycones have been observed previously in ruminants receiving red clover (Sakakibara et al., 2004b). Quercetin and even rutin were identified in milk from goats receiving hawthorn and borage, respectively (De Feo et al., 2006). Moreover, several flavonoids have been reported to be recovered in slight amounts (up to 7% dose) in the urine of monogastrics, including man (Hollman and Arts, 2000).

Ultraviolet-absorbing compounds may also contain products from AA degradation. Phenylalanine is another source of PA (Martin, 1973) and may also produce CA (Hansen and Crawford, 1968). Tyrosine is transformed into PPA (Martin, 1973) or may be converted to phenol, 4-ethylphenol, or *p*-cresol by *Clostridium tetanomorphum* (Brot et al., 1965). Microbial degradation of tryptophan led to indolyl compounds such as skatole and indole (Kemmer et al., 1997). However, most of the aromatic nitrogen- and sulfur-containing compounds contributing to the UAC pool are volatile (Moio et al., 1993) and therefore were eliminated in our process.

Only a few compounds were identified, but they represent one-quarter to one-half of the total area. The other compounds could not be identified within the scope of this study. They had spectra that could have originated from several kinds of molecules, but with a large proportion of catechol-like spectra. Of all the compounds tested, catechol had an intermediate relationship spectra area/concentration; therefore, it was chosen as standard to quantify the unknown UAC. The

high amounts of phenolics found in milks from the GP and GH diets were expected from the high polyphenol content of the forages. The different pattern of UAC in milks from MS, RS, and RH is probably attributable to their cell wall origin. As stated previously, MS cell wall is very rich in bioavailable aromatic compounds. The high amount of UAC in milk from CCH is striking given the low ingested amount of polyphenols. Considering that CH represented only 35% DM of the diet, and that composition in bioavailable aromatic compounds (cell wall + polyphenols) and digestibility are roughly similar for cocksfoot and rye grass, the expected amount of UAC should have been half that measured. The amount of polyphenols from the concentrates (data not shown) was too small to explain the difference, and so it is possible that rumen degradation and transfer to milk were more efficient for the concentrate-rich diet.

Discrimination of the Diets by Milk UAC

The cluster analysis of our data is close to that observed for other micronutrients transferred in the milk from the same diets, namely terpenes (Martin et al., 2005) and polyunsaturated fatty acids (Ferlay et al., 2006). In all cases, GP, in contrast to CCH, was the most separated from the other diets. Terpene analysis provides some differences with UAC in the discrimination of forages related to their initial pattern and preservation characteristics but, like UAC, specific compounds from diets are a good tool to discriminate milks. Carotenoids also depend on diet (Nozière et al., 2006) but they give only a rough classification between milks from forage- or concentrate-rich diets (Prache et al., 2007). Thus, in addition to volatile and lipophile compounds, the hydrophile UAC provide a complementary and suitable tool for traceability of the diet. Its measurement requires only simple laboratory tools. The use of the 3 kinds of micronutrients may give a powerful tool for traceability of milk.

Significance of Milk UAC for Human Nutrition and Health

Although few compounds were identified in this work, the great diversity of UAC observed and the relatively high amounts argue for a nutritional role. Phenolic and heterocyclic compounds have an effect on the flavor of milk (Schutte, 1999; O'Connell and Fox, 2001). The antioxidant power of phenolic compounds is often higher than that of ascorbic acid but generally lower than that of the parent polyphenols (Tabart et al., 2009). The overall antioxidant power of milk, which depends on phenolic compounds, vitamin E, and carotenoids, was clearly highest in milk from GP diet (Martin et al.,

2002). An additional nutritional role of UAC might therefore be to prevent milk oxidation (Havemose et al., 2004) or butter oxidation (Soulti and Roussis, 2007).

Simple phenolic compounds that may be found in milk, such as phenol or carvacrol, are toxic, whereas others are known to have beneficial effects (Clifford, 2000). Whether UAC have a beneficial or deleterious effect (Lule and Xia, 2005) needs to be assessed with in vitro and in vivo trials. A better knowledge of the relationships between plant polyphenols and UAC in milk and of the biological effects of UAC will make it possible to select plant mixtures to achieve an optimal quality of milk.

CONCLUSIONS

Large differences in polyphenol composition of diet is reflected by large differences in UAC composition in milk. Ultraviolet-absorbing compounds are therefore a tool to establish the traceability of milks according to diet. This study needs, however, to be extended to other forages to determine the effect of several factors such as taxon, conservation method, and type or localization of permanent pasture. The identity of the several compounds separated must be assessed using specific and accurate methods. In addition, the metabolism of plant polyphenols in the ruminant organism and their recovery yield in milk should be elucidated. The nutritional and health significance of the compounds warrant complementary studies to determine whether any ration or plant mixture will be of particular benefit for humans.

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