JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 5485–5494 5485
DOI:10.1021/jf1000293

Variation in Content and Composition of Phenolic Compounds in Permanent Pastures According to Botanical Variation

Aurelie Reynaud,[†,‡] Didier Fraisse,[§] Agnes Cornu,[†] Anne Farruggia,[†] Estelle Pujos-Guillot,[#] Jean-Michel Besle,[†] Bruno Martin,[†] Jean-Louis Lamaison,[§] Denis Paquet,[‡] Michel Doreau,[†] and Benoit Graulet*[,†]

[†]INRA, UR1213 Herbivores, Centre de Clermont-Ferrand/Theix, F-63122 St-Genès-Champanelle, France, [‡]Danone Research, Centre de Recherche Daniel Carasso, F-91767 Palaiseau, France, [§]Laboratoire de Pharmacognosie, Faculté de Pharmacie, Université d'Auvergne, B.P. 38, F-63000 Clermont-Ferrand, France, and [#]INRA, Plate-forme d'exploration du métabolisme, UMR 1019 Nutrition Humaine, Centre de Clermont-Ferrand/Theix, F-63122 St-Genès-Champanelle, France

Phenolic compounds contribute to the micronutrient composition of pasture, which in turn may affect animal product composition. To assess the importance and variations in content of these compounds, the polyphenolic and botanical compositions of 24 permanent pastures located in one lowland and two upland regions were studied at equivalent stages of growth. Phenolic fractions were characterized and quantified using HPLC-PDA-ESI-QToF, and the total content was determined by colorimetry over each whole pasture. A rise in altitude was accompanied by a marked increase in total phenolic content, linked to changes in botanical composition, but did not have any influence on the distribution according to molecular class. For all of the pastures, significantly different patterns due to qualitative and quantitative differences among the 92 separate peaks were observed with 31 compounds identified. The involvement of certain plants in the variations of content and composition in phenolic compounds of pastures was statistically evaluated.

KEYWORDS: Pasture; phenolic; flavonoid; HPLC-PDA-MS; botanical composition

INTRODUCTION

Permanent pasture, with 76 million hectares, covers about 46% of the agricultural area used in Europe (*1*) and is one of the major components of the ruminant diet. By their variety, the plants in this type of pasture contribute to the nutritional and sensorial properties of dairy and meat products (*2*, *3*). These specific characteristics are related to plant microconstituents such as terpenes (*4*), polyunsaturated fatty acids (*4*), carotenoids (*4*), and phenolic compounds. The latter have been extensively studied in human nutrition (*5*), but little is known about their occurrence (content and composition) in the ruminant diet, especially in pastures.

Phenolic compounds are secondary metabolites that are derivatives of the pentose phosphate, shikimate, and phenylpropanoid pathways in plants (*6*). They are composed of at least one aromatic ring, bearing one or more hydroxyl groups. Ranging from the simplest molecular forms such as phenolic acids to flavonoids then to polymerized compounds including lignins or tannins (*6*, *7*), phenolic structures appear to be specific to plant species, botanical family, or environmental feature (*2*). Ubiquitous in plants, these compounds are an integral part of both human and animal diets. Until recently, most of the interest in phenolic compounds concerned the adverse effects caused by their ability, especially true for tannins, to bind and precipitate macromolecules such as dietary protein, carbohydrate, and digestive enzymes, thereby reducing food digestibility (*6*). However, more recently interest in food phenolic compounds has increased greatly, owing to their antioxidant capacity and their potential beneficial implications for human health, in particular for the prevention of cancer, cardiovascular diseases, and other pathologies (*6*). Phenolic compounds found in milk seem particularly interesting because they are present in significant amounts (several milligrams per liter) and, due to biotransformation by rumen microbes, could contain original and nutritionally interesting molecules (*8*) compared to other phenolic compound sources in human nutrition. Despite ruminal conversion of dietary phenolic compounds, those of milk may sometimes reflect the ruminant diet (*9*).

Little is known about the soluble phenolic compound composition of permanent pastures in relation to their botanical composition. In the Alps, Jeangros et al. (*10*) showed, by colorimetry, that the total content in soluble phenolics was up to 1.7-fold higher in subalpine permanent pastures (above 1600 m) than in mountain pastures (between 900 and 1520 m), due to quantitative and qualitative changes in botanical composition. Indeed, as the altitude increased, there was a greater percentage of dicotyledon plants, especially families rich in soluble phenolics such as Rosaceae or Asteraceae. Several studies have also highlighted the richness of dicotyledon plants in these compounds (*11*−*13*). Using photodiode array detection (PDA) coupled to high-performance liquid chromatography (HPLC), Fraisse et al. (*7*) studied

*Corresponding author (telephone + 33 473624541; fax + 33 473624273; e-mail benoit.graulet@clermont.inra.fr).

Published on Web 04/15/2010

Table 1. General Characteristics of the Permanent Pastures: Location, Environmental Conditions, Forage Practices[a]

							fertilization	
region	altitude (m)	soil	rainfall[b] (mm/month)	sunniness[b] (h/month)	temp[b] (°C)	utilization (animal unit/ha)[c]	N mineral[d]	organic[e]
HN	**111**[f] (10−210)[g]	chalk, clay or sandstone	**94**	**134**	**13.7**	**1.5** (1.0−2.2)	**57** (30−110)	3/8
IS	**609** (320−910)	alluvial and/or glacial deposits, pebbles	**192**	**192**	**15.9**	**0.9** (0.3−1.8)	**20** (0−50)	2/8
MV	**683** (470−1000)	gneiss or volcanic basaltic rock	**100**	**200**	**15.0**	**0.9** (0.5−1.8)	**40** (0−100)	0/8

[a] Abbreviations: temp, temperature. [b] In May. [c] At the moment of collection; value >1.5 indicates pasture intensively used, else extensively. [d] N units/ha/year; value <30 indicates low; value between 30 and 80 indicates moderate, value >80 indicates high. [e] Parcels per region where the organic fertilization was used. [f] Means per region (in bold). [g] Range (extreme values in parentheses).

the polyphenolic content and composition of an upland permanent pasture according to the period of harvesting. The 10 phenolic compounds identified were phenolic acids such as cholorogenic and 3,5-dicaffeoylquinic acids and flavonoids including homoorientin, luteolin-7-glucoside, or rutinoside. Specific analysis of nine major plant species highlighted the high phenolic acid content of certain plants such as *Achillea millefolium* (Asteraceae) and conversely the high flavonoid content of other species such as *Galium verum* (Rubiaceae).

The first aim of this study was to better characterize the soluble phenolic compound composition of permanent pastures by coupling mass spectrometry (MS) and PDA to HPLC. The second aim was to compare the botanical and polyphenolic compositions of permanent pastures located in three regions, one lowland and two upland, at an equivalent growth stage, and then to evaluate statistically the implication of certain plants on the variations of phenolic compound content and composition in pastures.

MATERIALS AND METHODS

Chemicals. Standard compounds (gallic, syringic, protocatechuic, chlorogenic, caffeic, *p*-coumaric, chicoric, and rosmarinic acids, verbascoside, catechin, epicatechin, epigallocatechin gallate, rutin, myricitrin, quercetin-3-glucuronide, apigenin, luteolin, luteolin-7-*O*-glucoside, homoorientin, eriodictyol-7-*O*-glucoside, hesperidin, daidzein, genistein, formononetin, biochanin A, and coumestrol) were obtained from Extrasynthese (Genay, France). *neo*- and *crypto*-chlorogenic, 1,5-, 3,4-, and 3,5-dicaffeoylquinic acids, and schaftoside were isolated as described by Duband et al. (*14*) and Carnat et al. (*15*). Sodium carbonate and Folin−Ciocalteu's phenol reagent were purchased from Sigma-Aldrich Chimie (Saint-Quentin Fallavier, France). Acetone, acetonitrile, and formic acid were purchased from VWR (Fontenay sous Bois, France). All solvents were of HPLC grade, and water was of Milli-Q quality (Millipore Corp., Bedford, MA).

Plant Material Sampling, Botanical Characterization, and Preparation. Samples were taken in various regions of France (dairy regions) during spring 2007 (between May 10 and 31) on lowland permanent pastures in Haute-Normandie (HN), upland permanent pastures in Isère (IS), and Monts du Vivarais (MV). The pastures, eight per region, were chosen to represent a wide diversity of environmental conditions (topography, soil, climate) and farming practices (intensity of utilization and fertilization) (**Table 1**).

The botanical composition of the selected parcels was determined by two linear surveys, each located in an area of homogeneous vegetation, on a 25 m transect using a point quadrat method (*16*). The plants growing in 20 cm^2 around a vertical stick were identified and named using *Flora Europea* (*17*). This determination was recorded for each meter, thus at 50 points along the two transects. The numbers of plant species and botanical families and the Shannon index (*18*) were also calculated. Pasture samples (one handful) were collected in each 20 cm^2 area at about 3 cm above the soil because a previous observation had shown that for this cutting height the fraction collected corresponded to the potential intake of the cows at high stocking rate (*7*, *12*). Each handful was separated into two botanically representative average samples (two half-handfuls). Plants in the first set of 50 half-handfuls were pooled and sorted by species. The 50 half-handfuls of the second set were pooled to have a representative sample of the parcel for analytical purposes. All samples were frozen the same day and stored at −20 °C. After freeze-drying, the first set was then used to estimate the relative dry biomass of each individual species and stored at −20 °C. These biomass data were analyzed to determine the phytosociological membership of the pasture studied (*19*, *20*). After freeze-drying, the second set was ground with a cutter mill, sieved to pass through a 0.5 mm mesh sieve, and stored at −20 °C until phenolic compound analysis.

Colorimetric Assay of Total Phenolics. The total soluble phenolic content of the pastures was determined by a modification of the method using the Folin−Ciocalteu reagent developed for quantification in plant-derived products by Georgé et al. (*21*). For the assay, DM was determined on all samples of studied pastures at 103 °C for 24 h. Using 300 mg of freeze-dried and ground plant material, a first extraction was made with 7 mL of acetone/water (7:3, v/v) for 15 min, and then 7 and 6 mL of acetone/water (7:3, v/v) were used for re-extraction of the pellet. Acetone of pooled extracts was evaporated under nitrogen, and the residual volume of water was adjusted precisely to 6.000 g. Raw extracts (RE) were diluted 4-fold with water. Each diluted RE (2 mL) was settled on an Oasis HLB cartridge (Waters Corp., Milford, MA) (*21*). Interfering water-soluble components were recovered with 2 × 2 mL of water. The recovered volume of the washing extract (WE) was carefully measured for each sample.

Appropriate dilutions of RE and WE were assayed for phenolic compounds as follows: 100 μL of Folin−Ciocalteu's reagent/water (1:1, v/v) was added to 500 μL of each extract. The mixture was incubated for 2 min at room temperature, and then 400 μL of sodium carbonate (75 g/L) was added. The mixture was incubated for 30 min at 30 °C before deposit into a 96-well plate. The specific absorbance at 760 nm was then measured with an Infinite M-200 spectrophotometer plate-reader (Tecan France SAS, Lyon, France) controlled by Magellan software version 6.5. Quantification was carried out using a standard curve with increasing concentrations of gallic acid. Linearity was obtained between 10 and 70 mg/L corresponding to absorbance values between 0.2 and 2.8. Total phenolic contents of samples, determined by subtracting gallic acid equivalent in WE from that of RE, were expressed as milligrams of gallic acid equivalents per gram of product DM.

Characterization and Quantification of Soluble Phenolic Compounds in Permanent Pastures by HPLC-PDA-ESI-QToF. For the assay, DM was determined on all samples of studied pastures at 103 °C for 24 h. Using 200 mg of freeze-dried and ground plant material, three successive extractions were made with 8 mL of ethanol/water (4:1, v/v) for 20 min at 90 °C. The supernatants were collected and pooled, and the final volume was adjusted to 25 mL. Part of this volume (5 mL) was evaporated under nitrogen at 60 °C, and the dry residue was dissolved with 1.5 mL of ethanol/water (4:1, v/v) and then filtered through a 0.2 μm Acrodisc syringe filter with GHP membrane (Pall Corp.). Characterization of the phenolic compounds in the extracts was based on accurate mass measurement using a quadrupole/time-of-flight (QToF) mass spectrometer, in combination with spectroscopic analysis using a PDA detector. The chromatographic system was a Waters Alliance 2695 HPLC (Waters Corp.), equipped with a binary solvent delivery system and an autosampler. Separation was performed on a 125 mm × 2 mm i.d., 5 μm, Supersphere column 60 RP-8 (Merck KGaA, Darmstadt, Germany). The mobile phase consisted of (A) 0.01% formic acid in purified water and (B) acetonitrile/water (7:3, v/v) containing 0.01% formic acid. The linear gradient elution was optimized as follows: 0−2% B (0−0.5 min), 2−12% B (0.5−4 min), 12−20% B (4−29 min), 20−26% B (29−34 min), 26−70% B (34−49 min), 70−70% B (49−50 min), and the re-equilibration time of gradient elution was 10 min. The flow rate was 0.3 mL/min and the

injection volume 20 μL. The column and autosampler were maintained at 35 and 10 °C, respectively. Eluted compounds were first detected online at 210−600 nm using a Waters 2996 PDA, before entering a QToF micromass spectrometer (Waters Corp.) equipped with an electrospray ionization (ESI) source and a separate lock spray. Before each series of analyses, the mass spectrometer was calibrated using a solution of 0.1% orthophosphoric acid in acetonitrile/water (1:1, v/v). During sample analysis, the capillary voltage was set at 3 kV and the cone voltage at 30 V. The source temperature was set at 120 °C, with cone gas flow at 50 L/h, desolvation temperature at 250 °C, and nebulization gas flow at 400 L/h. After several preliminary tests on standard molecules representing each group of phenolic compounds, negative ion mode was chosen because ionization of these molecules was found to be better overall this way, in particular for phenolic acids. Data were acquired in continuum full scan (m/z 100−1000) mode, using a scan time of 0.9 s and an interscan delay of 0.1 s. Leucine enkephalin (Sigma) at a concentration of 0.25 ng/μL in acetonitrile/water (1:1, v/v) with 0.1% formic acid was used as a lock mass, with an infusion flow of 10 μL/min via a lock spray interface. All of the operations, acquisition, and analysis of data were made using Masslynx software version 4.0 or Millenium software version 4.0 for mass and PDA data, respectively.

The HPLC-MS continuum data were transformed to centroid data using the "accurate mass calculator" tool provided by Masslynx 4.0 software. All of the data were then processed using MetAlign software (*22*−*24*) (Plant Research International, Wageningen, The Netherlands) to yield a data matrix containing retention times, accurate masses, and normalized peak intensities. The compounds separated in the chromatographic system were identified on the basis of their absorbance spectrum, the exact mass of the precursor ion, in-source fragments, and comparison to standards.

An estimation of total phenolic compound content was established as follows. For the identified compounds, quantification was performed by comparison to standard compounds, using either the peak area of the extracted chromatogram at m/z $[M - H]^-$ when MS data were available or else the peak area at 275 nm. Unidentified peaks were first classified into different families of phenolic compounds according to their UV spectrum (*25*) and then quantified at 275 nm by comparison to standard molecules representing each group: gallic, syringic, or protocatechuic acids (for hydroxybenzoic acids), chlorogenic acid (hydroxycinnamic acids), catechin or epigallocatechin gallate (flavanols), rutin (flavonols), apigenin or luteolin (flavones), hesperidin (flavanones), genistein or daidzein (isoflavones), and coumestrol (coumestans).

Statistical Analysis. Statistica software (version 7.1, StatSoft, Inc., Tulsa, OK) was used for data analyses. Botanical and polyphenolic compositions and total phenolic contents were analyzed by one-way analysis of variance (Kruskal−Wallis test) followed by a post hoc test (*26*). $P < 0.05$ was accepted as statistically significant. The quantitative relationships between phenolic compounds and/or botanical families and plants were characterized by Pearson correlation coefficients. Finally, the description of the relationships between the botanical and polyphenolic compositions of the pastures was established by principal component analysis (PCA). All variables were active. These variables, both plant specific dry biomasses and phenolic compound concentrations, were chosen to discriminate the pastures according to the factor "region".

RESULTS

Botanical Composition of Permanent Pastures. A total of 155 different plant species was identified in the 24 pastures studied. The total number of observed species in lowland pastures (HN) was about 2.5-fold lower than in upland pastures (IS and MV) (40 vs 108 and 97, respectively). The mean numbers of plant species and botanical families per parcel were >2-fold higher in IS and MV than in HN (**Table 2**). On the basis of the Shannon index, HN pastures were significantly less diversified than IS and MV pastures.

The identified plant species represented on average from 90.2 to 95.1% of the total dry biomass collected in samples, depending on the region in question, with Poaceae as dominant family (**Table 3**). With respect to the most frequently occurring species of this family, "stem elongation" was established as the mature stage of pastures.

Table 2. Diversity Indicators in the Permanent Pastures of Three Regions[a]

	HN	IS	MV	SEM	*P*
no. of identified plant species	16 b	34 a	35 a	2.6	***
no. of accounted botanical families	5 b	13 a	12 a	1.0	***
Shannon index	2.9 b	3.6 a	3.7 a	0.17	**

[a] Values are means of eight pastures per region. Mean values within a row with different lower case letters are significantly different at $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

The proportion of monocotyledons was higher in HN pastures than in those of IS, with the value in MV pastures being intermediate ($P < 0.05$; **Table 3**). Whatever the region considered, *Lolium perenne* was the main Poaceae observed (on average, 18.9% of the total biomass). Several Poaceae had a significantly higher percentage in lowland pastures than in upland pastures. This was especially true for *Poa trivializ*, which represented on average 18.1% of the total biomass in HN pastures and only 1.1% in those of upland pastures ($P < 0.001$). A few other Poaceae such as *Phleum pratense* subsp. *pretense*, *Agrostis stolonifera*, and *Elymus repens* were present in HN pastures but absent or rarely identified in the two other regions. Conversely, several Poaceae had a significantly higher percentage in upland pastures than in lowland pastures. Indeed, *Anthoxanthum odoratum* was only identified in some upland pastures but never in lowland pastures, *Bromus erectus* only present in IS pastures, and *Bromus hordeaceus* subsp. *hordeaceus* was mainly found in MV pastures. Two other plant species, *Poa pratensis* and *Trisetum flavescens*, were present in upland pastures but absent or rarely identified in HN. For these two plants, the highest average percentage was noted either in IS (*P. pratensis*) or in MV (*T. flavescens*). Finally, other differences in the proportion of monocotyledon plants in pastures of the three regions were not significant, although *Poa augustofolia*, *Festuca nigrescens*, *Festuca arundinacea*, *Vulpia bromoides*, and *Arrhenatherum elatius* were observed only in some upland pastures and never in HN pastures.

The proportion of dicotyledons was higher in upland than in lowland pastures ($P < 0.01$). Whatever the considered region, Fabaceae and Asteraceae were the two main dicotyledon families observed (on average, 8.8 and 5.5% of the total biomass, respectively). Ranunculaceae were more abundant in HN than in MV, with the amount in IS pastures being intermediate ($P < 0.05$). Conversely, Fabaceae were more abundant in MV than in HN, the value being intermediate in IS ($P < 0.05$). Plantaginaceae and Rosaceae were only found in upland pastures ($P < 0.01$). Other differences in the proportion of dicotyledon families in pastures of the three regions were not significant, although some dominant families such as Lamiaceae, and also Geraniaceae and Rubiaceae (each of these two families representing on average 0.5% of the total biomass in upland pastures), were observed only in some upland pastures and never in HN pastures. Considering plant species, *Ranunculus acris* was more abundant in HN than in MV or IS pastures. Conversely, several dicotyledon species were only or mainly present in upland pastures (*Achillea millefolium*, *Plantago lanceolata*, *Sanguisorba minor* subsp. *minor*, *Trifolium pretense*, *Vicia sativa* subsp. *sativa*; $P < 0.05$). Finally, other differences in the proportion of dicotyledon plant species were not significant, although *Rhinanthus alectorolophus*, *Crepis setosa*, *Trifolium dubium*, and *Trifolium striatum* were observed only in some upland pastures and never in HN pastures.

Phytosociological Analysis of Vegetation Observed. When the 24 pastures were studied, four vegetation communities were described according to the method of Braun-Blanquet

Table 3. Biomass of the Identified Major Plant Species in the Permanent Pastures of the Three Regions[a]

plant species (family)	% of the total dry biomass (% of pasture sample DM) HN	IS	MV	SEM	*P*
		Monocotyledons			
Agrostis capillaris (Poaceae)	9.0	2.6	9.3	2.76	ns
Agrostis stolonifera (Poaceae)	7.7 a	0.0 b	0.3 ab	2.12	**
Anthoxanthum odoratum (Poaceae)	0.0	0.9	1.0	0.43	*
Arrhenatherum elatius (Poaceae)	0.0	1.6	<0.1	0.52	ns
Bromus erectus (Poaceae)	0.0 b	10.0 a	0.0 b	2.87	**
Bromus hordeaceus subsp. *hordeaceus* (Poaceae)	0.2 b	0.2 b	5.4 a	1.46	*
Cynosurus cristatus (Poaceae)	<0.1	0.3	2.4	1.03	ns
Dactylis glomerata (Poaceae)	4.3	6.2	5.6	1.96	ns
Elymus repens (Poaceae)	2.2	<0.1	0.0	0.94	*
Festuca arundinacea (Poaceae)	0.1	2.7	0.0	0.80	ns
Festuca nigrescens (Poaceae)	0.0	0.0	2.3	0.87	ns
Festuca rubra subsp. *rubra* (Poaceae)	0.1	3.7	4.0	1.71	ns
Holcus lanatus (Poaceae)	8.6	0.5	2.2	3.04	ns
Lolium perenne (Poaceae)	19.1	18.3	19.2	5.31	ns
Phleum pratense subsp. *pratense* (Poaceae)	5.8 a	0.0 b	0.0 b	1.12	**
Poa angustifolia (Poaceae)	0.0	0.4	3.5	1.94	ns
Poa pratensis (Poaceae)	0.5 b	4.6 a	2.4 ab	1.27	*
Poa trivialis (Poaceae)	18.1 a	1.1 b	1.0 b	2.12	***
Trisetum flavescens (Poaceae)	0.0 b	2.0 ab	2.1 a	1.04	*
Vulpia bromoides (Poaceae)	0.0	<0.1	2.1	1.06	ns
total mentioned monocotyledon species	**75.8 a**	**55.5 b**	**63.1 ab**	**4.44**	*
Poaceae	76.4 a	58.3 b	66.0 ab	4.34	*
total identified monocotyledon species	**76.4 a**	**58.3 b**	**67.2 ab**	**4.30**	*
		Dicotyledons			
Achillea millefolium (Asteraceae)	0.2 b	0.6 ab	1.9 a	0.51	*
Crepis setosa (Asteraceae)	0.0	2.4	0.0	1.39	ns
Plantago lanceolata (Plantaginaceae)	0.0 b	4.5 a	3.7 a	2.14	**
Ranunculus acris (Ranunculaceae)	3.9 a	0.1 b	<0.1 b	0.95	*
Rhinanthus alectorolophus (Scrophulariaceae)	0.0	2.9	0.0	1.69	ns
Sanguisorba minor subsp. *minor* (Rosaceae)	0.0	1.8	<0.1	0.61	*
Taraxacum officinale (Asteraceae)	2.5	2.4	3.1	1.50	ns
Trifolium dubium (Fabaceae)	0.0	0.3	2.0	0.63	ns
Trifolium pratense (Fabaceae)	0.3 b	2.6 a	1.1 ab	0.61	*
Trifolium repens (Fabaceae)	4.7	4.2	4.8	2.20	ns
Trifolium striatum (Fabaceae)	0.0	0.3	1.7	0.92	ns
Vicia sativa subsp. *nigra* (Fabaceae)	0.0	1.1	0.6	0.37	*
total mentioned dicotyledon species	**11.6**	**23.2**	**19.0**	**3.97**	**ns**
Asteraceae	3.6	6.4	6.5	2.09	ns
Caryophyllaceae	0.5	0.5	1.4	0.42	ns
Fabaceae	4.9 b	9.9 ab	11.4 a	2.14	*
Lamiaceae	0.0	1.4	0.2	0.48	ns
Plantaginaceae	0.0 b	4.7 a	3.8 a	2.11	***
Ranunculaceae	4.2 a	1.1 ab	0.4 b	1.05	*
Rosaceae	0.0 b	3.0 a	0.6 ab	0.62	**
Scrophulariaceae	0.3	3.1	0.4	1.72	ns
total mentioned dicotyledon families	**13.7 b**	**30.2 a**	**24.9 ab**	**3.99**	*
total identified dicotyledon species	**13.7 b**	**33.7 a**	**27.8 a**	**4.12**	**
total identified species	**90.2**	**92.0**	**95.1**	**2.17**	**ns**
total dry biomass of pasture sample	**100**	**100**	**100**		**ns**

[a] Values are means of eight pastures per region. Mean values within a row with different lower case letters are significantly different at $P < 0.05$; *, $P < 0.05$, **, $P < 0.01$; ***, $P < 0.001$; ns, not significant. Only the identified plant species and botanical families having a mean biomass (mean of total parcels) >5‰ of total dry biomass are mentioned.

(*19*) and the phytosociological classification of Julve (*20*) (**Figure 1**).

The two first vegetation communities of the *Arrhenatheretea eliatoris* class Br-Bl. 1949 were found in seven, four, and seven pastures of HN, IS, and MV, respectively, occurring at altitudes ranging from 10 to 1000 m for the first vegetation community and in a parcel of HN at an altitude of 180 m for the second community (**Table 4**). The geological substratum mainly consisted of chalk, clay, or sandstone in HN, pebbles or glacial deposits in IS, and gneiss or volcanic basaltic rock in MV. Globally, these pastures were moderately fertilized and trampled, but some of them, in particular in HN and MV, were heavily fertilized and trampled. In May, the pastures of this class were the least sunny of the study, they were present on a colder but also drier soil than the pastures belonging to the *Festuco valesiacae*−*Brometea erecti* class Br-Bl. & Tx. 1943 *em.* Royer 1987. Within these 19 pastures, a total of 126 species were counted. Among them, 38 plant species were characteristic of the *A. elatioris* class (38 and 7 plants for the first and second vegetation communities, respectively) and 11 were characteristic of the *Cynosurion cristati* alliance (species

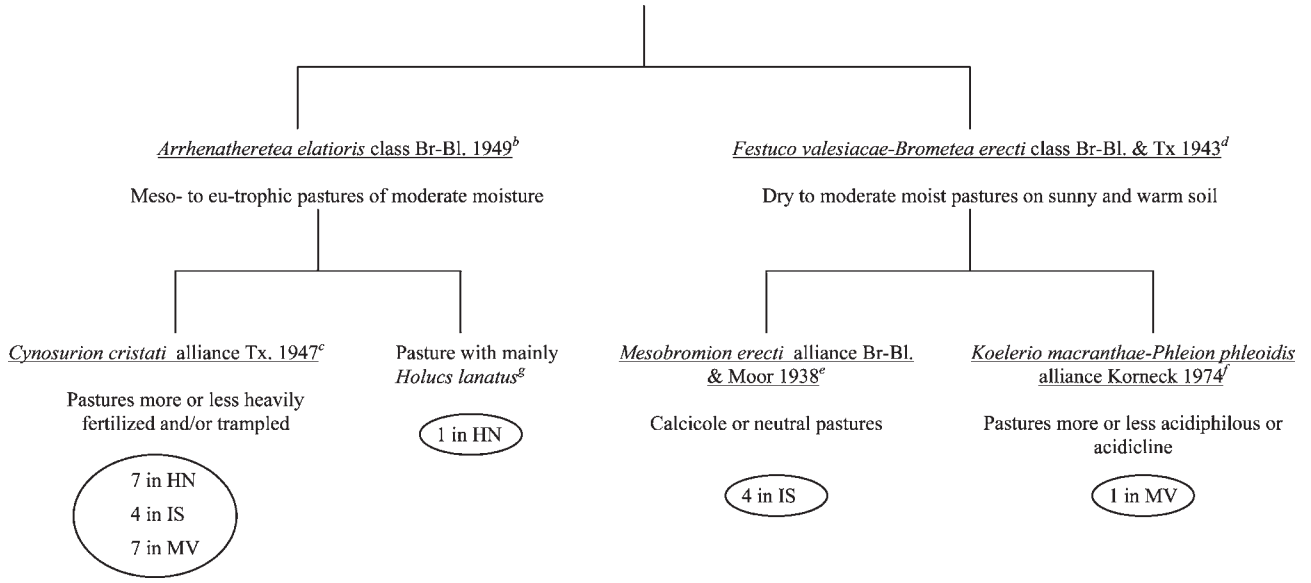

Figure 1. Phytosociological position of pastures studied. [a] The presence of some characteristic species helps us to determine the phytosociological membership of pastures. [b] Characteristic plant species of the *Arrhenatheretea elatioris* class: *Achillea millefolium* (Asteraceae); *Arrhenatherum elatius* (Poaceae); *Bromus hordeaceus* subsp. *hordeaceus* (Poaceae); *Dactylis glomerata* (Poaceae); *Holcus lanatus* (Poaceae); *Plantago lanceolata* (Plantaginaceae); *Poa pratensis* (Poaceae); *Poa trivializ* (Poaceae); *Ranunculus acris* (Ranunculaceae); *Taraxacum officinale* (Asteraceae); *Trifolium dubium* (Fabaceae); *Trifolium pratense* (Fabaceae); *Trisetum flavescens* (Poaceae); *Vicia sativa* subsp. *nigra* (Fabaceae). [c] Characteristic plant species of the *Cynosurion cristati* alliance: *Cynosurus cristatus* (Poaceae); *Festuca rubra* susbp. *rubra* (Poaceae); *Lolium perenne* (Poaceae);*Phleum pratense* subsp. *pratense* (Poaceae); *Trifolium repens* (Fabaceae). [d] Characteristic plant species of the *Festuco valesiacae-Brometea erecti* class: *Bromus erectus* (Poaceae); *Galium verum* (Rubiaceae); *Poa angustifolia* (Poaceae); *Poa bulbosa* (Poaceae); *Potentilla heptaphylla* (Rosaceae); *Salvia pratensis* (Lamiaceae); *Sanguisorba minor* subsp. *minor* (Rosaceae). [e] Characteristic plant species of the *Mesobromion erecti* alliance: *Knautia arvensis* (Dipsacaceae); *Ranunculus bulbosus* (Ranunculaceae); *Plantago media* (Plantaginaceae); *Rhinanthus alectorolophus* (Scrophulariaceae). [f] Characteristic plant species of the *Koelerio macranthae−Phleion phleoidis* alliance: *Armeria alliacea* (Plumbaginaceae); *Trifolium incarnatum* subsp. *molinierii* (Fabaceae). [g] *Holcus lanatus* was the main observed species in this pasture with 47.3% of the total dry biomass identified.

Table 4. General Characteristics of the Vegetation Communities Described: Environmental Conditions, Forage Practices[a]

							fertilization	
vegetation community	altitude (m)	soil	rainfall[b] (mm/month)	sunniness[b] (h/month)	temp[b] (°C)	utilization (animal unit/ha)[c]	N mineral[d]	organic[e]
Cynosurion cristati	**421**[f] (10−1000)[g]	chalk, clay or sandstone, pebbles or glacial deposits, gneiss or basaltic rock	**113**	**172**	**15.0**	**1.2** (0.5−1.9)	**40** (0−110)	4/18
PP with *Holcus lanatus*	**180**	sandstone	**94**	**134**	**13.7**	**2.2**	**55**	1/1
Mesobromion erecti	**748** (500−910)	calcareous alluvial and/or glacial deposits	**216**	**197**	**15.0**	**0.5** (0.3−0.7)	**25** (0−50)	0/4
Koelerio macranthae−Phleion phleoidis	**470**	coarse-grained gneiss	**109**	**233**	**17.7**	**1.0**		**65** 0/1

[a] Abbreviations: temp, temperature. [b] In May. [c] At the moment of collection; value >1.5 indicates pasture intensively used, else extensively. [d] N units/ha/year; value <30 indicates low, value between 30 and 80 indicates moderate, value >80 indicates high. [e] Number of parcels per vegetation community where the organic fertilization was used. [f] Means per vegetation community (in bold). [g] Range (extreme values in parentheses).

identified in the first vegetation community). The sum of these characteristic species represented on average 77.4% of the total dry biomass identified for the first vegetation community and 83.5% for the second. Finally, the 77 other differential species (neither typical of class nor typical of alliance) were of different phytosociological origin: they were grouped in 7 classes including *Festuco valesiacae−Brometea erecti* class.

The two last vegetation communities of the *Festuco valesiacae−Brometea erecti* class were found in four pastures of IS at altitudes ranging from 500 to 910 m for the third vegetation community and in a parcel of MV at an altitude of 470 m for the fourth community. The geological substratum mainly consisted of calcareous alluvial and/or glacial deposits in IS and coarse-grained gneiss in MV. These pastures were lowly to moderately fertilized and were extensively used. Within these five pastures, a total of 97 plant species were counted. Among them, 15 plant species were characteristic of the *Festuco valesiacae−Brometea erecti* class (14 and 4 plants for the third and fourth vegetation communities, respectively), 10 were typical of the *Mesobromion erecti* alliance Br-Bl. & Moor 1938 (species identified in the third vegetation community), and 2 were typical of the *Koelerio macranthae−Phleion phleoidis* alliance Korneck 1974 (species identified in the fourth vegetation community). The sum of these characteristic species represented on average 42.0% of the total dry biomass identified for the third and 32.1% for the fourth vegetation community. Finally, the 70 other differential species were grouped in 6 classes including *Sedo-Scleranthetea* Br-Bl. 1955 *em.* Mull. 1961 (with xerophilous and acidiphilous species as

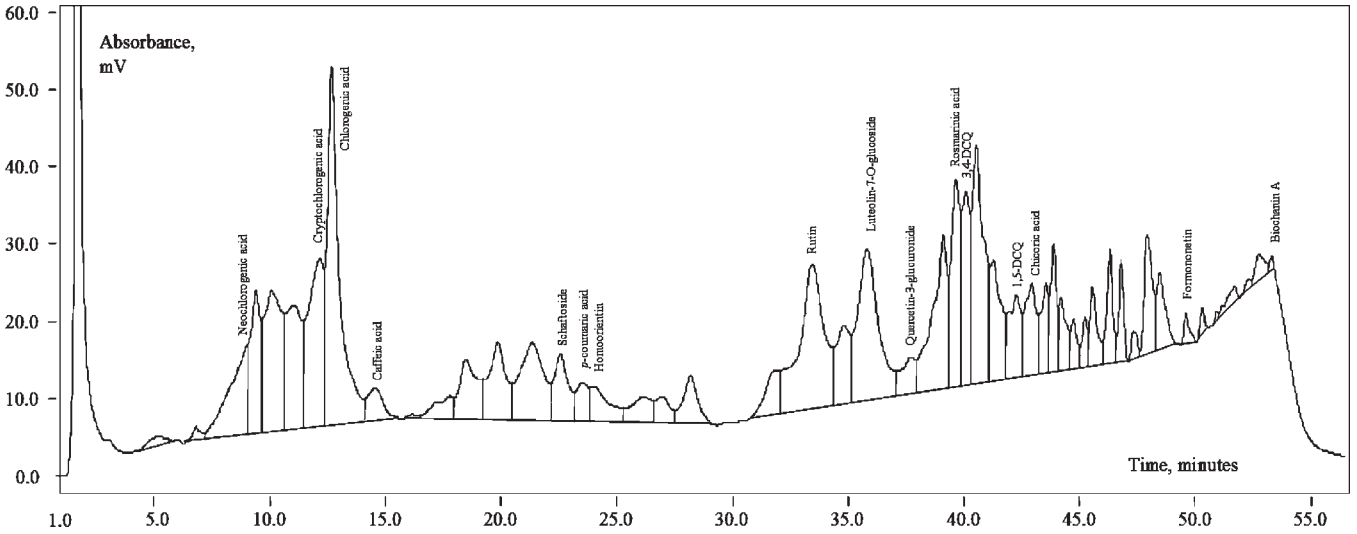

Figure 2. HPLC of phenolic compounds from permanent pasture at 275 nm.

Table 5. Phenolic Compounds Detected by HPLC-PDA-ESI-QToF[a]

peak	RT ± SD (min)	$(M - H)^-$ (*m*/*z*)	elementary formula	calcd mass	Δppm	postulated structure	IR	exptl MS fragments	absorbance max (nm)
1	8.82 ± 0.03	353.0858	$C_{16}H_{18}O_9$	353.0873	−1.5	neochlorogenic acid	a	191, 353	326
2	12.16 ± 0.17	353.0872	$C_{16}H_{18}O_9$	353.0873	−0.1	cryptochlorogenic acid	a	191, 353	326
3	12.28 ± 0.07	289.0719	$C_{15}H_{14}O_6$	289.0712	−0.7	catechin	b	289	
4	12.89 ± 0.08	353.0860	$C_{16}H_{18}O_9$	353.0873	−1.3	chlorogenic acid	a	191, 353	326
5	14.94 ± 0.09	179.0359	$C_9H_8O_4$	179.0344	1.5	caffeic acid	a	135, 179	324
6	15.64 ± 0.01	289.0693	$C_{15}H_{14}O_6$	289.0712	−1.9	epicatechin	b	289	
7	15.94 ± 0.36	197.0430	$C_9H_{10}O_5$	197.0450	−2.0	syringic acid	a	197	275
8	22.75 ± 0.12	563.1432	$C_{26}H_{28}O_{14}$	563.1401	3.1	schaftoside	a	563	271, 336
9	23.65 ± 0.13	163.0385	$C_9H_8O_3$	163.0395	−1.0	*p*-coumaric acid	a	119, 163	310
10	24.20 ± 0.10		$C_{25}H_{24}O$	515.1190		cynarin	c		327
11	24.31 ± 0.10	447.0912	$C_{21}H_{20}O_{11}$	447.0927	−1.5	homoorientin	a	447	270, 350
12	28.72 ± 0.14	449.1116	$C_{21}H_{22}O_{11}$	449.1084	3.2	eriodictyol-7-*O*-glucoside	a	287, 449	284
13	33.66 ± 0.32	463.0878	$C_{21}H_{20}O_{12}$	463.0876	0.2	myricitrin	b	463	
14	33.82 ± 0.13	609.1448	$C_{27}H_{30}O_{16}$	609.1455	−0.7	rutin	a	609	256, 354
15	35.77 ± 0.10	623.1981	$C_{29}H_{36}O_{15}$	623.1976	0.5	verbascoside	a	623	332
16	36.46 ± 0.25	447.0934	$C_{21}H_{20}O_{11}$	447.0927	0.7	luteolin-7-*O*-glucoside	a	447	257, 350
17	37.91 ± 0.12		$C_{21}H_{18}O_{13}$	477.0669		quercetin-3-glucuronide	c		257, 353
18	39.22 ± 0.12	609.1843	$C_{28}H_{34}O_{15}$	609.1819	2.4	hesperidin	a	609	285
19	39.79 ± 0.06	359.0786	$C_{18}H_{16}O_8$	359.0767	1.9	rosmarinic acid	a	359	329
20	40.24 ± 0.06	515.1219	$C_{25}H_{24}O$	515.1190	2.9	3,4-dicaffeoylquinic acid	a	515	326
21	40.71 ± 0.03	607.1676	$C_{28}H_{32}O_{15}$	607.1663	1.3	diosmin	d	607	253, 346
22	42.45 ± 0.34	515.1242	$C_{25}H_{24}O$	515.1190	5.2	1,5-dicaffeoylquinic acid	a	515	326
23	42.92 ± 0.08		$C_{22}H_{18}O_{12}$	473.0720		chicoric acid	c		327
24	43.50 ± 0.49	515.1239	$C_{25}H_{24}O$	515.1190	4.9	3,5-dicaffeoylquinic acid	a		326
25	43.67 ± 0.03	253.0520	$C_{15}H_{10}O_4$	253.0501	1.9	daidzein	a	253	250
26	45.93 ± 0.20	285.0400	$C_{15}H_{10}O_6$	285.0399	0.1	luteolin	b	285	
27	48.24 ± 0.10	269.0676	$C_{15}H_{10}O_5$	269.0450	22.6	apigenin	a	269	268, 338
28	47.60 ± 0.03	269.0459	$C_{15}H_{10}O_5$	269.0450	0.9	genistein	a	269	261
29	47.88 ± 0.02	267.0405	$C_{15}H_8O_5$	267.0293	11.2	coumestrol	a	267	245, 343
30	49.72 ± 0.12	267.0646	$C_{16}H_{12}O_4$	267.0657	−1.1	formononetin	a	267	250
31	53.46 ± 0.05	283.0613	$C_{16}H_{12}O_5$	283.0606	0.7	biochanin A	a	283	261

[a] Abbreviations: RT ± SD, mean retention time ± standard deviation; calcd, calculated; exptl, experimental; max, maxima; IR, reliability of the identification; IR = a, RT + MS fragments + absorbance maxima, in accordance with those of standards; IR = b, RT + MS fragments in accordance with those of standards; IR = c, RT + absorbance maxima in accordance with those of standards; IR = d, RT + MS fragments + absorbance maxima, in accordance with published data.

Trifolium striatum (Fabaceae) and *Arrhenatheretea elatioris* classes.

Identification of Soluble Phenolic Compounds in Plant Material. HPLC analysis revealed the complexity of the polyphenolic composition of permanent pastures (**Figure 2**), with 92 separate peaks for all pastures among which 31 compounds were identified (**Table 5**).

Some compounds were identified with a very good degree of confidence, as indicated by the identification reliability (IR) score of "a" in **Table 5**. For example, the identification of chromatographic peak 4 was based on comparison of its retention time (RT), absorbance spectrum, and in-source MS fragments with those of the chlorogenic acid (5-caffeoylquinic acid) standard. In the mass spectrum, a peak at *m*/*z* 353.0860 $[M - H]^-$, corresponding to chlorogenic acid, and its fragment at *m*/*z* 191.05 (quinic acid) were observed. The λ_{max} of this compound was 326 nm. On the other hand, for most of the compounds identified

with IR = a (chlorogenic acid, caffeic acid, syringic acid, *p*-coumaric acid, homoorientin, eriodictyol-7-*O*-glucoside, rutin, verbascoside, luteolin-7-*O*-glucoside, hesperidin, daidzein, formononetin, genistein, coumestrol, apigenin, and biochanin A), the parent ion mass corresponded to within 5 ppm of the theoretical mass of the standard, except for coumestrol and apigenin ($\Delta = 11.2$ and 22.6 ppm, respectively). The latter two compounds were found in pastures in very small amounts as we will show later, the mass error being consequently quite acceptable.

Catechin, epicatechin, myricitrin, quercetin-3-glucuronide, chicoric acid, cynarin, and luteolin were identified with a good degree of confidence. For example, peak 26 was identified as luteolin thanks to the retention time, with the precursor mass (m/z 285.0400 $[M - H]^-$) corresponding within 5 ppm to that of the standard (IR = b; **Table 5**). Peak 10 was identified as cynarin (1,3-dicaffeoylquinic acid) by comparison of retention time and absorbance spectrum to those of the standard, but no mass value was measured (no response); the IR score was "c" in **Table 5** because of the risk of coelution.

Peak 21 was identified as diosmin (luteolin-4′-methylether-7-rutinoside) but with a lower degree of confidence than observed for the previous compounds, based not on standards but on published data (IR = d; **Table 5**). The absorbance spectrum was typical of flavones, and the parent mass (m/z 607.1676) corresponded within 5 ppm to the calculated mass for that of diosmin (m/z 607.1663 $[M - H]^-$).

Composition in Soluble Phenolic Compounds and Variations between Pastures. Comparison of chromatographic profiles at 275 nm between pastures of the three regions did not underscore any statistical difference in the number of separate peaks (on average, 52). When the colorimetric data were compared to those of chromatography, a significant relationship was observed (Pearson's coefficient, 0.55, $P < 0.01$) and the total phenolic values were close (mean ratio, 1.15 ± 0.45). The variability around the mean could be explained by the great difference between the two methodological approaches, especially because the Folin−Ciocalteu assay is based on oxidoreductive properties of molecules, whereas the chromatographic quantification resulted in spectroscopic absorbance values of phenolic compounds. Other factors differing between the two approaches could also explain this variability, around the mean as the expression mode of the results, the method of extraction, especially the nature and composition of solvents. Total phenolic content evaluated by colorimetry was higher in upland pastures than in HN ($P < 0.01$; **Table 6**), whereas the total phenolic content estimated by chromatography was higher in IS than in HN pastures and intermediate in MV ($P < 0.05$).

Contrary to the total phenolic compound content, the distribution of these compounds in the various molecular classes did not vary significantly between regions. Indeed, about half of the phenolic compounds were phenolic acids, including on average 97% of hydroxycinnamic acids, whereas the other half were flavonoids, notably flavones and flavonols (58 and 37% of the total flavonoids, respectively), with the isoflavonoids representing <1% of the total phenolic compound content. Within the flavonoids, the proportion of phenolic compounds in the two main molecular families varied between regions: flavones were more present in lowland than in upland pastures (88 and 43%, respectively) and the reverse was true for flavonols (6 and 52%, respectively) ($P < 0.001$).

The identified fraction represented from about half to two-thirds of the total phenolic content depending on the region. Quantitatively the largest amounts of phenolic compounds identified were quercetin-3-glucuronide (on average, 16% of the total phenolics), homoorientin (9%), chlorogenic acid (8%) and its *neo*- and *crypto*-isomers (7 and 4%, respectively), 1,5- and 3,4-dicaffeoylquinic acids (1 and 3%, respectively), rutin (3%), rosmarinic acid (2%), chicoric acid (1%), and verbascoside (1%).

The mean contents in flavonoids, phenolic acids, and total phenolics were higher in IS than in HN pastures, with the amounts being intermediate in MV pastures ($P < 0.05$). The variations of flavonoid contents were quantitatively associated with those of flavonols (Pearson's coefficient, 0.95; $P < 0.001$) and especially of quercetin-3-glucuronide (Pearson's coefficient, 0.95; $P < 0.001$) and rutin (Pearson's coefficient, 0.62, $P < 0.01$). The variations of phenolic acid contents were positively correlated to those of chlorogenic (Pearson's coefficient, 0.87, $P < 0.001$) and rosmarinic acids (Pearson's coefficient, 0.43, $P < 0.05$). Finally, several other differences in phenolic compound (1,5- and 3,4-dicaffeoylquinic acids) concentration between regions seemed to positively influence the lower content in total phenolics of lowland pastures (Pearson's coefficient, 0.63; $P < 0.01$; Pearson's coefficient, 0.35, $P < 0.1$, respectively).

Some phenolic compounds were mainly or only present in one region. Indeed, HN pastures had higher diosmin ($P < 0.001$; **Table 6**) and hesperidin contents ($P < 0.05$) than pastures of the other two regions, even if the differences between regions were quantitatively minor. Myricitrin and coumestrol were detected in only some pastures of this region and cynarin in IS pastures. Conversely, syringic acid was absent from IS pastures, whereas epicatechin and eriodictyol-7-*O*-glucoside were absent from HN pastures and verbascoside rarely present from HN pastures ($P < 0.001$). Finally, the concentration in *p*-coumaric acid was atypical compared to other phenolic compounds, because it was significantly higher in HN and IS than in MV pastures ($P < 0.01$).

DISCUSSION

Amount of Total Soluble Phenolic Compounds in Permanent Pastures. In this study, total phenolic content evaluated by colorimetry was 1.5-fold higher in upland permanent pastures than in lowland pastures ($P < 0.01$), probably due to quantitative and qualitative changes in botanical composition. In agreement with Jeangros et al. (*10*), total phenolic content was correlated positively with the proportion of Rosaceae and negatively with that of Poaceae ($P < 0.001$). Moreover, for the first time to our knowledge, two other botanical families (Plantaginaceae and Rubiaceae), quantitatively important in upland pastures but absent in HN, were shown to be statistically linked to variations of total phenolics between regions ($P < 0.01$). Other differences in the proportion of dicotyledon families seemed to influence the lower content in phenolic compounds of lowland pastures. Indeed, some families described in the Swiss studies (*10*, *12*) as being rich in soluble phenolics, were either absent in HN pastures (Rosaceae, Geraniaceae, and Plantaginaceae) or present in low amounts (Asteraceae and Fabaceae) by comparison to upland pastures. Conversely, Ranunculaceae, mainly found in HN pastures, were reported as poor in soluble phenolics by Jeangros et al. (*10*) with values close to those of Poaceae.

In our study, the total phenolic content of 24 pastures studied was systematically lower (maximal value = 15.7 g/kg DM for an IS pasture) than the content determined by Fraisse et al. (*7*) for an upland pasture (1100 m) at a comparable period of the year and the values reported in Swiss studies (*10*, *12*, *27*) in lowland pastures (below 650 m), in mountain pastures (between 900 and 1600 m), and at subalpine level (above 1600 m). The lower contents obtained in this study could be partly due to optimization of the method using the Folin−Ciocalteu reagent to specifically determine the total phenolic content by taking the water-soluble reducing interferents into consideration (*21*). Nevertheless, it seemed difficult to make comparisons among all of these values

Table 6. HPLC Analysis of Phenolic Compounds in the Three Regions and Total Phenolics (Chromatography and Colorimetry)[a]

	g/kg pasture DM				
	HN	IS	MV	SEM	*P*
phenolic acids					
syringic acid	<0.01	0.00	<0.01	0.001	ns
other hydroxybenzoic acids	0.06 b	0.15 a	0.15 a	0.020	**
total hydroxybenzoic acids	**0.06 b**	**0.15 a**	**0.15 a**	**0.020**	**
neochlorogenic acid	0.76	0.44	0.44	0.104	ns
cryptochlorogenic acid	0.33	0.41	0.38	0.075	ns
chlorogenic acid	0.49	1.00	0.80	0.156	ns
caffeic acid	<0.01	<0.01	<0.01	0.001	ns
p-coumaric acid	0.02 a	0.01 a	<0.01 b	0.002	**
verbascoside	<0.01 b	0.13 a	0.12 a	0.041	***
rosmarinic acid	0.02	0.62	0.04	0.181	ns
chicoric acid	0.06	0.08	0.25	0.072	ns
cynarin	0.00	0.10	0.00	0.043	ns
1,5-dicaffeoylquinic acid	0.07 b	0.17 ab	0.20 a	0.037	*
3,5-dicaffeoylquinic acid	<0.01 b	0.01 ab	0.02 a	0.004	**
3,4-dicaffeoylquinic acid	0.11 b	0.34 ab	0.47 a	0.091	*
other hydroxycinnamic acids	1.28	1.92	1.69	0.238	ns
total hydroxycinnamic acids	**3.15**	**5.24**	**4.44**	**0.592**	**ns**
total phenolic acids	**3.21 b**	**5.39 a**	**4.59 ab**	**0.591**	*
flavonoids					
catechin	<0.01	0.01	<0.01	0.003	ns
epicatechin	0.00	<0.01	<0.01	0.001	ns
other flavanols	0.02	0.02	0.07	0.020	ns
total flavanols	**0.02**	**0.03**	**0.08**	**0.020**	**ns**
rutin	0.06 b	0.55 a	0.27 a	0.096	***
myricitrin	0.00	0.00	<0.01	0.004	ns
quercetin-3-glucuronide	0.07 b	3.95 a	1.97 a	0.766	**
other flavonols	0.04	0.03	0.09	0.038	ns
total flavonols	**0.17 b**	**4.53 a**	**2.34 a**	**0.828**	***
apigenin	<0.01 b	<0.01 ab	<0.01 a	0.001	*
schaftoside	<0.01	<0.01	<0.01	0.001	ns
luteolin	<0.01	0.02	0.01	0.005	ns
luteolin 7-*O*-glucoside	0.02	0.22	0.13	0.061	ns
homoorientin	0.82	0.67	0.68	0.143	ns
diosmin	0.04 a	<0.01 b	<0.01 b	0.005	***
other flavones	1.51	1.02	1.59	0.274	ns
total flavones	**2.40**	**1.95**	**2.42**	**0.365**	**ns**
eriodictyol 7-*O*-glucoside	0.00	0.01	<0.01	0.004	ns
hesperidin	0,04a	<0.01b	<0.01b	0.009	***
other flavanones	0.10	0.17	0.10	0.031	ns
total flavanones	**0.13**	**0.18**	**0.11**	**0.031**	**ns**
total flavonoids	**2.73 b**	**6.69 a**	**4.95 ab**	**1.047**	*
isoflavonoids					
daidzein	<0.01	<0.01	<0.01	0.001	ns
genistein	<0.01	<0.01	<0.01	0.003	ns
formononetin	<0.01	0.02	<0.01	0.006	ns
biochanin A	<0.01	0.02	<0.01	0.009	ns
other isoflavones	0.03	0.10	0.06	0.026	ns
total isoflavones	**0.04**	**0.15**	**0.07**	**0.040**	**ns**
coumestrol	0.00	0.00	<0.01	0.001	ns
total coumestans	**0.00**	**0.00**	**<0.01**	**0.001**	**ns**
total isoflavonoids	**0.04**	**0.15**	**0.08**	**0.040**	**ns**
unclassified compounds	0.26	0.17	0.19	0.031	ns
total identified phenolic compounds	**2.94 b**	**8.81 a**	**5.85 a**	**1.106**	**
total phenolics (chromatography)	**6.25 b**	**12.41 a**	**9.82 ab**	**1.364**	*
total phenolics (Folin−Ciocalteu)	**7.25 b**	**11.19 a**	**10.02 a**	**0.680**	**

[a] Mean values within a row with different lower case letters are significantly different at $P < 0.05$; *, $P < 0.05$, **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

because of differences in the samples collected (maturity stage and environment), in the choice of the reference molecule used for quantification (catechin used by Fraisse et al. (*7*) vs gallic acid for other authors), and in the method of extraction, especially the nature of solvents (methanol acidified with 1% of HCl for the Swiss studies vs ethanol/water (1:1, v/v) used by Fraisse et al. (*7*) or acetone/water (7:3, v/v) in the present study).

Distribution of Soluble Phenolic Compounds in the Different Molecular Families and Classes. Whatever the considered pastures, phenolic compounds were phenolic acids for about half, including almost exclusively hydroxycinnamic acids, and flavonoids for the other half, mainly flavones and flavonols. These results were in agreement with those of Fraisse et al. (*7*) for a comparable harvesting period. On the other hand, not many

isoflavonoids were present in our study, representing on average < 1% of the total phenolic content (0.09 g/kg DM). According to the literature, this class of phytoestrogens is mainly found in plant species of the Fabaceae family such as subterranean (*Trifolium subterraneum*), red (*Trifolium pratense*), and white clover (*Trifolium repens*) or alfalfa (*Medicago sativa*) (*28*, *29*). Subterranean and red clovers are the two pasture plant species having the highest content in these compounds because they may contain up to 5% (*28*) and between 1 and 2.5% (*30*) DM of estrogenic isoflavonoids, respectively. Conversely, for the two other species of Fabaceae, the value is < 0.06% (*30*). In our study, subterranean clover and alfalfa were not present in the pastures. The mean content in isoflavonoids was correlated positively with the proportion of Fabaceae ($P < 0.01$), in particular red clover ($P < 0.001$). Moreover, in agreement with the literature (*30*, *31*), the major identified isoflavonoids were formononetin and biochanin A.

Relationships between the Botanical and Polyphenolic Compositions of Permanent Pastures. The PCA revealed three groups within the 24 pastures studied linked to polyphenolic and botanic profiles (**Figure 3A**). The first two axes represented 51.8% of total variability. The first axis PC1 (34.2%) made a distinction between the more diversified pastures of IS on the one hand and the less diversified pastures of HN on the other. The second axis PC2 (17.6%), less explanatory, made it possible only to discriminate upland pastures according to the occurrences of specific dicotyledon species and phenolic compounds.

The first group (on the upper left part of **Figure 3A**) included the HN permanent pastures belonging to the *Arrhenatheretea elatioris* class. These pastures were the richest in diosmin and hesperidin and, conversely, the poorest in chlorogenic acid, 1,5-dicaffeoylquinic acid, and rutin (**Figure 3B**). The diosmin content was correlated positively with the dry biomass of *Poa trivialis* (Poaceae) and the concentration in hesperidin with the percentage of *Ranunculus acris* (Ranunculaceae). On the contrary, the low level of floristic diversity of these pastures seemed to have a negative influence on the contents in rutin, chlorogenic acid, and 1,5-dicaffeoylquinic acid.

The second group of pastures (upper right part of **Figure 3A**) included four IS permanent pastures of the *Mesobromion erecti* alliance. These pastures were the richest in total flavonols, quercetin-3-glucuronide, and rosmarinic acid (**Figure 3B**). The concentration in quercetin-3-glucuronide, just like that in flavonols, increased with the higher biomass of Rubiaceae. The content in rosmarinic acid correlated positively with the dry biomass of *Salvia pratensis* (Lamiaceae) (this phenolic compound has been already reported in the genus *Salvia* in refs *32* and *33*) and also the dry biomass of *Bromus erectus* (Poaceae).

The last group included the remaining upland pastures (in the center part of **Figure 3A**), that is, IS and MV pastures of the *Cynosurion cristati* alliance and the MV pasture belonging to the *Koelerio macranthae*−*Pheleion phleoidis* alliance. These pastures were the richest in 3,5- and 3,4-dicaffeoylquinic acids and verbascoside and, conversely, the poorest in *p*-coumaric acid (**Figure 3B**). In agreement with the literature, the percentage of *Achillea millefolium* (Asteraceae) seemed to influence positively the contents of 3,5-dicaffeoylquinic acid (*7*, *34*) and 3,4-dicaffeoylquinic acid (*34*), and the dry biomass of *Plantago lanceolata* correlated positively with the content in verbascoside (Pearson's coefficient = 0.74, $P < 0.001$) in agreement with the literature (*35*). Conversely, and for the first time to our knowledge, the concentration in *p*-coumaric acid correlated negatively with the dry biomass of this plant species.

In conclusion, this work enabled several new phenolic compounds of permanent pastures to be characterized, leading to the identification of up to two-thirds of the total phenolics in plants. The systematic distribution of phenolic compounds in one molecular family and class made it possible to show that (a) in permanent pastures, phenolic compounds are mainly composed of hydroxycinnamic acids, flavones, and flavonols; (b) the higher content in phenolic compounds in upland than in lowland pastures did not result in a modification of the overall composition and distribution between phenolic acids and flavonoids but was due to an increase in the concentration in both families; however, within the flavonoids, there were more flavones in lowland pastures, whereas upland pastures were richer in flavonols; and (c) differences in phenolic compounds between pastures

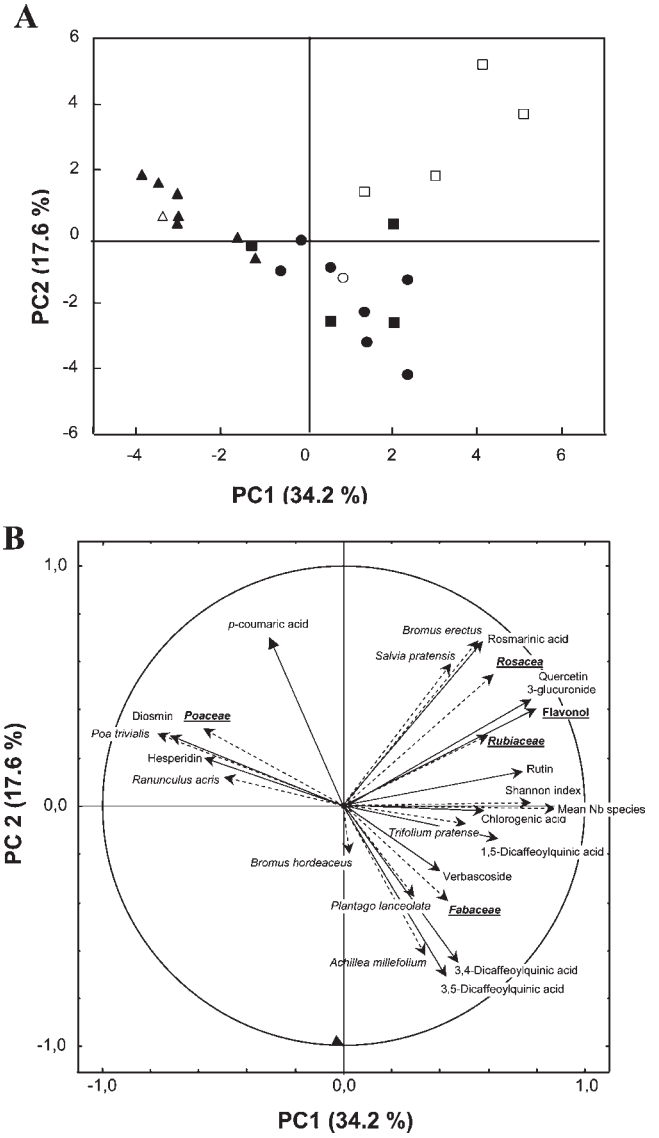

Figure 3. Statistical relationships performed by principal component analysis using botanical descriptors and the content in phenolic compounds of the vegetation: (**A**) positioning of individuals (pastures) on the principal components (PC) 1 × 2 plot; (**B**) positioning of the botanical (in italic) and chemical variables according to axes 1 × 2. All variables are active. The families either botanical or molecular are in bold and underlined. Arrows concerning botanical variables are dotted, whereas those concerning the phenolic compounds are solid. Symbols: (▲) HN pastures of the *Cynosurion cristati* alliance; (△) HN pasture with mainly *Holcus lanatus*; (■) IS pastures of the *C. cristati* alliance; (□) IS pastures of the *Mesobromion erecti* alliance; (●) MV pastures of the *C.* alliance; (○) MV pasture of the *Koelerio macranthae*−*Phleion phleoidis* alliance.

from different regions were linked to variations in the botanical composition at the level of various plant species, especially certain dicotyledons. The principal component analysis even allowed a distinction to be drawn between IS permanent pastures of the *Mesobromion erecti* alliance and other pastures of the same region because its botanical composition is highly specific and resulted in the presence of characteristic phenolic compounds. However, other vegetation communities of the same region (in HN and in MV) were not distinguishable according to their polyphenolic composition. Thus, the precise determination of the botanical composition of the pastures seems to be more relevant than a botanically less detailed phytosociological classification.

ACKNOWLEDGMENT

We kindly thank the staff of Danone, who coordinated the relationship between the dairy company and producers and thus the farmers involved in this study. We also thank Dr. M. Frain (Association pour le Développement de l'Enseignement et de la Recherche en Auvergne) for his expertise, helpful suggestions, and comments on phytosociological aspects, and Isabelle Kerveillant, Josiane Portelli, Bernard Lyan, and Jean-François Martin for their excellent technical assistance.

LITERATURE CITED

(1) Fischer, G.; Prieler, S.; van Velthuizen, H.; Berndes, G.; Faaij, A.; Londo, M.; de Wit, M. Biofuel production potentials in Europe: sustainable use of cultivated land and pastures, part II: land use scenarios. *Biomass Bioenerg*. **2010**, *34*, 173–187.

(2) Farruggia, A.; Martin, B.; Baumont, R.; Prache, S.; Doreau, M.; Hoste, H.; Durand, D. Quels intérêts de la diversité floristique des prairies permanentes pour les ruminants et les produits animaux? *INRA Prod. Anim*. **2008**, *21*, 181–199.

(3) Lucas, A.; Coulon, J. B.; Grolier, P.; Martin, B.; Rock, E. Nutritional quality of dairy products and human health. In *Indicators of Milk and Beef Quality*; Hocquette, J. F., Gigli, S., Eds.; Academic Publishers: Wageningen, The Netherlands, 2005; pp 163−178.

(4) Martin, B.; Hurtaud, C.; Graulet, B.; Ferlay, A.; Chilliard, Y.; Coulon, J. B. Herbe et qualités nutritionnelles et sensorielles des produits laitiers. *Fourrages* **2009**, *199*, 291–310.

(5) Scalbert, A.; Manach, C.; Morand, C.; Remesy, C.; Jimenez, L. Dietary polyphenols and the prevention of diseases. *Crit. Rev. Food Sci. Nutr*. **2005**, *45*, 287–306.

(6) Bravo, L. Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutr. Rev*. **1998**, *56*, 317–333.

(7) Fraisse, D.; Carnat, A.; Viala, D.; Pradel, P.; Besle, J. M.; Coulon, J. B.; Felgines, C.; Lamaison, J. L. Polyphenolic composition of a permanent pasture: variations related to the period of harvesting. *J. Sci. Food Agric*. **2007**, *87*, 2427–2435.

(8) Setchell, K. D. R.; Brown, N. M.; Lydeking-Olsen, E. The clinical importance of the metabolite equol − a clue to the effectiveness of soy and its isoflavones. *J. Nutr*. **2002**, *132*, 3577–3584.

(9) King, R. A.; Mano, M. M.; Head, R. J. Assessment of isoflavonoid concentrations in Australian bovine milk samples. *J. Dairy Res*. **1998**, *65*, 479–489.

(10) Jeangros, B.; Scehovic, J.; Troxler, J.; Bachmann, H. J.; Bosset, J. O. Comparaison de caractéristiques botaniques et chimiques d'herbages pâturés en plaine et en montagne. *Fourrages* **1999**, *159*, 277–292.

(11) Jeangros, B.; Scehovic, J.; Schubiger, F. X.; Lehmann, J.; Daccord, R.; Arrigo, Y. Valeur nutritive des plantes des prairies. 4. Composés phénoliques. *Rev. Suisse Agric*. **2001**, *33*, 147–151.

(12) Lebreton, P.; Jeangros, B.; Gallet, C.; Scehovic, J. Sur l'organisation phytochimique de formations prairiales permanentes. *Can. J. Bot*. **2001**, *79*, 510–519.

(13) Scehovic, J. Effet de quelques plantes de prairies permanentes sur la qualité des associations végétales. *Rev. Suisse Agric*. **2000**, *32*, 195–200.

(14) Duband, F.; Carnat, A. P.; Carnat, A.; Petitjean-Freytet, C.; Clair, G.; Lamaison, J. L. [Aromatic and polyphenolic composition of infused peppermint, *Mentha* x *piperita* L.]. *Ann. Pharm. Fr*. **1992**, *50*, 146–55.

(15) Carnat, A.; Heitz, A.; Fraisse, D.; Carnat, A. P.; Lamaison, J. L. Major dicaffeoylquinic acids from *Artemisia vulgaris*. *Fitoterapia* **2000**, *71*, 587–9.

(16) Daget, P.; Poissonet, J. *Analyse Phytologique des Prairies, Applications Agronomiques*; CNRS-CEPE: Montpellier, France, 1969; Vol. 48, p 67.

(17) Tutin, T. G.; Heywood, V. H.; Burges, N. A.; Moore, D. M.; Valentine, D. H.; Walters, S. M. et al. *Flora Europaea*; Cambridge University Press: Cambridge, U.K., 1964−1993; 6 vols.

(18) Shannon, C. E.; Weaver, W. *The Mathematical Theory of Communication*; University of Illinois Press: Urbana, IL, 1949; p 117.

(19) Braun-Blanquet, J. *Plant Sociology, The Study of Plant Communities*; McGraw-Hill: New York, 1932; p 439.

(20) Julve, P. *Synopsis Phytosociologique de la France* (*Communautés de Plantes Vasculaires*); Lejeunia, revue de Botanique; Université de Liège: Liège, Belgium, 1993; Vol. 140, p 160.

(21) Georgé, S.; Brat, P.; Alter, P.; Amiot, M. J. Rapid determination of polyphenols and vitamin C in plant-derived products. *J. Agric. Food Chem*. **2005**, *53*, 1370–1373.

(22) America, A. H.; Cordewener, J. H.; van Geffen, M. H.; Lommen, A.; Vissers, J. P.; Bino, R. J.; Hall, R. D. Alignment and statistical difference analysis of complex peptide data sets generated by multidimensional LC-MS. *Proteomics* **2006**, *6*, 641–53.

(23) De Vos, R. C.; Moco, S.; Lommen, A.; Keurentjes, J. J.; Bino, R. J.; Hall, R. D. Untargeted large-scale plant metabolomics using liquid chromatography coupled to mass spectrometry. *Nat. Protoc*. **2007**, *2*, 778–91.

(24) Tikunov, Y.; Lommen, A.; de Vos, C. H.; Verhoeven, H. A.; Bino, R. J.; Hall, R. D.; Bovy, A. G. A novel approach for nontargeted data analysis for metabolomics. Large-scale profiling of tomato fruit volatiles. *Plant Physiol*. **2005**, *139*, 1125–37.

(25) Markam, K. R. Ultraviolet−visible absorption spectroscopy. In *Techniques of Flavonoids Identification*; Markam, K. R., Ed.; Academic Press: New York, 1982; pp 297−311.

(26) Siegel, S.; Castellan, N. J. The case of key independant samples. In *Nonparametric Statistics for Behavioral Sciences*, 2nd ed.; McGraw-Hill Humanities: New York, 1988; pp 190−222.

(27) Scehovic, J. Tanins et autres polymères phénoliques dans les plantes de prairies: détermination de leur teneur et de leur activité biologique. *Rev. Suisse Agric*. **1990**, *22*, 179–184.

(28) Adams, N. R. Detection of the effects of phytoestrogens on sheep and cattle. *J. Anim. Sci*. **1995**, *73*, 1509–15.

(29) Andersen, C.; Nielsen, T. S.; Purup, S.; Kristensen, T.; Eriksen, J.; Soegaard, K.; Sorensen, J.; Frette, X. C. Phyto-oestrogens in herbage and milk from cows grazing white clover, red clover, lucerne or chicory-rich pastures. *Animal* **2009**, *3*, 1189–1195.

(30) Saloniemi, H.; Wahala, K.; Nykanenkurki, P.; Kallela, K.; Saastamoinen, I. Phytoestrogen content and oestrogenic effect of legume fodder. *Proc. Soc. Exp. Biol. Med*. **1995**, *208*, 13–17.

(31) Sakakibara, H.; Viala, D.; Ollier, A.; Combeau, A.; Besle, J. M. ICoFF 3: Isoflavones in several clover species and in milk from goats fed clovers. In *BioFactors*; Niki, E., Terao, J., Eds.; IOS Press: Amsterdam, The Netherlands, 2004; Vol. 22, pp 237−239.

(32) Harborne, J. B. *Phytochemical Dictionary. A Handbook of Bioactive Compounds from Plants*; Taylor and Francis: London, U.K., 1993; 791 pp.

(33) Petersen, M.; Simmonds, M. S. J. Molecules of interest − rosmarinic acid. *Phytochemistry* **2003**, *62*, 121–125.

(34) Benedek, B.; Geisz, N.; Jager, W.; Thalhammer, T.; Kopp, B. Choleretic effects of yarrow (*Achillea millefolium* s.l.) in the isolated perfused rat liver. *Phytomedicine* **2006**, *13*, 702–706.

(35) Fons, F.; Gargadennec, A.; Gueiffier, A.; Roussel, J. L.; Andary, C. Effects of cinnamic acid on polyphenol production in *Plantago lanceolata*. *Phytochemistry* **1998**, *49*, 697–702.

Received for review January 5, 2010. Revised manuscript received March 26, 2010. Accepted March 30, 2010. A.R. was supported by a doctoral fellowship (CIFRE 025/2007), jointly financed by the Danone Research Company and ANRT (Association Nationale de la Recherche Technique).