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A gas chromatography-mass spectrometry-based metabolomic approach for the characterization of goat milk compared with cow milk

Paola Scano,* Antonio Murgia,* Filippo M. Pirisi,† and Pierluigi Caboni†[1]
*Department of Chemical and Geological Sciences, and
†Department of Life and Environmental Sciences, University of Cagliari, 72-09124 Cagliari, Italy

ABSTRACT

In this work, the polar metabolite pool of commercial caprine milk was studied by gas chromatography-mass spectrometry and multivariate statistical data analysis. Experimental data were compared with those of cow milk and the discriminant analysis correctly classified milk. By the same means, differences due to heat treatments (UHT or pasteurization) on milk samples were also investigated. Results of the 2 discriminant analyses were combined, with the aim of finding the discriminant metabolites unique for each class and shared by 2 classes. Valine and glycine were specific to goat milk, talose and malic acid to cow milk, and hydroxyglutaric acid to pasteurized samples. Glucose and fructose were shared by cow milk and UHT-treated samples, whereas ribose was shared by pasteurized and goat milk. Other discriminant variables were not attributed to specific metabolites. Furthermore, with the aim to reduce food fraud, the issue of adulteration of caprine milk by addition of cheaper bovine milk has been also addressed. To this goal, mixtures of goat and cow milk were prepared by adding the latter in a range from 0 to 100% (vol/vol) and studied by multivariate regression analysis. The error in the level of cow milk detectable was approximately 5%. These overall results demonstrated that, through the combined approach of gas chromatography-mass spectrometry and multivariate statistical data analysis, we were able to discriminate between milk typologies on the basis of their polar metabolite profiles and to propose a new analytical method to easily discover food fraud and to protect goat milk uniqueness. The use of appropriate visualization tools improved the interpretation of multivariate model results.

Key words: goat milk, gas chromatography-mass spectrometry, metabolomics, heat treatment, food fraud

INTRODUCTION

Goat (*Capra hircus*) milk and related dairy products have nowadays gained a valuable industry niche (Dubeuf et al., 2004). Many commercial parameters indicate that the diffusion of goat dairy products is increasing as whole milk, fermented milk derivatives, dried or evaporated milk, and for the production of cheese. The nutritional and health benefits of goat milk are of the utmost relevance for people affected by food allergies, with bovine milk proteins the dominant food cause. Although controversial, superior digestibility of goat milk compared with cow milk, attributed to the higher content of the α_{s2}-CN variant rather than α_{s1}-CN, lower naturally homogenized fat globule size, and the higher proportion of medium-chain triacylglycerols has been commonly accepted. Despite the huge number of reports on its nutraceutical properties (Silanikove et al., 2010; Ceballos et al., 2009), the use of goat milk for nutraceutical needs still deserves in-depth discussion and documentation (Haenlein, 2004). Milk is a very complex mixture of several components in different physical states. Milk composition is influenced by a range of different factors (e.g., diet, genetics, number and stage of lactation, seasonal variation, SCC, and milk processing; Goetsch et al., 2011). These factors may have remarkable quantitative effects on milk nutrients as well as on the physical and technological properties of milk (e.g., coagulation properties, heat stability, and fermentation quality of the milk; Dubeuf et al., 2004). Whereas lipids and lactose are the 2 major caloric nutrients, milk also contains a wide variety of bioactive compounds, including immunoglobulins and other immune proteins, peptides, nucleotides, oligosaccharides, and metabolites (Raynal-Ljutovac et al., 2008; Sundekilde et al., 2013). Sugars, free amino acids, organic acids, and other low-molecular-weight compounds compose the metabolite pool of milk. The different origin and sources of these compounds contribute to the variability of milk metabolite profiles. Milk metabolites often reflect metabolic activity in the mammary gland or metabolism in the whole organism, or both; they may also originate from enzymatic reactions or from microorganisms present in

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[1]Corresponding author: caboni@unica.it

raw milk, or both (Sundekilde et al., 2013). Moreover, before entering the market, milks undergo different heat treatments that determine their commercial value and their quality; these treatments can modify the overall metabolite composition of milk. It was found that levels of monosaccharides in milk change because of thermal processing (Mendoza et al., 2005) and during storage (Troyano et al., 1996). The characterization of the metabolite profile in a biological matrix is well performed by metabolomics; this science is based on the use of analytical methods, such as GC-MS (Marincola et al., 2012), nuclear magnetic resonance (Locci et al., 2011), and direct analysis in real time-mass spectrometry (Hrbek et al., 2014), coupled with multivariate statistical data analysis (**MVA**). Metabolomic studies have been extensively applied in the areas of nutrition sciences and food matrices, such as milk (Chen et al., 2004; Boudonck et al., 2009; Klein et al., 2010; Marincola et al., 2012; Harzia et al., 2013; Sundekilde et al., 2013; Hrbek et al., 2014).

The risk linked to food fraud is increasing due to the global and composite nature of food supply chains. With the aim to reduce this risk and in view of detecting economically driven adulterations, in this study, the issue of adulteration of caprine milk by the addition of cheaper bovine milk was addressed. The commercial value of goat milk is much higher than that of cow milk due to lower productivity and little market demand; therefore, the addition of cow milk to goat milk can allow economic advantages and becomes a fraud when the mixture is sold with label. Taking into account this option, in this paper, an attempt to assess whether the metabolomic approach could be suitable tool for discovering such fraud was carried out. The literature reports several attempts to find suitable methods to detect milk adulteration. Quantification of cow milk adulteration of goat milk, based on solvent separation of whey proteins, followed by HPLC with electrospray ionization mass spectrometry, was performed by Chen et al. (2004); levels as low as 5% of cow milk were detected. Levieux and Venien (1994) proposed an ELISA to detect cow β-LG at 5 ng/mL. Antonilli et al. (2005), by inspection of the ratios of some FAME, offered some parameters suitable for discovering such fraud. Proton nuclear magnetic resonance low-molecular-weight metabolite fingerprinting was applied for the quantification of the relative amount of cow and sheep milk in mixtures (Lamanna et al., 2011).

In this work, for the first time, the goat milk metabolite profile, composed by polar and hydrophilic low-molecular-weight compounds, was characterized by the means of GC-MS and compared, through the application of discriminant multivariate analysis, with cow milk. Differences in milk metabolite profiles correlated with heat treatments: UHT and pasteurization were also investigated. Moreover, milk mixtures of goat milk with increasing quantities of cow milk were prepared and their GC-MS metabolic profiles used to construct a suitable model, based on orthogonal projections to latent structures (**OPLS**) regression, to detect adulteration of goat milk with cow milk.

MATERIALS AND METHODS

Chemicals and Reagents

Methanol, chloroform, hexane, pyridine, methoxamine hydrochloride, potassium chloride, *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide, lactic acid, valine, butyric acid, urea, glycine, succinic acid, fumaric acid, serine, malic acid, proline, alanine, creatinine, glutamine, phosphoric acid, fructose, glucose, galactose, gluconic acid, palmitic acid, inositol and stearic acid were purchased from Sigma-Aldrich (Milan, Italy). Bidistilled water was obtained from a Milli-Q purification system (Millipore S.p.A., Milan, Italy) before use.

Samples

Seventeen commercial samples of goat whole milk (**G1**–**G17**) and 14 samples of cow whole milk (**C1**–**C14**) were acquired in local markets; all samples were within the expiration date. Seventeen milk samples were subjected to UHT and 14 samples were subjected to pasteurization processes. Furthermore, 9 mixtures were prepared by adding different aliquots (%, vol/vol) of cow milk to goat milk as follows: 0, 5, 10, 20, 40, 50, 60, 80, and 100%.

Extraction and Derivatization

To obtain rupture of the milk micelles, 15 mL of sample was sonicated for 15 min; 100 μL of milk was transferred to an Eppendorf tube and then 250 μL of methanol and 125 μL of chloroform were added. Samples were vortexed every 15 min 4 times and then 380 μL of chloroform and 90 μL of aqueous 0.2 *M* potassium chloride were added. The suspension was centrifuged at 13,572 × *g* for 10 min at 4°C. After centrifugation, the aqueous layer was transferred to a glass vial and dried by a gentle nitrogen stream and derivatized with 50 μL of pyridine containing methoxamine hydrochloride at 10 mg/mL. After 17 h, 100 μL of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide was added and after 1 h, samples were resuspended with 600 μL of hexane.

GC-MS Analysis

One microliter of derivatized samples was injected splitless into a 6850 gas chromatograph coupled with a 5973 Network mass spectrometer (Agilent Technologies Inc., Santa Clara, CA). The injector temperature was 200°C. The gas flow rate through the column was 1 mL/min. The fused silica capillary column was a 0.25-μm DB5-MS column (30 m × 0.25 mm i.d.; J&W Scientific Inc., Folsom, CA). The initial temperature program was as follows: 3 min of isothermal heating at 50°C, which was then increased to 250 at 3°C/min and held at 250°C for 25 min. The transfer line and the ion source temperatures were 280 and 180°C, respectively. Ions were generated at 70 eV with electron ionization and were recorded at 1.6 scans/s over the mass range m/z 50 to 550. The GC-MS data analysis was conducted by integrating each resolved chromatogram peak. Identification of metabolites was performed using the standard NIST08 mass spectra library (http://www.nist.gov/srd/mslist.cfm), a library developed at the Max Planck Institute of Golm (Germany), and, when available, by comparison with authentic standards.

Multivariate Statistical Data Analysis

A 31 × 40 matrix composed of the analyzed milk samples (31 samples) and the chromatographic peak areas (40 variables) was constructed together with a **Y** matrix with sample information: milk typologies (goat and cow) and heat treatments (pasteurization and UHT). In addition, a second **X** matrix (9 × 41) was constructed; it was composed of the 9 mixtures, the 40 **x** variables and 1 continuous **y** variable consisting of the percentages of cow milk added. For both of them, the median fold change normalization row-wise was selected for adjusting peak intensities between samples to a common scale. This centering method assumes that measured peak intensities are directly proportional to concentrations of metabolites in solution. Under this assumption, the change in intensity of a profile due to variable sample dilution or inconsistency of GC-MS acquisition parameters, or both, are expected to be uniform across all peaks and thus a fixed scaling factor is used (Veselkov et al., 2011). When a variable presented skew distribution it was logarithmically transformed and the improvement of the symmetry evaluated using the skewness test statistics as implemented in SIMCA-P software (version 13.0; MKS Umetrics AB, Umeå, Sweden). Prior to analysis, each data matrix was mean centered and unit variance scaled column-wise. The obtained matrices were submitted to MVA. Principal components analysis (**PCA**), the partial least squares (**PLS**) method, PLS-discriminant analysis (**PLS-DA**), and their orthogonal extensions (OPLS and **OPLS-DA**; Eriksson et al., 2013) were performed with SIMCA-P software. The quality of the models was evaluated on the basis of the cumulative parameters R^2X (variation in **X** explained by the model), R^2Y (amount of **Y** explained by the model), and Q^2 (i.e., the cumulated cross-validation for R^2Y; or R^2X for the PCA), estimated by the default leave-one-seventh-out cross-validation in the corresponding PLS-DA model. Models were tested for overfitting using the y-table permutation test (n = 400) as implemented in the SIMCA-P+ program. Jackknifed standard errors were calculated from all rounds of cross-validation. Accuracy for the PLS model, in which the leave-one-out cross-validation was performed, was evaluated by the root mean square error in cross-validation (Eriksson et al., 2013). Results of the OPLS-DA models were also compared and reported as a shared and unique structures (**SUS**) plot; this is a 2-dimensional scatter plot of the loading correlation vectors of the predictive components of 2 separate models (Wiklund et al., 2008).

RESULTS AND DISCUSSION

A total of 31 commercial samples of goat and cow whole milks were studied by GC-MS; from the analysis of the chromatograms, we selected a total of 40 polar metabolites reported in Table 1. Twelve compounds were not identified and they will be hereafter named **U1** to **U12**. The chemical composition analysis revealed that the aqueous fraction obtained by the extraction procedure was rich in short-chain hydroxylated carboxylic acids, such as lactic acid, succinic acid, fumaric acid, malic acid, 2-hydroxyglutaric acid, and gluconic acid; long-chain stearic and palmitic acids were also found. Among free amino acids, serine, valine, glycine, alanine, proline, and glutamine were detected. Also the saccharides D-glucose, fructose, talose, inositol, and galactose were identified. In Table 2, we reported the normalized areas of each metabolite as means and standard deviations over all samples for the 2 typologies of milk. In this work, aimed at observing also the variability of the detected metabolites within each milk typology, we also reported the coefficient of variation (SD/mean). The reported coefficient of variation for each variable gives a measure of the dispersion of the variable among samples in a way that does not depend on the variable measurement unit; this allowed us to directly compare the coefficients of variation, higher values of which indicated a greater dispersion in the variable. By the Student's t-test, we also tested the null hypothesis (the means are not significantly different among the 2 sets of samples) and we reported the results in Table 2. In this table, we observed that, with

a high level of confidence ($P < 0.01$), the null hypothesis was rejected for a great number of metabolites (27 out of 40). The sum of coefficient of variation values was 27.8 and 18 for goat and cow milk samples, respectively, indicating a greater variability of the metabolite concentrations within caprine milk. The data we reported in Table 2 were the results of univariate tests that analyze each variable separately. Conversely, in biological samples, the variables are often interconnected and only a multivariate approach is able to fully describe these systems, taking into consideration several variables and their relationship simultaneously. Under this perspective, we constructed a matrix composed of the normalized area of chromatographic data for the analyzed samples and we submitted it to MVA. Initially, for sample distribution overview, to detect outliers, deviating features, and common trends, we performed a PCA; the first 2 principal components accounted for 45% of the total variance. We report the results in the score plot, shown in the top panel of Figure 1; here, we observed that samples of cow and goat milk clustered in different areas of the plot and goat samples were very scattered. We concluded that cow milk samples had similar characteristics that differed from those of goat milk, and that goat milk, based on the detected variables, had a more heterogeneous composition, as already observed from the analysis of results in Table 2. Based on Hotelling's T^2 test at 99% confidence, sample G12 was identified as an outlier, and because no evident reasons for its deviating features were found and, moreover, its behavior was in trend with sample G15, we decided to keep this sample

Table 1. The GC-MS characteristics of milk metabolites

Compound	Retention time (min)	EI-MS[1] [m/z (amu), with relative abundance (%) in parentheses]	Trivial name	Abbreviation
2-Hydroxypropanoic acid	9,352	147 (100), 73 (80), 117 (74)	Lactic acid	Lac
Valine	9,713	72 (100), 75 (31), 73 (27)	Valine	Val
Butanoic acid	10,879	147 (100), 117 (52), 73 (53)	Butyric acid	BA
Unknown 1	11,665	73 (100), 228 (90), 184 (39)		U1
Urea	12,139	147 (100), 189 (62), 171 (58)	Urea	Urea
Unknown 2	12,533	158 (100), 73 (64), 159 (15)		U2
Glycine	13,02	174 (100), 73 (34), 248 (18)	Glycine	Gly
Butanedioic acid	13,064	147 (100), 73 (41), 75 (17)	Succinic acid	Suc
2-Butenedioic acid	13,495	245 (100), 147 (40), 73 (37)	Fumaric acid	Fum
Serine	13,782	204 (100), 73 (63), 218 (55)	Serine	Ser
Unknown 3	14,610	73 (100), 174 (74), 248 (58)		U3
Hydroxybutanedioic acid	15,430	73 (100), 74 (86), 147 (74)	Malic acid	Mal
L-Proline	15,820	156 (100), 73 (55), 147 (20)	Proline	Pro
Alanine	15,883	84 (100), 174 (86), 75 (47)	Alanine	Ala
Creatinine	16,279	115 (100), 73 (67), 329 (33)	Creatinine	Crn
2,3,4-Trihydroxybutyric acid	16,399	73 (100), 147 (65), 292 (43)	2,3,4-Trihydroxybutyric acid	3-HBA
2-Hydroxypentanedioic	16,470	73 (100), 147 (49),129 (41)	Hydroxyglutaric acid	HGA
Glutamine	16,992	246 (100), 73 (46), 128 (21)	Glutamine	Gln
Unknown 4	18,366	117 (100), 73 (54), 160 (14)		U4
Unknown 5	18,419	254 (100), 357 (51), 73 (45)		U5
Unknown 6	18,497	117 (100), 73 (71), 147 (25)		U6
Glycerophosphoric acid	18,691	357 (100), 299 (95), 73 (72)	Phosphoglycerate	PG
Unknown 7	18,793	73 (100), 292 (69), 147 (46)		U7
Phosphoric acid	18,884	292 (100), 174 (28), 217 (20)	Phosphoric acid	PA
Unknown 8	19,422	73 (100), 147 (51), 117 (23)		U8
Unknown 9	19,489	73 (100), 217 (60), 147 (40)		U9
D-Fructose	19,929	73 (100), 103 (68), 217 (51)	Fructose	Frc
Unknown 10	19,990	73 (100), 331 (54), 147 (33)		U10
Unknown 11	20,144	204 (100), 73 (50), 205 (20)		U11
D-Glucose	20,203	73 (100), 319 (80), 205 (61)	Glucose	Glc
D-Galactose	20,601	73 (100), 333 (87), 292 (59)	Galactose	Gal
Talose	20,955	204 (100), 73 (52), 191 (46)	Talose	Tal
D-Gluconic acid	21,243	73 (100), 147(47), 333 (40)	Gluconic acid	GcA
Hexadecanoic acid	21,29	73 (100), 117 (75), 313 (73)	Palmitic acid	PmA
Inositol	22,050	73 (100), 217 (72), 305 (72)	Inositol	Ino
D-Ribose	22,240	73 (100), 315 (44), 299 (34)	Ribose	Rib
D-Mannitol	22,553	73 (100), 319 (70), 205 (50)	Mannitol	Man
Octadecanoic acid	23,077	73 (100), 341 (51), 117 (49)	Stearic acid	StA
Unknown 12	24,182	73 (100), 315 (82), 217 (45)		U12
Unknown 13	24,317	73 (100), 387 (82), 299 (54)		U13

[1]Electron impact-mass spectrometry.

for further analysis. The role of the variables in sample distribution can be evinced by the analysis of the loading plot shown in the bottom panel of Figure 1. Here, considering that samples clustered based on their animal provenience along the first principal component, we assumed that variables in the right side of the plot (ribose, glycine, and U9, among others) characterized goat milk and sugars (glucose, fructose, and talose) characterized cow milk. The deviating samples, G12 and G15, were high in succinate, a metabolite entering the Krebs cycle. To classify goat and cow milk based on their metabolite profiles, we performed an OPLS-DA. This discriminant analysis, with a high degree of confidence, correctly classified samples and well separated the 2 classes of milk, producing a model with $R^2(X) = 0.43$, $R^2(Y) = 0.95$, and $Q^2(Y) = 0.87$, with 1 predictive component and 1 orthogonal component; the resulting score plot is shown in the top panel of Figure 2. The analysis of loading values along the predictive component, (bottom panel of Figure 2), indicated that the most important variables in discriminating the 2 classes [i.e., those having the largest values (negative or positive)] were glycine, ribose, and valine for goat milk (positive values in the bottom panel of Figure 2), and U5, U11, glucose, and talose for cow milk. In the orthogonal direction, interclass variability was dominated by the difference of the metabolite profiles due to the heat treatments. Consequently, we also compared UHT

Table 2. Metabolite composition (peak area, %), as calculated by GC-MS, of goat and cow milk[1]

	Goat (n = 17)			Cow (n = 14)			
Compound	Mean	SD	CV	Mean	SD	CV	*P*-value[2]
Lactic acid	5.47	4.75	0.87	1.79	0.62	0.35	0.007**
Valine	0.56	0.47	0.84	0.10	0.04	0.37	0.001**
Butyric acid	0.20	0.11	0.56	0.33	0.13	0.38	0.004**
Unknown 1	0.84	0.26	0.31	0.55	0.23	0.41	0.004**
Urea	62.50	10.42	0.17	49.15	6.09	0.12	0.000**
Unknown 2	0.25	0.40	1.63	0.08	0.10	1.26	0.13
Glycine	4.99	2.40	0.48	1.20	0.24	0.20	0.000**
Succinic acid	1.34	2.54	1.89	0.31	0.14	0.46	0.14
Fumaric acid	0.15	0.16	1.07	0.23	0.08	0.32	0.081
Serine	0.09	0.10	1.12	0.05	0.03	0.56	0.10
Unknown 3	0.04	0.01	0.32	0.02	0.01	0.30	0.000**
Malic acid	0.24	0.30	1.23	0.66	0.20	0.30	0.000**
Proline	0.70	0.37	0.52	0.73	0.21	0.29	0.80
Alanine	0.15	0.10	0.63	0.38	0.31	0.80	0.007**
Creatinine	1.04	0.63	0.60	0.68	0.27	0.40	0.054
2,3,4-Trihydroxybutyric acid	0.14	0.11	0.78	0.09	0.05	0.49	0.16
Hydroxyglutaric acid	2.28	1.00	0.44	1.34	0.52	0.39	0.003**
Glutamine	1.86	0.89	0.48	3.05	0.65	0.21	0.000**
Unknown 4	0.85	0.61	0.71	0.88	0.23	0.26	0.86
Unknown 5	1.21	0.44	0.36	8.65	2.04	0.24	0.000**
Unknown 6	0.27	0.22	0.80	0.25	0.05	0.20	0.77
Phosphoglycerate	2.16	1.31	0.61	3.17	2.02	0.64	0.10
Unknown 7	0.13	0.11	0.88	0.23	0.10	0.45	0.015**
Phosphoric acid	0.24	0.12	0.49	0.20	0.26	1.32	0.52
Unknown 8	0.54	0.21	0.39	1.70	0.62	0.37	0.000**
Unknown 9	4.81	1.28	0.27	2.78	0.64	0.23	0.000**
Fructose	0.09	0.08	0.87	0.21	0.04	0.22	0.000**
Unknown 10	0.55	0.19	0.35	0.64	0.26	0.40	0.28
Unknown 11	1.50	1.02	0.68	8.60	3.05	0.35	0.000**
Glucose	0.76	0.51	0.67	4.18	1.71	0.41	0.000**
Galactose	0.80	0.35	0.44	1.19	0.22	0.18	0.001**
Talose	0.53	0.52	0.99	4.48	2.71	0.60	0.000**
D-Gluconic acid	0.11	0.13	1.11	0.16	0.05	0.30	0.26
Palmitic acid	0.21	0.18	0.86	0.36	0.37	1.02	0.15
Inositol	0.80	0.37	0.46	0.50	0.09	0.19	0.005**
Ribose	0.17	0.07	0.42	0.05	0.04	0.90	0.000**
Mannitol	0.21	0.19	0.87	0.27	0.08	0.31	0.34
Stearic acid	0.07	0.04	0.59	0.13	0.14	1.11	0.10
Unknown 12	0.42	0.20	0.48	0.23	0.10	0.44	0.004**
Unknown 13	0.70	0.39	0.56	0.38	0.11	0.29	0.006**

[1]Means, SD, and CV over all samples.

[2]Probability associated with the Student's *t*-test.

**$P < 0.01$.

and pasteurized samples by an OPLS-DA. It resulted in a model with 1 predictive component and 1 orthogonal component and with $R^2(X) = 0.44$, $R^2(Y) = 0.83$, and $Q^2(Y) = 0.68$; the latter value indicated a higher uncertainty in class separation compared with that of the model concerning milk animal origin. We reported the score plot and the loading values in the top and bottom panel of Figure 3, respectively. Loading values indicated that U7 and U11, together with the sugars glucose and fructose had higher levels in UHT samples, whereas

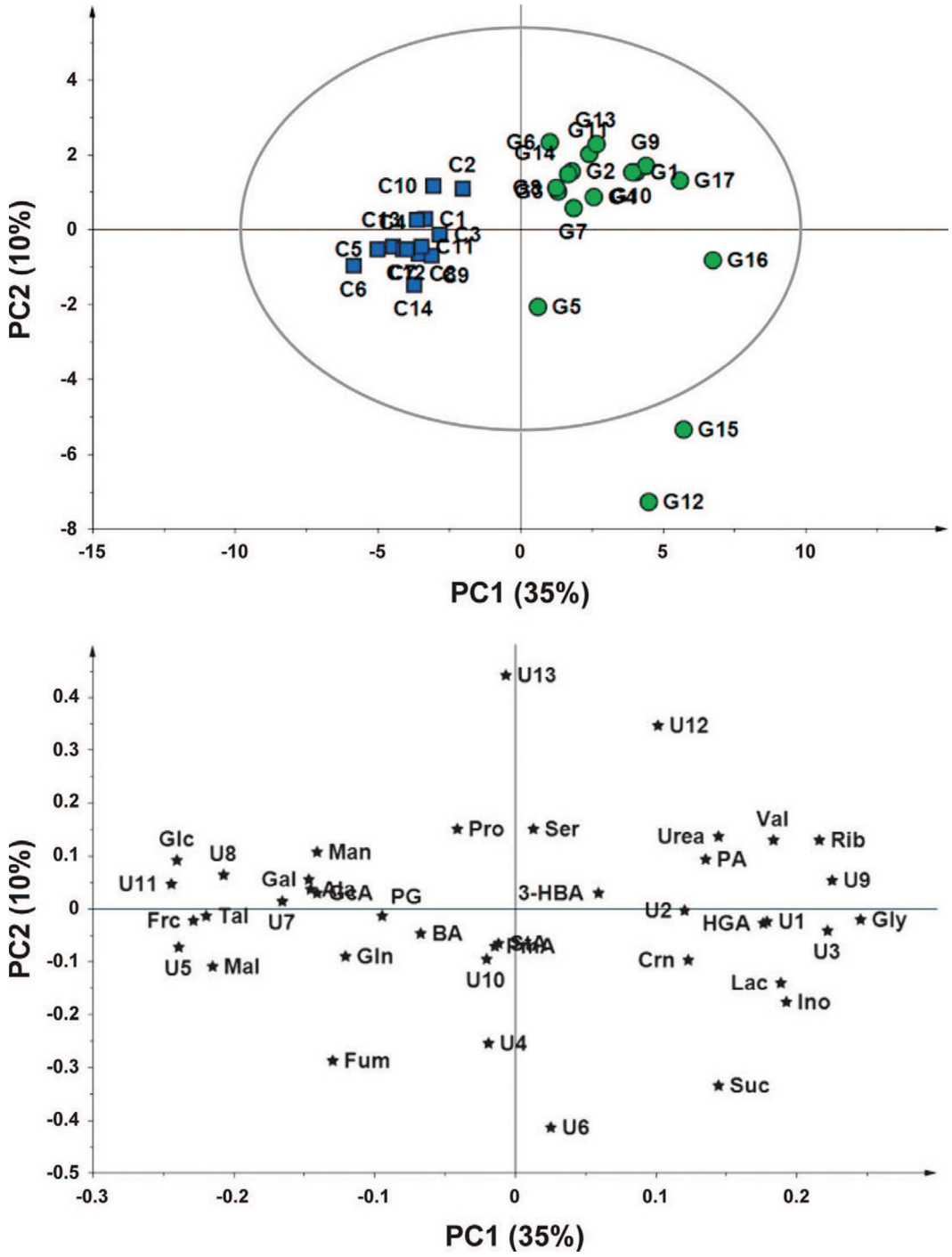

Figure 1. Principal components (PC) analysis of milk samples: PC1 versus PC2 score plot (top) of goat (G in green circles) and cow (C in blue squares) milk. The explained variance is reported in parentheses. The ellipse encloses the 95% Hotelling's T^2 confidence region. The bottom panel shows the corresponding loading plot. Metabolites are abbreviated as proposed in Table 1. Color version available in the online PDF.

hydroxyglutaric acid, U9, and ribose had higher levels in pasteurized samples. To target metabolites due solely to milk typology (goat vs. cow) and solely to heat treatments (UHT vs. pasteurization) and to avoid confounding overlapping, we projected the correlation loading vector of the predictive component in the goat and cow OPLS-DA model versus that of the OPLS-DA model concerning heat treatments. In such a way, we obtained the SUS plot reported in Figure 4. This 2-dimensional plot helped us to visualize both the shared

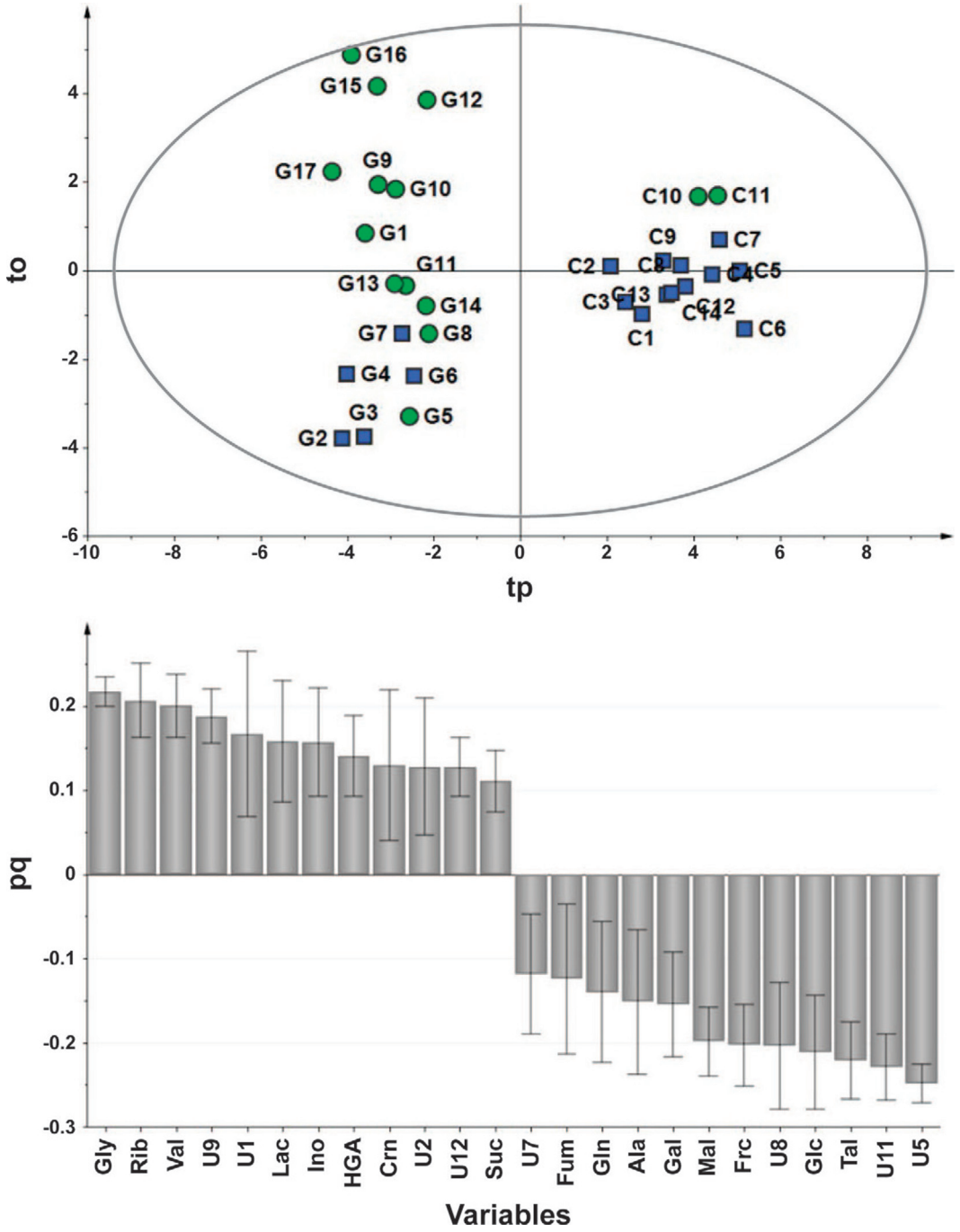

Figure 2. Orthogonal projections to latent structures-discriminant analysis of milk samples from different animal origin: score plot (top) of goat (G) and cow milk (C) samples, including pasteurized (green circles) and UHT-treated (blue squares) samples; tp = predictive component; to = first orthogonal component. The bottom panel shows loading plot values along the predictive component, with error bars indicating the jackknifed confidence interval. Only metabolites with the highest loading values are reported and aligned in ascending order, with positive values for goat milk. Metabolites are abbreviated as proposed in Table 1. Color version available in the online PDF.

and unique information of the 2 OPLS-DA models. The variables lined up along the diagonal running from the lower left corner to the upper right corner represented the shared structure between the 2 compared OPLS models. Conversely, variables that were not located along this diagonal represented structures that were unique to each class of the 2 compared models, always under the assumption that correlation loading vectors

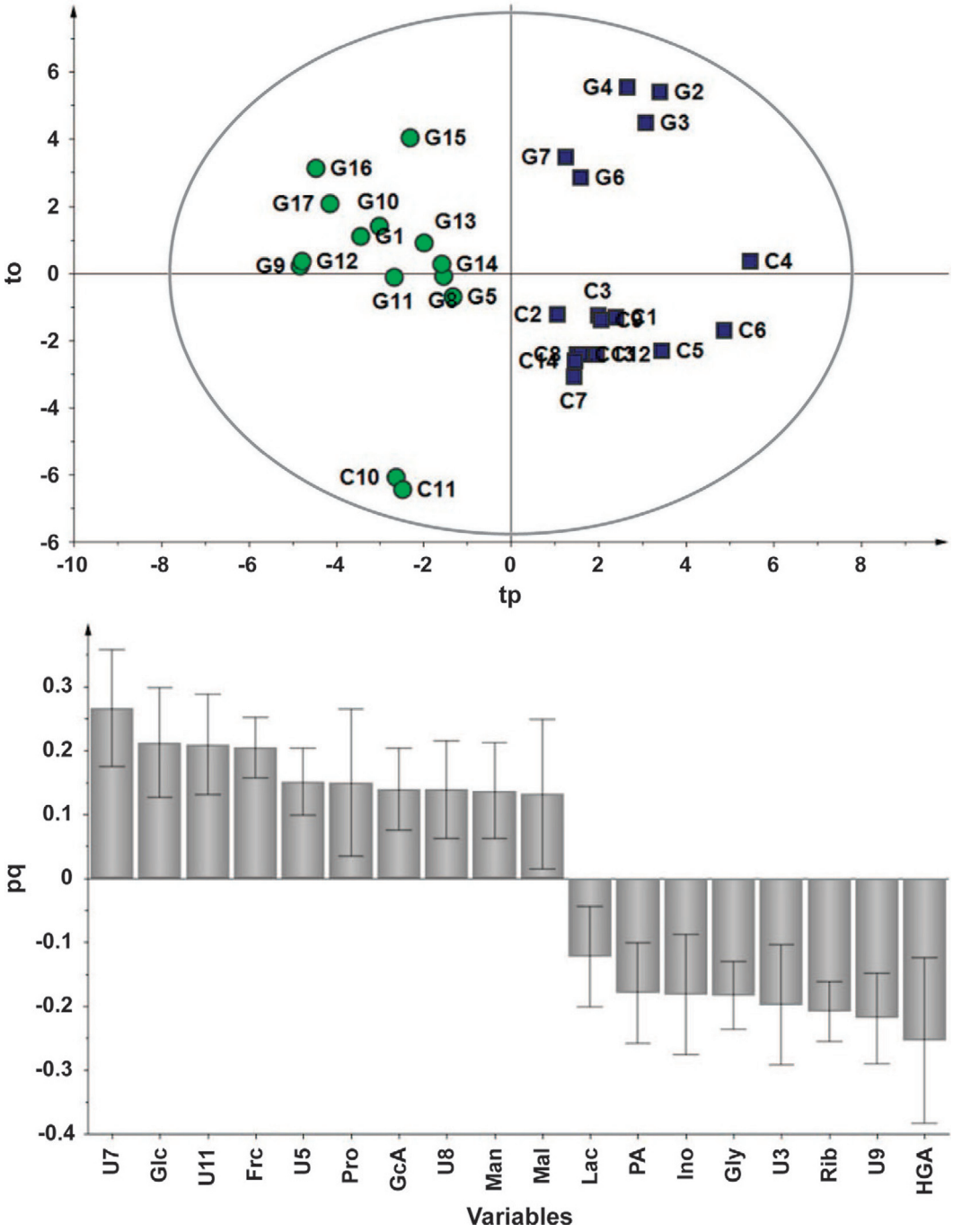

Figure 3. Orthogonal projections to latent structures-discriminant analysis of milk samples classified by heat treatments: score plot (top) of goat (G) and cow milk (C) samples, including pasteurized (green circles) and UHT-treated (blue squares) samples; t1 = predictive component; to = first orthogonal component. The bottom panel shows loading plot values along the predictive component, with error bars indicating the jackknifed confidence interval. Only metabolites with the highest loading values are reported and aligned in ascending order, with positive values for UHT milk. Metabolites are abbreviated as proposed in Table 1. Color version available in the online PDF.

have significant values for the studied classes of samples. We observed that the metabolites with the highest correlations with goat milk were valine and glycine; it is worth recalling that valine, together with leucine and isoleucine, enters in metabolic pathways involved in the production of branched-chain FA, of which caprine milk is particularly rich (Massart-Leën and Massart, 1981). An important characteristic of goat milk is the unique flavor, attributed to differences in the fat fraction (Amigo and Fontecha, 2011); contribution to milk taste can be also given by valine (bitter) and glycine (sweet). The SUS plot also indicated that U5, talose, U8, and malic acid were the metabolites unique to bovine milk. The UHT samples resulted high in U7 and pasteurized samples resulted high in hydroxyglutaric acid (Figure 4). Ribose and U9 were shared by goat and pasteurized milk. Glucose, fructose, and U11 were shared by UHT and cow samples. This latter observation is in agreement with previous findings that indicate a higher presence of lactose in cow milk compared with goat milk (Amigo and Fontecha, 2011) and that, following UHT treatments, lactose is hydrolyzed to monosaccharide-reducing sugars, which, reacting with amino groups, can give rise to undesired Amadori compounds (Mendoza et al., 2005). We also observed that other metabolites, although lower in correlation, exhibited interesting features; for example, proline exhibited a correlation value of approximately 0 for the goat versus cow OPLS-DA model but had a correlation of 0.5 with UHT treatment class. In this regard, it has been reported that, in UHT milk, casein exhibits lower proline content (Tamime, 2009); a release of proline could then take place, thus increasing its presence as a free amino acid in the bulk.

The above-reported results proved that the GC-MS-based metabolomic approach was able to discriminate the 2 milk typologies (goat and cow) based on their metabolites. To test the predictive potentiality of the GC-MS profile for fraud detection, we submitted to MVA the analytical data of the prepared mixtures of goat and cow milk (see Materials and Methods section). A single-Y OPLS technique was used to construct the model that correlated the chromatographic data of the mixtures to the percentages of cow milk added as the **y** dependent variable. The obtained OPLS model had $R^2(Y) = 0.996$ and $Q^2 = 0.879$; the accuracy for prediction in cross-validation of percentage of cow milk added, indicated by the root mean square error in cross-validation value, was approximately 5%. A detectability of addition of cow milk as low as 5% can be considered a satisfactory result when such a fraud has to be detected; lower quantities are economically meaningless. This strategy can be proposed as a valid, fast, and cheap tool against commercial fraud regarding

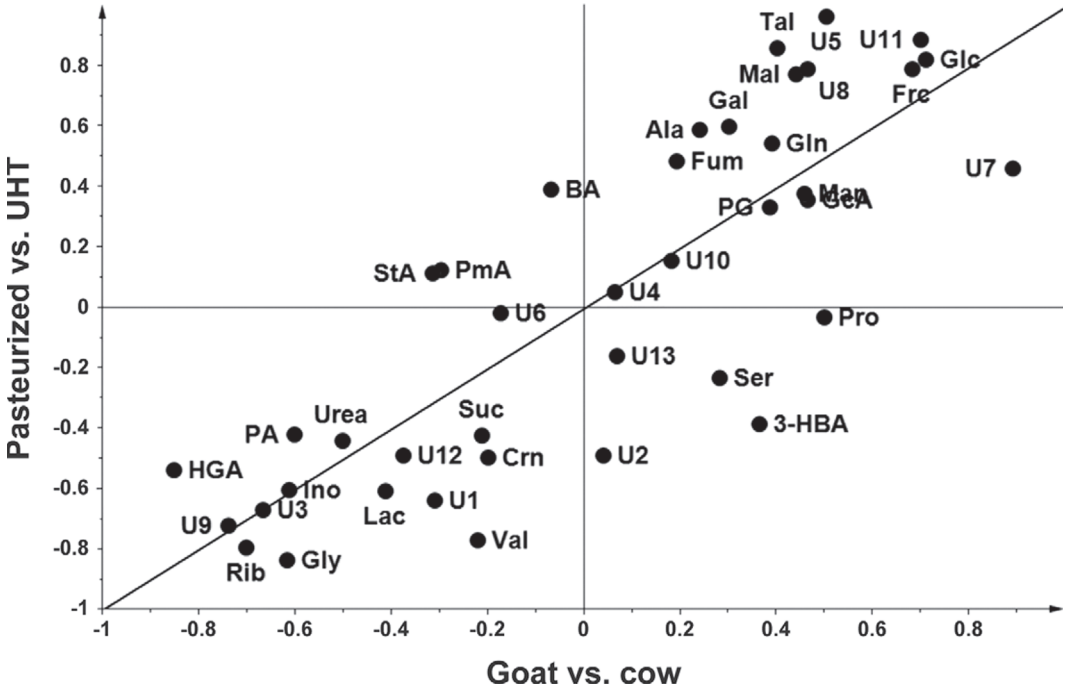

Figure 4. Shared and unique structures plot of the orthogonal projections to latent structures-discriminant analysis models of milk samples. The horizontal axis contains the loading correlation values of goat versus cow model and the vertical axis contains the loading correlation values of the pasteurized versus UHT model. Variables in the lower left corner are shared in goat and pasteurized samples; variables in the upper right corner are shared in cow and UHT samples. Metabolites are abbreviated as proposed in Table 1.

mixtures of milk having different commercial values. As a final remark, we want to recall the extreme variability of goat milk reported by several authors (Haenlein, 2004; Amigo and Fontecha, 2011; Sabahelkheir et al., 2012); therefore, studies of caprine milk and derivatives could still be far from delineating standardizable results.

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