Detection of bovine milk adulteration in caprine milk with *N*-acetyl carbohydrate biomarkers by using ^1H nuclear magnetic resonance spectroscopy

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ABSTRACT

In a return to tradition, the popularity of caprine milk is on the rise. However, particularly in countries with developed dairy industries based on bovine milk, there is the risk of adulteration with bovine milk, which is a cheaper alternative. Thus, a rapid, robust, and simple method for the detection of bovine milk added to caprine milk is necessary, and ^1H nuclear magnetic resonance spectroscopy appears to provide a solution. A matrix of 115 pure and artificially adulterated pasteurized milk samples was prepared and used to discover biomarkers of bovine milk that are independent of chemical and biological variation caused by factors such as genetics, diet, or seasonality. Principal component analysis and orthogonal projections to latent structures discriminant analysis of pure bovine milk and pure caprine milk revealed spectral features that were assigned to the resonances of 4 molecules. Of these, the peaks corresponding to protons in the *N*-acetylglucosamine and *N*-acetylgalactosamine acetyl moieties showed significant applicability for our method. Receiver operating characteristic curve analysis was used to evaluate the performance of the peak integrals as biomarkers of adulteration. This approach was able to distinguish caprine milk adulterated with 5% of bovine milk with 84.78% accuracy and with 10% of bovine milk an excellent 95.65% accuracy. This study demonstrates that *N*-acetyl carbohydrates could be used as biomarkers for the detection of bovine milk in caprine milk and could help in protecting caprine milk authenticity.

Key words: biomarker, caprine milk, food authenticity, nuclear magnetic resonance

INTRODUCTION

The importance of goats as part of the human diet, during infancy as well as adulthood, has been recognized since the beginning of domestication (Clark and Mora García, 2017). However, the production level of caprine milk is much lower than that of bovine and buffalo milk. Worldwide population of goat has been rising dramatically since the 1960s (Miller and Lu, 2019). In 2016, the production of caprine milk represented only 1.9% of entire production of the dairy industry. In the future, however, things may change. If the current growing trend continues, caprine milk production is expected to increase by approximately 9.7 million tonnes (+53%) by 2030 (FAOSTAT, 2018; Pulina et al., 2018). Although caprine milk is similar to bovine milk in composition, it is becoming popular because of 2 specific characteristics: lower allergenic properties and better digestibility (Lara-Villoslada et al., 2004; Chen et al., 2019; Li et al., 2020). The growing demand for caprine milk and the distinct differences in rearing cows and goats, such as the liters of milk produced per animal, prices per liter of milk, and seasonality, are major reasons for fraud to be committed with caprine milk (Haenlein, 2004; Clark and Mora García, 2017). This fraud is worrying because many consumers, especially children, are allergic to bovine milk protein (Haenlein, 2001; Ballabio et al., 2011; Watson et al., 2017).

Adulteration is a major current issue for the food industry and security authorities on a global scale because fraudsters continually develop new ways to elude protection methods. This situation led to the constitution of the Administrative Assistance and Cooperation System, which has been used by members of the European Union Food Fraud Network to exchange information since 2015. In milk, the adulteration options have expanded from water, whey, and urea manipulation to include the addition of a diverse list of unfamiliar substances or other species of milk with greater availability (Azad and Ahmed, 2016; Poonia et al., 2017; Romero

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Gonzalez et al., 2019). For instance, the tragic incident in 2008 when milk powder was adulterated with melamine, or the adulteration of water buffalo mozzarella with sodium hydroxide in Italy, are 2 examples of such (Pei et al., 2011; Aiello et al., 2019). Given the increase in the fraud mentioned, the development of new, simple, rapid, affordable, and effective methods for the quantification and detection of milk adulteration is a priority to ensure the protection of consumers.

To date, several analytical approaches have been developed for the detection of bovine milk in milk from other species, despite the great similarity between different dairy matrices and the intrinsic variability within each. These methods include GC and liquid chromatography (LC), immunological tests, capillary electrophoresis, and PCR analysis based on detecting genomic DNA. In the past, substantial progress has also been made with spectroscopic techniques. For instance, Brandao et al. (2017) proposed a novel method for the discriminant analysis of powdered bovine and caprine milk by fluorescence techniques. Pereira et al. (2020) employed near-infrared spectroscopy and partial least squares algorithms for the identification and quantification of caprine milk adulteration with added bovine milk. Also, a method based on matrix-assisted laser desorption/ionization time-of-flight MS was used to identify marker peptides of bovine milk that were usable for the detection of adulteration (Calvano et al., 2012).

In addition to the above-mentioned spectroscopic techniques, in recent decades, nuclear magnetic resonance (NMR) spectroscopy has also proved to be a very powerful tool for dairy commodities fraud detection, despite the fact that milk is considered to be among the most complicated foods to study (Sundekilde et al., 2013; Sobolev et al., 2019). Nuclear magnetic resonance spectroscopy has gained popularity in the dairy science field because of its many advantages, such as minimal sample preparation, nondestructive application, rapidity, reproducibility, easy automation, remarkable selectivity, and ability to look at all of the components in a mixture at once (Mannina et al., 2012; de Moura Ribeiro et al., 2017; Shi et al., 2018).

A few previous studies have mentioned that metabolite profiles could be used to discriminate between bovine milk and milk from other species. Andreotti et al. (2000) were able to distinguish pure bovine milk and pure buffalo milk through quantitative determination of the fatty acid content by ¹³C NMR spectroscopy. A combination of ¹H NMR spectroscopy and an artificial neural single-layer network was used by Lamanna et al. (2011) to quantify the relative amounts of bovine milk in mixtures with ovine milk. Several biomarkers for distinguishing pure milk from bovines and other

ruminants were revealed by Yang et al. (2016) using ¹H NMR spectroscopy and LC–MS spectrometry. Li et al. (2017) successfully developed a ¹H NMR spectroscopic method in combination with mathematical models for the detection of milk adulteration. The limit of quantification was 5% (vol/vol) for bovine milk in caprine milk.

However, to our knowledge, the previously published NMR studies demonstrated the applicability of profiling by multivariate analysis but were not designed and validated with the intention of practical use. The objective of this study was to find biomarkers of bovine milk that are robust and not prone to chemical and biological variation as a result of pasteurization processes, genetic background, diet, or seasonality to develop a robust and rapid method that would be useful for the inspection authorities of the Czech Republic.

MATERIALS AND METHODS

Milk Samples

Milk samples were obtained from 6 dairy goat flocks and 6 dairy cow herds. The goats were of the following average breeding ratio: 59% white shorthaired goat, 29% brown shorthaired goat, 12% Anglo-Nubian goat; 83% of the cows were Holstein, and 17% were Czech Fleckvieh. The herds were selected to cover the typical breeding profiles of dairy farms in the Czech Republic (Bucek et al., 2019; Kvapilík et al., 2019).

Bulk tank samples of raw milk were collected from cooling tanks (<8°C) on the dairy farms twice in May and twice in August during the year 2019 and were transported under cold conditions (<6°C) to the laboratory. A total of 23 pure bovine milk and 23 pure caprine milk samples were taken. These samples were thermally treated by pasteurization in a batch process in a water bath to reach 72°C for 20 min. Then, a matrix of adulterated samples was prepared with random combinations of pure bovine and caprine milk; series with 1%, 5%, and 10% adulteration were prepared from each pair (Table 1). The total number of pure and adulterated milk samples was 23 × 5.

Table 1. Ratios of bovine and caprine milk in artificially adulterated samples

Sample identification	Bovine		Caprine	
	%	mL	%	mL
Caprine 100%	0	0	100	250
Bovine 1%	1	2.5	99	247.5
Bovine 5%	5	12.5	95	237.5
Bovine 10%	10	25	90	225
Bovine 100%	100	250	0	0

Other series of milk samples were obtained during the year 2020. This series was used as an external validation data set to verify the capability of the biomarker discovered by the blind test. Bulk tank samples of pure caprine and bovine milk were collected from the same goat flocks and dairy cow herds mentioned above. The blind test was performed with 20 pure and adulterated milk samples in 4 categories: caprine 100%, bovine 5%, bovine 10%, and bovine 100% (Table 1). Each category contained 5 samples. Equivalent pasteurization was applied.

Baseline Sample Characteristics

The samples were characterized in terms of their physicochemical and microbiological properties to guarantee the reproducibility and validity of the findings. The composition of the milk (fat, crude protein, casein, lactose monohydrate, total dry matter (solids), nonfat solids, and urea) was determined by indirect infrared spectroscopy with Michelson interferometry and Fourier transformations by using a Bentley DairySpec FT instrument (Bentley Instruments). The SCC was determined by fluoro-opto-electronic flow cytometry on a Somacount 300 instrument (Bentley Instruments). The active acidity of the milk was measured by using a pH meter (pH phenomenal pH 1100L; VWR), and the titratable acidity was determined by titration of 100 mL samples of milk with 0.25 mol/L NaOH with phenolphthalein as the indicator. Results were calculated as Soxhlet Henkel degrees ($^{\circ}\text{SH}$).

The classical plate method, performed according to the International Standard Organization (ISO 4833-1:2013; ISO, 2013), was used to determine the total count of mesophilic microorganisms (**TCM**). Milk samples and the appropriate dilutions in a volume of 1 mL were plated on GTK-M agar (MILCOM Corp.), then the Petri dishes were incubated at 30°C for 72 h. The number of colonies was expressed as colony-forming units in 1 mL of sampled milk, in accordance with ISO 7218:2007 (ISO, 2007).

The fermentation ability of milk (yogurt test) was determined by the titratable acidity according to the above-mentioned method. The test was performed with a mixed thermophilic yogurt culture, YC-180 Yo-Flex 50 U, containing *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *lactis*, and *Lactobacillus delbrueckii* ssp. *bulgaricus*; the inoculum was prepared according to the manufacturer's specifications (Chr. Hansen). The starter culture was grown at 43°C for 3.5 h. The rennet coagulation time of lactoproteins (time until the formation of visible protein flakes) was determined with the addition of bacterial rennet (Fromase 220 TL BF; Royal DSM) in 50 mL of milk at 32°C in a water bath. The

time was adjusted empirically to approximately 5 min of exposure. After 1 h of syneresis, the quality of the curd was checked according to the fitness of the curd cake formed, in centimeters, by dropping the inserted body under standardized conditions.

NMR Spectroscopy

Heat-treated samples were mixed with chloroform (1/1, vol/vol). The chloroform contained ethanol as stabilizer (0.5–1.0%) (PENTA, Prague, Czech Republic). After 1 min of agitation with an IKA MS 3 instrument, centrifugation at $18,000 \times g$ was applied at 4°C for 15 min to separate the milk fat (Rotanta 460 R; Hettich). The supernatant (720 μL) was added to a new centrifuge tube and mixed with 80 μL of phosphate buffer (pH 7.4; 1.5 M phosphate salt buffer, 5 mM sodium 3-trimethylsilyl-2,2,3,3,3- d_5 -propionate (TSP), 0.2% NaNO_3). The mixture was thoroughly shaken for 1 min. After centrifugation at 4°C for 15 min at $24,400 \times g$, 600 μL of the supernatant were transferred into an NMR tube (5 mm; Norell ST500; Norell Inc.).

The NMR spectra were acquired on a Bruker Avance III HD spectrometer, operating at 500.23 MHz. The spectrometer was equipped with a 5-mm broad-band fluorine observation SmartProbe and autosampler (Bruker BioSpin) and was controlled by Topspin 3.57 software (Bruker BioSpin). The Bruker standard pulse sequence for one-dimensional nuclear Overhauser enhancement spectroscopy with water peak suppression (*noesypr1d*) at a temperature of 298 K (25°C) was applied. The ^1H NMR spectra were measured by using the following acquisition parameters: relaxation delay = 1 s; acquisition time = 4.00 s; mixing time = 100 ms; spectral width of 8,000 Hz; over 32,000 data points obtained by using 128 scans and 4 dummy scans. Based on standard Bruker routines, the NMR conditions were optimized by using automated tuning, locking, gaining, shimming, and calibration of 90° pulses. The free induction decay was multiplied by a 0.3 Hz line broadening function.

For multivariate analysis, NMR spectra were Fourier-transformed (**FT**), phased, zero-filled to 64,000 data-points, aligned to the TSP at 0.0 ppm, and baseline corrected by using the Splines algorithm in the MestReNova 14.0.1 software package (Mestrelab Research). The spectra were then normalized to the average intensity of the internal standard, and their resolution was reduced to 232 bins with equal width of 0.04 ppm, excluding the solvent signals (1.02–1.3 and 3.66–3.70 ppm) and water signal (4.70–4.98 ppm).

Peaks from the discriminating bins were assigned as described below. The FT spectral datapoints in the ranges of 2.05 to 2.07 ppm (*N*-acetyl carbohydrates),

2.42 to 2.66 ppm (citrate), 5.34 to 5.38 ppm (unknown compound), and 6.18 to 6.20 ppm (orotate) were exported into Microsoft Office Excel 2016, in which the regions were precisely baseline corrected by using a zero-degree polynomial function, the peaks were integrated, and the integral values were normalized to the integral of TSP.

Metabolites in the milk profiles were assigned in the ¹H NMR spectra by using authentic standards (betaine, carnitine, fucose, galactose-1-phosphate, lactulose, *N*-acetylgalactosamine, *N*-acetylglucosamine, taurine, and tyrosine), all purchased from Sigma–Aldrich and Alfa Aesar. Signatures from these authentic standards were constructed in Chenomx NMR Suite 8.6. professional edition software (Chenomx Inc.) and were used for quantification and confirmation. All other compounds were confirmed by using the Chenomx built-in library ver. 10.0, the Human Metabolome Database (<https://hmdb.ca/>; Wishart et al., 2018), and previous references (Klein et al., 2010; Sundekilde et al., 2013; Yanibada et al., 2018; Garwolińska et al., 2020).

Statistics

The characteristics of the experimental bulk tank samples of pure bovine and pure caprine milk were compared by univariate statistical methods by using the 2-tailed Student's *t*-test in Microsoft Excel 2016 (Microsoft Corp.).

Multivariate statistical analyses were carried out with the online processing tool for metabolomics data, MetaboAnalyst 4.0 (<https://www.metaboanalyst.ca/>; Chong et al., 2019). Principal component analysis (PCA) and orthogonal projections to latent structures discriminant analysis (OPLS-DA) were applied to the mean-centered binned data of the pure bovine and caprine milk samples. Before the multivariate analysis, data were scaled by using auto scaling. The PCA was used to examine the overall arrangement structure in terms of the similarities and differences between samples. To determine the key features in the spectra for distinction between caprine and bovine milk, OPLS-DA was performed to improve the significant metabolite variations associated with complex exposures, as well as to reduce the complexity of the model. Furthermore, OPLS-DA was conducted on the 4 peak integral values of the major biomarkers of bovine milk to select the most robust, with a significant difference between 100% caprine milk and the lowest possible percentage of bovine milk. Validation of the OPLS-DA models was performed by the random permutation test based on 1,000 permutations. Characteristics evaluating the OPLS-DA included the goodness of fit (R^2Y) and predictive ability (Q^2). A color-coded ¹H NMR spectrum corresponding plot

based on the *p*(corr) value from the above-mentioned OPLS-DA was constructed in MATLAB R2020a software (MathWorks Inc.).

The ability of the biomarker to represent the level of adulteration was assessed from the validation correlation coefficient by using leave-one-out cross-validation (LOOCV). Based on the LOOCV process, *n*–1 correlation coefficients were obtained. This set of values was used to obtain the estimation of correlation coefficient as a mean value with its confidence interval.

The univariate receiver operating characteristic (ROC) curve analysis was used to calculate the performance of the *N*-acetyl carbohydrate signal as a biomarker. The ROC curve enabled the decision about the cutoff point separating positive (pure bovine milk or caprine milk adulterated by bovine) and negative (pure caprine milk) cases. Sensitivity, specificity, and accuracy were determined by true positive (TP; number of samples correctly identified as pure bovine milk or caprine milk adulterated by bovine milk), true negative (TN; number of samples correctly identified as pure caprine milk), false positive (FP; number of samples incorrectly identified as pure bovine milk or caprine milk adulterated by bovine milk), and false negative (FN; number of samples incorrectly identified as pure caprine milk) results. The ability of the biomarker to distinguish between positives and negatives was evaluated by measuring the area under the curve (AUC). The AUC values were evaluated according to the following scale: an AUC of 1.0 indicated a perfect test, 0.9 to 0.99 was an excellent test, 0.8 to 0.89 was a good test, 0.7 to 0.79 was a fair test, and <0.7 was not a useful test (Carter et al., 2016). To evaluate the ability of the test to classify the positive and negative values, the data set was split randomly into training and validation sets by using *k*-fold cross-validation. The *k*-fold cross-validation was performed by dividing the data set into 9 groups chosen randomly (*k* = 9). Eight of these 9 groups were used as the training data set to set the ROC curve and to assess the AUC values. The last group was the validation data set. This procedure was repeated 9 times; each time, a different group was used for validation. The sensitivity, specificity, and accuracy were computed to measure the quality of the classification process based on the cutoff point provided by the ROC curve to distinguish between pure caprine milk and caprine milk adulterated by bovine milk. The optimum cutoff point was set by the highest sum of sensitivity and specificity. The utility of the *N*-acetyl carbohydrate signal for the detection of caprine milk adulterated by bovine milk was evaluated in a blind test according to the formula mentioned by Dalabasmaz et al. (2019). Unless stated otherwise, statistical analysis was performed with the IBM SPSS Statistics software

(Version 26.0; SPSS Inc.). The level of significance was set at 0.05 for all tests.

RESULTS AND DISCUSSION

Characteristics of Experimental Samples of Caprine and Bovine Milk

The mean values, variability, and baseline statistical comparisons for pure bovine and pure caprine milk are given in Table 2. Based on analysis of the quality and hygiene indicators, components, and properties of the milk, the samples were found to be good representatives of local milk and suitable for further analysis. The values obtained as characteristics of bovine and caprine milk correspond in general terms to the values given in reference sources (Morand-Fehr et al., 2007; Park et al., 2007; Pirisi et al., 2007; Raynal-Ljutovac et al., 2008).

Biomarkers for Detection of Bovine Milk in Caprine Milk

To select potential biomarkers from the ¹H NMR spectra, PCA, and OPLS-DA were performed on 0.04-ppm spectral bins. For this purpose, 46 pure bovine and caprine milk samples were used. The PCA results showed that the metabolite profiles of these milk samples are separated in the 2 classes (Figure 1A). The first 2 principal components provide 22.2 and 16.0% of the total variance. The value of variance is 38.2%, which is attributable to many independent factors in the data set. For better discrimination of the significant biomarkers, an OPLS-DA supervised method was applied to the same data used for the PCA (Figure 1A). The score plot of the OPLS-DA demonstrated clear separation of the caprine and bovine milks with a goodness of fit $R^2Y = 0.989$ ($P < 0.001$) and predictive ability $Q^2 = 0.964$ ($P < 0.001$; Figure 1B). These characteristics obtained from the random permutation test indicated that the model had very high explanatory and predictive ability with good concordance of data. The corresponding coefficient color-coded plot based on the $p(\text{corr})$ correlation coefficients revealed bins that were more abundant in bovine milk and may serve as biomarkers of adulteration (2.06, 2.50–2.66, 5.34, and 6.18 ppm; Figure 1C).

One-dimensional ¹H NMR spectra were subject to metabolite profiling. Fifty-two metabolites were identified in either caprine, bovine milk, or both. The representative spectra for all of the analyzed milk samples are shown in Figure 2, with the corresponding proton chemical shifts, metabolite assignments, and sources of identified metabolites listed in Table 3. The discriminating bins from the OPLS-DA contained signals from

Table 2. Composition and quality indicators for the original experimental bulk tank milk samples from goats (n = 23) and cows (n = 23) as the basic materials for the identification of caprine milk adulteration by bovine milk (milk multicomponent matrix)

Item	Unit	Caprine milk					Bovine milk					P-value
		Arithmetic mean	Geometric mean	SD	Minimum	Maximum	Arithmetic mean	Geometric mean	SD	Minimum	Maximum	
TCM ¹	cfu/mL	48 × 10 ⁴	62 × 10 ³	19 × 10 ⁵	20 × 10 ²	90 × 10 ⁵	54 × 10 ³	12 × 10 ³	13 × 10 ⁴	10 × 10 ²	57 × 10 ⁴	NS
Log TCM		4.80		0.74	3.30	6.95	4.09		0.72	3.00	5.76	<0.01
SCC	10 ³ cells/mL	1,329	970	993	195	3,824	228	154	252	13	1,190	<0.001
Log SCC		2.99		0.38	2.29	3.58	2.19		0.40	1.11	3.08	<0.001
Fat	g/100 mL	3.83		0.74	2.61	6.09	4.25		0.95	2.11	6.32	NS
CP content	%	3.23		0.195	2.76	3.64	3.38		0.21	2.85	3.76	<0.05
Casein content	%	2.75		0.19	2.34	3.14	2.71		0.25	1.95	3.12	NS
Lactose	%	4.61		0.18	4.38	5.07	5.05		0.08	4.85	5.26	<0.001
SNF content	%	8.51		0.29	8.02	8.98	9.01		0.23	8.33	9.37	<0.001
TS content	%	12.27		0.83	10.52	14.13	13.15		0.95	10.77	14.61	<0.01
Urea concentration	mg/100 mL	63.93		19.86	35.15	114.77	35.11		7.23	22.66	48.83	<0.001
pH value		6.59		0.09	6.47	6.83	6.65		0.03	6.59	6.72	<0.01
Titration acidity	°SH	5.97		1.41	3.67	8.95	7.00		1.07	4.72	8.82	<0.01
RCT ²	s	84		34	44	178	487		55	356	579	<0.001
Crude firmness	cm	1.87		0.08	1.71	2.00	1.91		0.04	1.80	2.00	NS
Milk fermentation ability	°SH	33.11		5.79	17.39	41.19	30.75		3.28	25.35	39.62	NS

¹Total count of mesophilic microorganisms.

²Rennet coagulation time of lactoproteins.

4 compounds: *N*-acetyl carbohydrates (2.05–2.07 ppm; singlet), citrate (2.48–2.70 ppm; 2 doublet signals), an unknown compound (5.34–5.38; quartet), and orotate (6.19–6.20 ppm; singlet). The peak of the *N*-acetyl carbohydrates was further assigned, by spiking with authentic standards, as the signal of the acetyl moieties of 2 related compounds: *N*-acetylglucosamine and *N*-acetylgalactosamine.

The integrals of these 4 signals were submitted as variables to another OPLS-DA, in which the $p(\text{corr})$ correlation coefficients for all combinations of bovine–caprine pairs were compared (Supplemental File

S1 Table 1; <https://doi.org/10.5281/zenodo.4779047>; Rysova et al., 2021). It has been found that the *N*-acetyl carbohydrate peak had the highest scores by far and, therefore, had utility as a single highly specific biomarker (Figure 3).

Role of *N*-Acetyl Carbohydrates in Bovine and Caprine Milk

In this study, it was found that bovine milk was rich in *N*-acetylglucosamine and *N*-acetylgalactosamine, whereas these compounds were almost completely

Figure 1. (A) Principal component analysis (PCA) score plot, (B) orthogonal projections to latent structures discriminant analysis (OPLS-DA) score plot, and (C) OPLS-DA corresponding color-coded coefficient plot of median proton nuclear magnetic resonance (^1H NMR) spectra for pure bovine ($n = 23$) and caprine milk ($n = 23$). The filled red circles represent the 95% confidence region for caprine milk, and the filled blue circles represent the 95% confidence region for bovine milk. The color of the signals in the coefficient plot signifies the contribution of the metabolites toward class segregation between pure bovine milk and pure caprine milk. The color of the bar indicates the correlation loading of the bin $p(\text{corr})$ value. The size of the bar denotes the $p(\text{corr})$ multiplied by SD.

Figure 2. Median proton nuclear magnetic resonance (^1H NMR) spectra of pure caprine milk (top) and pure bovine milk (bottom) including metabolite assignment (see Table 3). Spectra were referenced to the internal sodium salt (TSP) signal at 0 ppm. Solvent signals ($\text{C}_2\text{H}_6\text{O}$; 1.06–1.46 and 3.64–3.71 ppm) and water suppression artifacts (4.75–4.98 ppm) were removed.

lacking in caprine milk. These compounds are likely part of the oligosaccharide fraction of the milk; their role in milk is largely unknown and has been shown to vary between species and dairy breeds (Sischo et al., 2017; Giorgio et al., 2018). It has been shown that the total concentration of oligosaccharides in caprine milk (0.25–0.30 g/L) is low compared with that in human milk (5–8 g/L); however, the total content should be even lower in bovine and ovine milk (0.03–0.06 and 0.02–0.04 g/L, respectively; Martinez-Ferez et al., 2006). Therefore, the presence of *N*-acetyl carbohydrates in higher quantities in bovine milk suggests the presence of some specific oligosaccharides with a higher degree of *N*-acylation, rather than reflecting the total content. Recent studies have suggested that *N*-acetyl carbohydrates may be affected by the stage of lactation in dairy cows, as evidenced by Tomassini et al. (2019). It was found that the *N*-acetylglucosamine peak increased in late lactation, suggesting significant qualitative and quantitative changes in oligosaccharide synthesis during lactation. These results were also confirmed by Zhu et al. (2020a). The presence of *N*-acetylglucosamine and *N*-acetylgalactosamine in bovine milk in connection to freeze-drying and subsequent storage was also described by Zhu et al. (2020b). The *N*-acetyl carbohydrates in caprine milk have also been studied in relation to the milk metabolome and the anti-adhesive effect of these compounds on bacterial, viral, and protozoan pathogens, preventing them from binding to mammary tissue. Salama et al. (2020) stated that the *N*-acetyl carbohydrate content increased in response to infection to boost the mammary immune system. In a previous study, *N*-acetyl carbohydrates were also used as 1 of 10

metabolites for the multivariate model for the detection of bovine milk in caprine milk. Li et al. (2017) reported higher amounts of this group of compounds in bovine milk ($P < 0.05$) but did not specify the type of sugar moiety. *N*-Acetyl carbohydrates can also originate from catabolism of glycoprotein. As part of the glycoprotein, oligosaccharides are not detectable by NMR spectroscopy, however, during the proteolysis, glycans can be cleaved off from the protein structure and become detectable. Increased amounts of *N*-acetylglucosamine and *N*-acetylgalactosamine may be associated with longer milk coagulation time (Sundekilde, 2012; Sundekilde et al., 2014). The role of *N*-acetylcarbohydrates in caprine and bovine milk remains unclear; other analysis is needed to further understand it.

ROC Curve Analysis and Optimization of Classification Threshold

Before the evaluation of ROC curve analysis, the significance of the relationship between the *N*-acetyl carbohydrates and the percentage of bovine milk was confirmed by the validation correlation coefficient ($r = 0.9344$ with 95% CI from 0.9342 to 0.9347) based on the LOOCV process. The previously mentioned values indicate that there is high positive correlation between bovine milk and *N*-acetyl carbohydrates. Arithmetic mean, standard error, minimum and maximum of peaks integral values of *N*-acetyl carbohydrates are given in Supplemental File S1 Table 2 (<https://doi.org/10.5281/zenodo.4779047>; Rysova et al., 2021).

To determine the performance of *N*-acetyl carbohydrates as a biomarker, the matrix of adulterated

Table 3. Chemical shifts (ppm), assignments, and sources of median proton nuclear magnetic spectra (¹H NMR) signals of 52 metabolites in caprine and bovine milk (spectra in Figure 2)

No.	Metabolite	¹ H Chemical shift (ppm)	Assignment
1	2-Oxoglutarate ¹	2.99	CH ₂
2	Acetate ²	1.90	CH ₃
3	Acetone ²	2.22	2 × CH ₃
4	Alanine ^{1,2}	1.47	CH ₃
5	Betaine ³	3.26	3 × CH ₃
6	Butyrate ²	3.88	CH ₂
		1.54	CH ₂
		2.13	CH ₂
7	Caprate ¹	1.27	CH ₂
		2.16	CH ₂
		0.85	CH ₃
8	Caprylate ¹	1.27	CH ₂
		2.16	CH ₂
		0.85	CH ₃
9	Carnitine ³	2.44	CH ₂
		3.21	3 × CH ₃
		3.43	N-CH ₂
10	<i>cis</i> -Aconitate ²	5.69	CH
11	Citrate ²	2.52	CH ₂
		2.65	CH ₂
12	Creatine ^{1,2}	3.02	CH ₃
		3.92	CH ₂
13	Creatinine ^{1,2}	3.03	CH ₃
		4.05	CH ₂
		3.13	N-CH ₂
14	Ethanolamine ^{1,2}	8.44	CH
15	Formate ²	1.25–5.21	Multiple
16	Fucose ³	3.47–5.26	Multiple
17	Galactose ²	5.50	CH-1
18	Galactose-1-phosphate ³	4.02	CH
19	Gluconate ^{1,2}	3.23–5.22	Multiple
20	Glucose ²	3.39–5.44	Multiple
21	Glucose-1-phosphate ²	3.26–5.22	Multiple
22	Glucose-6-phosphate ²	2.10	CH ₂
23	Glutamate ¹	2.32	CH ₂
		2.13	CH ₂
		2.43	CH ₂
24	Glutamine ¹	7.54	CH-2,6
		7.63	CH-3,5
		7.82	CH-4
25	Hippurate ²	3.19	3 × CH ₃
		4.05	O-CH ₂
		0.91	CH ₃
26	Choline ²	0.99	CH ₃
		4.12	CH
27	Lactate ²	3.28–5.22	Multiple
28	Lactose ²	4.01–4.43	Multiple
29	Lactulose ³	3.1	CH ₂
30	Malonate ²	3.26–5.39	Multiple
31	Maltose ²	2.14	S-CH ₃
32	Methionine ²	2.02–2.10	CH ₃
33	<i>N</i> -Acetyl carbohydrates ³	3.21	3 × CH ₃
34	<i>O</i> -Phosphocholine ²	4.16	O-CH ₂
35	Orotate ¹	6.19–6.20	CH
36	Phenylalanine ¹	7.29	CH-2,6
		7.41	CH-3,5
37	Phosphocreatine ^{1,2}	3.03	CH ₃
38	Pyruvate ¹	2.35	CH ₃
39	<i>sn</i> -Glycero-3-phosphocholine ^{1,2}	3.21	N-(CH ₃) ₃
40	Succinate ²	4.31	O-CH ₂
41	Sucrose ²	2.39	2 × CH ₂
42		3.46–5.39	Multiple

Continued

Table 3 (Continued). Chemical shifts (ppm), assignments, and sources of median proton nuclear magnetic spectra (¹H NMR) signals of 52 metabolites in caprine and bovine milk (spectra in Figure 2)

No.	Metabolite	¹ H Chemical shift (ppm)	Assignment
43	Taurine ³	3.26	S-CH ₂
		3.42	N-CH ₂
44	Threonine ¹	1.31	CH ₃
45	Tyrosine ³	6.88	CH-3,5
		7.18	CH-2,6
46	Uridine diphosphate galactose ^{1,2}	1.02–7.94	Multiple
47	Uridine diphosphate glucose ^{1,2}	3.46–7.94	Multiple
48	Uridine diphosphate <i>N</i> -acetylglucosamine ²	2.7–8.33	Multiple
49	Uracil ³	5.79	CH-5
50	Urea ²	5.79	NH ₂
51	Uridine ²	5.92	CH-2,10
		7.86	CH-11
52	Valine ²	0.98	CH ₃
		1.02	CH ₃

¹Literature (Klein et al., 2010; Sundekilde et al., 2013; Wishart et al., 2018; Yanibada et al., 2018; Garwolińska et al., 2020).²Chenomx Inc.³Standard.

samples with integrals of relative intensities of the region between 2.05 and 2.07 ppm was subjected to ROC curve analysis (Figure 4). Overall, 5 ROC analyses were conducted to examine accuracy in distinguishing the caprine 100% from bovine 1%, bovine 5%, bovine 10% and bovine 100%, including the analysis of bovine 5% and bovine 10% groups. Each group contained 23 observations. Before the analysis, each data set was divided into 9 subsets following the principles of *k*-fold cross-validation, as introduced in the Materials and Methods section.

The diagonal line for the pair of caprine 100% and bovine 1% indicates an AUC ranging from 0.454 to 0.528 with an accuracy of <50%; this result represents a chance result with no significance (Figure 4A2). The performance of the model for the pair of caprine 100% and bovine 1% was not further classified. As another pair, caprine 100% and bovine 5% were plotted against each other (Figure 4B2). Although the discriminatory power was not confirmed in the first model, the integrals of relative intensities of *N*-acetyl carbohydrates produced optimal detection of 5% bovine milk in caprine milk, with AUC values ranging from 0.908 to 0.952 ($P < 0.001$). The AUC values obtained from the ROC curve analysis can be considered as an excellent test, with 86.96% sensitivity and 82.61% specificity. Higher sensitivity than specificity means that the model better recognized adulterated milk (FP > FN). Further ROC curve analysis was performed to evaluate the AUC values for *N*-acetyl carbohydrates between the

caprine 100% and bovine 10% pair (Figure 4C2). The AUC values ranged between 0.992 and 1 ($P < 0.001$); thus, model can be described as an almost perfect test. The sensitivity and specificity in this case had identical values of 95.96%. According to the performance of the latter ROC curve analysis, the expected performance of the model between pure bovine milk and pure caprine milk was confirmed. The comparison of these 2 groups represented a perfect test with an AUC value of 1 ($P < 0.001$; Figure 4D2).

ROC curve analysis was also constructed to assess the cutoff points for 5 and 10% adulteration by bovine milk and for pure bovine milk (Table 4), which are related to the specificities and sensitivities listed above. The cutoff points were determined as the best trade-offs of sensitivity and specificity. However, it is not always necessary to use a compromise between sensitivity and specificity to determine the cutoff point; the specific aims of the analysis may require cutoff points to be adjusted for either greater sensitivity at the expense of specificity or the converse (Carter et al., 2016). For a lower detection limit than 5% bovine milk in caprine milk, different methods are clearly advantageous, such

as the immunochemical method developed by Hurley et al. (2006), which is capable of unbiased detection of 0.01% bovine milk in caprine milk or method based on the duplex polymerase chain reaction by Rodrigues et al. (2012) with detection limit of 0.5% of bovine milk. It should be noted that a content of bovine milk below 5% is unlikely to be a targeted adulteration but rather an accidental impurity, especially if the farm raises both large and small ruminants or the dairy factory is used to process various types of milk.

The last ROC curve analysis of evaluation of 5% bovine (negative cases) against 10% bovine (positive cases) was used to evaluate whether *N*-acetyl carbohydrates could differentiate the percentage of adulteration. The AUC was 0.871 to 0.914 ($P < 0.001$), a result referred to as a good test with an overlap of values into an excellent test (Figure 4E2). The values of 86.95% sensitivity and 78.26% specificity are related to the selected optimal cutoff point of 0.0862 that represents the integral of relative intensities of *N*-acetyl carbohydrates. Higher sensitivity than specificity indicates a better detectability of samples with a higher percentage of bovine milk, which also confirms the number of

Figure 3. Proton nuclear magnetic resonance spectra overlay of pure caprine milk ($n = 23$; red) and pure bovine milk ($n = 23$; blue) in the region of the *N*-acetyl carbohydrate signals.

Figure 4. (A1–E1) Confusion matrices of receiver operating characteristic (ROC) analysis (TP = true positive, TN = true negative, FP = false positive, FN = false negative). (A2–E2) The ROC curve analyses calculated from integrals of relative intensities of the 2.05–2.07 ppm spectral region resonance peaks of *N*-acetyl carbohydrates. (A3–E3) Box plots showing the separation of pairs based on the integrals (red = pure caprine milk; dark blue = pure bovine milk; pale blue = caprine milk with 1, 5, or 10% bovine milk); boxplots show the lower and upper quartile, median, minimum, and maximum.

Table 4. Area under the curve (AUC) and optimal cutoff point of 3 categories of milk samples determined by receiver operating characteristic (ROC) curve analysis

Sample identification	AUC	Optimal cutoff point ¹
Bovine 5%	0.908–0.952	0.060
Bovine 10%	0.992–1	0.072
Bovine 100%	1	0.337

¹Integrals of relative intensities of *N*-acetyl carbohydrates.

samples designated as FP. This result opens the way to finding the thresholds for other categories of adulteration (>10%).

The capability of *N*-acetyl carbohydrates to act as biomarkers for the detection of caprine milk adulteration with bovine milk was evaluated in a blind test according to the method of Dalabasmaz et al. (2019). The blind test was performed with 20 external milk samples in 4 categories, 5 samples in each: pure caprine milk, pure bovine milk, caprine milk with 5% bovine milk, and caprine milk with 10% bovine milk (Supplemental File S1 Table 3; <https://doi.org/10.5281/zenodo.4710195>; Rysova et al., 2021). Based on the suggested cutoff points shown in Table 4, the percentage of adulteration was predicted. The accuracy of prediction was calculated for the pairs that are mentioned in Figure 4B2, 4C2, 4D2. The accuracy obtained from the blind test reflected the results from the ROC curve analysis. The distinction between pure bovine milk and pure caprine milk, as well as the distinction between pure caprine milk and caprine milk adulterated with 10% bovine milk, was ideal, with 100% accuracy. The 5% adulterated caprine milk was predicted with 80% accuracy (details not shown), owing to the lower classification sensitivity of NMR spectroscopy over other analytical methods (Karoui and De Baerdemaeker, 2007; Kamal and Karoui, 2015).

NMR Spectroscopy in the Detection of Milk Adulteration

Recent literature has reported the ability of NMR spectroscopy to detect the addition of cheaper milk; a 2017 study (Li et al., 2017) showed detection of the adulteration of caprine milk with bovine milk at a concentration of 5% by incorporating 10 variables into a mathematical model. Although the authors of that study achieved a similar result to ours, their method was not intended for practical use, as evidenced by the fact that only milk treated at ultrahigh temperature was used. The detection of adulteration of milk from another small ruminant, specifically sheep, has also been reported (Lamanna et al., 2011). The combined approach of NMR profiling and an artificial neural

single-layer network was used to detect bovine milk in ovine milk. Although the correspondence between the mixture content and the artificial neural single-layer network was high, the lowest mixing ratio was only 20% bovine milk in ovine milk. No other previous studies have examined the feasibility of the NMR chemometric approach for detecting caprine milk adulteration by bovine milk.

CONCLUSIONS

In this study, an NMR chemometric approach combined with multivariate and univariate analysis identified *N*-acetylglucosamine and *N*-acetylgalactosamine as biomarkers for uncovering fraud with caprine milk. It was found that a high positive correlation exists between the *N*-acetyl carbohydrates and the percentage of bovine milk. Subsequent models constructed by using ROC curve analysis determined the threshold value of *N*-acetyl carbohydrates to detect 5% bovine milk in caprine milk with 84.78% accuracy and 10% bovine milk in caprine milk with 95.65% accuracy. The fact that most adulteration by cheaper milk occurs at between 10% and 50% makes this method particularly useful. The present findings show a promising utility of NMR spectroscopy for routine analysis in the control of caprine milk authenticity by inspection authorities in the Czech Republic.

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