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ORIGINAL PAPER

NMR spectroscopy as a screening tool to validate nutrition labeling of milk, lactose-free milk, and milk substitutes based on soy and grains

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Abstract Lactose-free milk and milk substitutes based on soy, oat, or rice are widely marketed to lactose-intolerant consumers. In this study, 400 MHz ^{1}H nuclear magnetic resonance (NMR) spectroscopy was used in the context of food surveillance to validate the "lactose-free" claims labeled on these beverages. Using soft independent modeling of class analogy (SIMCA) analysis, a qualitative classification according to the type of beverage (lactose-containing milk, lactose-free milk, oat, soy, and rice milk substitutes) was possible. Furthermore, quantitative data regarding nutrition labeling parameters were predicted from the same spectra using partial least squares (PLS) regression. The models obtained for carbohydrate, sugars, protein, fat, saturates, and energy (R^2=0.89–0.97) were suitable for a screening analysis. Using nicotinamide as an internal standard, quantitative determination of lactose with a detection limit of 0.03 g.L^{-1} was also possible using direct integration of the signals (linear range, 0.05–50.0 g.L^{-1}, R>0.999). The relative standard deviations for the lactose-free milks were below 10%. NMR spectroscopy was judged to be suitable for the rapid routine analysis of milk and milk substitutes.

核磁共振(NMR)法确证乳、脱乳糖乳和代乳食品营养标签的合法性

摘要 脱乳糖乳和以大豆、燕麦和谷物为基料的代乳食品是乳糖不耐症消费者广泛青睐的食品。本研究利用核磁共振波谱(400 MHz ^{1}H)检测那些标注"无乳糖"饮料的合法性。利用软独立建模(SIMCA)分类法对饮料的类型(含乳糖乳,脱乳糖乳以及大豆、燕麦和谷物为基料的代乳食品)进行了定性分类。而且,关于这些营养标注产品的定量数据可以从相同的光谱中利用偏最小二乘回归(PLS)来预测。该模型的建立对碳水化合物、糖、蛋白质、脂肪、饱和

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烃以及能量(R^2 = 0.90–0.97)都可以用来进行检测分析。以烟碱作为内标物,乳糖定量分析的检测限是0.03 g. L^{-2},可以直接对检测信号进行积分(线性范围为0.05–50.0 g.L, R>0.999)。脱乳糖乳产品检测的相对标准偏差低于10%,因此,NMR光谱法适合对乳以及代乳食品进行快速常规的分析。

Keywords NMR spectroscopy · Lactose · Lactose intolerance · Milk · Milk substitutes · Soy milk · Dairy products

关键词 核磁共振光谱 · 乳糖 · 乳糖不耐症 · 乳 · 代乳食品 · 豆奶 · 奶产品

1 Introduction

Due to health problems associated with cow's milk consumption, which include lactose intolerance (Montalto et al. 2006) or milk allergy (Crittenden and Bennett 2005), or due to general ethical considerations against drinking cow's milk (Beardsworth and Keil 1992), the market for milk substitutes has increased in recent years (Jelen and Tossavainen 2003). All these surrogate products are characterized as being white, milk-like emulsions with milk-similar properties and applications, most commonly based on soy or grains. Besides the milk-free substitutes, lactose-free products based on cow's milk are also available, in which the lactose has been enzymatically decomposed to glucose and galactose (Adhikari et al. 2010).

The most common nutrition claim found on this product category is "lactose-free." This is not surprising considering that more than 70% of the world's population are lactose intolerant, which varies by race and age (Montalto et al. 2006). Lactose intolerance occurs when the human body is unable to produce the lactase enzyme required to break down lactose to glucose and galactose for the body to metabolize. As a result, the lactose is fermented in the intestine where it can produce symptoms ranging from mild unpleasant conditions, such as abdominal gas or bloating, to severe diarrhea. On the other hand, the milk substitutes can also cause adverse health effects such as soy allergy or gluten intolerance (Hodge et al. 2009; Savage et al. 2010).

So far, most efforts have been dedicated to the qualitative, technological, and nutritional characterization of ordinary milks (pasteurized, ultra high temperature), whereas characterization of dietetic milks and milk substitutes has received very little attention (Adhikari et al. 2010). This study therefore aimed to develop a reliable analytical tool to control the absence of lactose in such products as well as to confirm further nutrition claims found on the labelings.

There are several methods capable of measuring the lactose content in raw milk. The most frequently used technique for lactose determination in milk and dairy products is an enzymatic assay based on spectrophotometric measurement, which is also the German reference method (Amtliche Sammlung von Untersuchungsverfahren 2010). Other approaches include biosensors (Ammam and Fransaer 2010), colorimetry (Bakos et al. 2002), near-infrared (NIR) spectroscopy (Kawasaki et al. 2008; Woo et al. 2002), Fourier transform infrared spectroscopy (Lefier et al. 1996), or chromatographic techniques (Eadala et al. 2009; Paredes et al. 2006; Schuster-Wolff-Bühring et al. 2011). Besides being comparably time-consuming, none of these methods appear to be appropriate for our purpose to provide a rapid screening of nutrition parameters. This

study will therefore evaluate nuclear magnetic resonance (NMR) spectroscopy, which is a powerful tool for the simultaneous identification and quantification of compounds in complex mixtures (Le Gall and Colquhoun 2003). Requiring little sample preparation, NMR allowed the direct and reliable determination of analytes in different food matrices, including beer (Lachenmeier et al. 2005), juices (Belton et al. 1998), grapes (Forveille et al. 1996), infant formulas (Lachenmeier et al. 2009), or pine nuts (Köbler et al. 2011). To the best of our knowledge, there have been only two papers published that give an insight into the potential of ^{1}H and ^{13}C NMR spectroscopy for quantitative analysis of lactose in conventional lactose-containing milk (Hu et al. 2007; Klein et al. 2010). ^{1}H NMR together with statistical approaches has been also recently used to differentiate milk according to its origin (Lamanna et al. 2011). This investigation will be the first to apply ^{1}H NMR spectroscopy with multivariate methods to characterize lactose-free milk and milk substitutes.

2 Materials and methods

2.1 Samples

Eighty-four milk samples and milk substitutes were analyzed using NMR. The sample collective comprised a wide range of styles, including 26 ordinary cow's milk and 24 lactose-free cow's milk. The milk substitutes were based on oat (n=10), rice (n=8), and soy (n=16). All samples were bought in August 2010 at local stores in Karlsruhe, Germany.

2.2 Sample preparation

For non-targeted analysis, 1 mL of milk or milk substitute sample is mixed with 100 μL of 3-(trimethylsilyl)-propionate acid-d_4 (TSP) solution [500 mg of the sodium salt of 3-(trimethylsilyl)-propionate acid-d_4 in 50 mL of D_2O]. The mixture (0.6 mL) is poured into an NMR tube and directly measured.

For quantification of lactose, 20 g of milk or milk substitute sample is placed into a centrifuge tube, and 1 mL of acetic acid (100%) is added. After 10 min, the solution is centrifuged for 10 min (20,000 rpm, 4 °C). One milliliter of the clear, aqueous phase is separated (the fat phase is discarded), and 100 μL of TSP solution (see above) as well as 100 μL of nicotinamide solution (500 mg in 50 mL of D_2O) are added. The mixture (0.6 mL) is poured into an NMR tube and directly measured. For calibration, lactose solutions are prepared [0.05, 0.1, 0.4, 0.6, 0.8, 1.0, 2.0, 3.0, 4.0, 5.0, 20.0 and 50.0 g. L^{-1}, containing 5% acetic acid (100%)] and prepared similarly to the samples with the exception of the centrifuge step, which is unnecessary for the calibrators.

2.3 ^{1}H NMR measurements at 400 MHz

All NMR measurements were performed on a Bruker Avance 400 Ultrashield spectrometer (Bruker Biospin, Rheinstetten, Germany) equipped with a 5-mm SEI probe with Z-gradient coils, using a Bruker automatic sample changer (B-ACS 120). All spectra were acquired at 300.0 K. For non-targeted analysis, NMR spectra were

acquired using the Bruker 1D noesygppr1d pulse sequence with 16 scans (NS) and two prior dummy scans. The sweep width was 20.0352 ppm, and the time domain of the free induction decay (FID) was 64k.

For acquisition of 2D J-resolved NMR spectra, the Bruker experiment jrespprgf was used. After application of 16 dummy scans (DS), eight FIDs (NS=4) were collected into a time domain of 8,192 (8.2k) complex data points using a 16.6595 ppm spectral width (SW) and a receiver gain (RG) of 28.5.

The data were acquired automatically under the control of ICON-NMR (Bruker Biospin, Rheinstetten, Germany), requiring about 12 min per sample. All NMR spectra were phased and baseline-corrected.

For non-targeted analysis, the full spectra were exported for multivariate data analysis. For targeted quantification of lactose in ordinary milks (1D spectra) and lactose-free milk (2D J-resolved spectra), the doublet at 4.44 ppm was integrated. Additionally, the doublet at 9.17 ppm of nicotinamide as internal standard was integrated. The samples were quantified using a linear calibration curve constructed with the lactose/nicotinamide ratios. Separate calibration curves were used for lactose-free products (2D J-resolved spectra) and normal milk (1D spectra; investigated range, 0.05–50.0 $g.L^{-1}$).

2.4 Non-targeted analysis and chemometrics

The resulting spectra were analyzed using the software Unscrambler X version 10.0.1 (Camo Software AS, Oslo, Norway). We tested several spectral regions for calculation: aliphatic (0.25–3 ppm), mid-field (3–6 ppm), aromatic (6–10 ppm) with 0.04 ppm bucket width and the region selective to lactose (4–5 ppm) with a bucket width of 0.01 ppm. Details on the bucketing process of NMR spectra for multivariate data analysis were previously described (Lachenmeier et al. 2005).

The technique of cross-validation was applied to determine the number of principal components (PCs) needed. Using partial least squares (PLS) regression, the NMR spectra were correlated with nutrition information from labeling, as no reference analyses could be conducted besides for lactose. PCA and PLS models were validated via full cross-validation.

Different classification methods integrated in the Unscrambler X software [soft independent modeling of class analogy (SIMCA), support vector machine (SVM), partial least square discriminant analysis (PLS-DA), and linear discriminant analysis (LDA)] were applied for classification. From these methods, SIMCA classification was evaluated for our data set in detail. SIMCA is based on building a PCA model for each class in a defined training set. The test set samples are then compared to the class models and assigned to classes according to their proximity to the training samples (Wold 1976). SIMCA is known as a supervised pattern recognition method as the individual PCA models define classification rules. The prediction ability of the SIMCA classification model was tested on 12 randomly chosen test set samples that were not included in the models.

2.5 Reference analysis for lactose

The analysis of lactose was performed using the German reference method (Amtliche Sammlung von Untersuchungsverfahren 2010) with a commercially

available lactose/D-galactose test kit (R-Biopharm AG, Darmstadt, Germany). The enzymatic analysis is based on the hydrolysis of lactose in the presence of the enzyme β-galactosidase and water. The formed D-galactose is oxidized by nicotinamide-adenine dinucleotid (NAD) to D-galactonic acid in the presence of β-galactose dehydrogenase. The amount of NADH formed is stoichiometric to the amount of lactose. The increase in NADH is measured by means of light absorbance at 340 nm. The enzymatic method was performed strictly following the manufacturer's test kit instructions.

2.6 Validation studies

For the validation, standard solutions as well as authentic milk (ordinary milk and lactose-free milk) samples were analyzed several times ($n=5$). The limit of detection (LOD) and quantification (LOQ) were determined according to DIN 32645 (1994) standard procedure. For all calculations, statistical significance was assumed at below the 0.05 probability level.

3 Results and discussion

3.1 Non-targeted analysis

Figure 1 shows the whole ^{1}H NMR spectra of lactose-containing and lactose-free milk as well as of a soy substitute. The spectra show extensive spectral overlap, especially in the aliphatic and mid-field regions. Although the investigated samples differ significantly in their composition, there are no single substances to which these differences can be attributed. Thus, a chemometric approach, such as PCA, is required to interpret the complex NMR signals of our beverages.

In our case, the best PCA model with regard to classification ability was obtained in the 6–3 ppm region. Figure 2 suggests that the samples can be separated into five groups: lactose-containing and lactose-free milk clusters are in the region of negative and positive PC2, respectively; oat and rice samples are in the positive values of PC1; and soy substitutes are located in the negative values of PC1 and positive PC3 values. It should be mentioned that, previously, this statistical method was used to discriminate NMR milk spectra into only two classes (Lamanna et al. 2011). Therefore, ^{1}H NMR combined with chemometrics can be used as a method to determine and monitor the type of milk and milk substitute samples.

3.2 Quantitative prediction of nutrition labeling parameters

Besides our major aims, the classification and quantification of lactose, it would be advantageous to control other nutrition labeling parameters such as carbohydrate, sugars, protein, fat, saturates, fiber content, and energy. As a first evaluation, if a quantitative approach is at all possible from the NMR spectra, we correlated different NMR ranges to the nutrition information provided by the manufacturers on the labeling. Parameters of the best-fitting PLS models are listed in Table 1. All models were verified through full cross-validation where one sample at a time is kept out of the calibration per iteration.

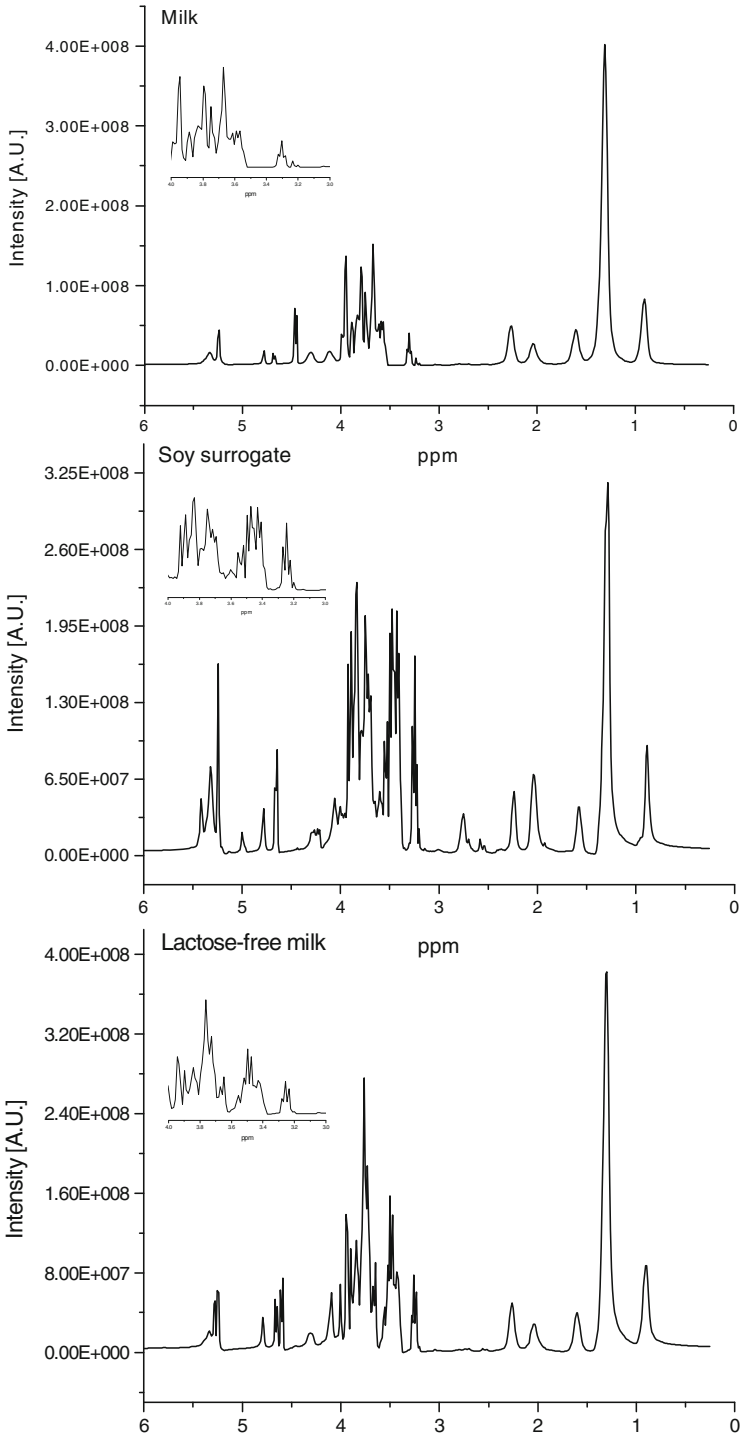

Fig. 1 ^{1}H NMR spectra of milk and lactose-free milk compared to a soy substitute. The *insert* shows ^{1}H NMR spectra in the 4–3 ppm region

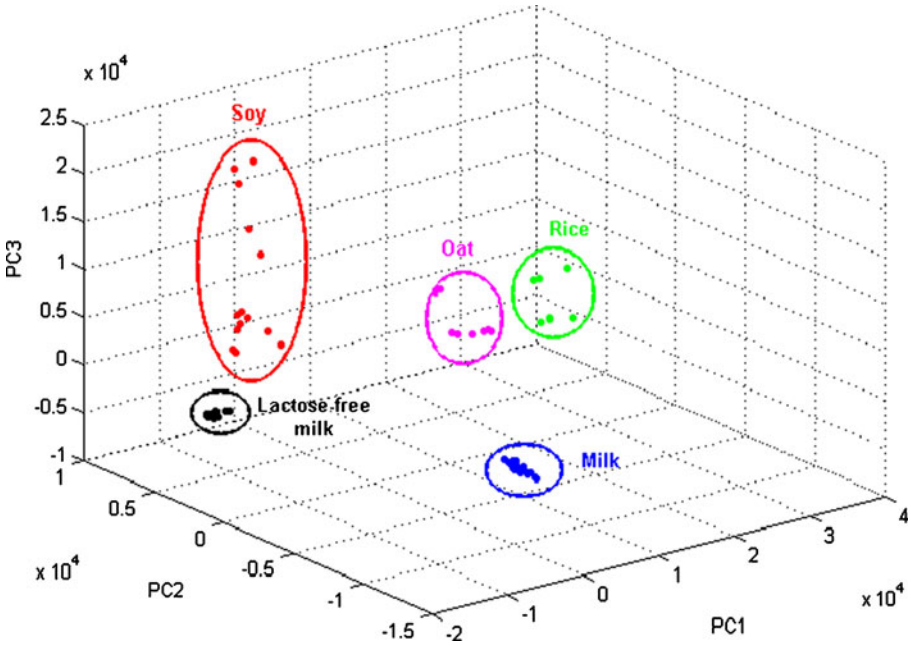

Fig. 2 Scatter plot of the PCA scores (6–3 ppm)

It came rather surprising that even without "real" reference values from own analyses, PLS models with comparably high correlations were obtained for carbohydrate, protein, fat, and saturates content (the values of R^2 are in the range of 0.95–0.97). Similar to our results in beer analysis, where we were able to quantify the major composition from NMR spectra (Lachenmeier et al. 2005), the main ingredients of milk show strong absorbances in the specified NMR ranges. The correlations for energy and sugars (R^2 are 0.90 and 0.89 correspondingly) also appear to be adequate for a screening procedure. Possibly, these models can be improved in the future by re-calibration using in-house reference analyses. An inadequate PLS model was only obtained for fiber

Table 1 PLS correlation between labeling parameters and NMR spectra

Parameter	Reference range	NMR range (ppm)	Calibration		Validation	
			RMSE[a]	R^2	RMSE[a]	R^2
Energy (kJ.100 mg^{-1})	79-296	3–0	14	0.90	17	0.86
Carbohydrate (g.100 mL^{-1})	0.2–11	6–3	0.42	0.97	0.48	0.96
Sugars (g.100 mL^{-1})	0.1–7.3	6–3	0.37	0.89	0.48	0.82
Protein (g.100 mL^{-1})	0.1–3.7	6–3	0.28	0.95	0.35	0.93
Fat (g.100 mL^{-1})	0.1–4.2	3–0	0.17	0.97	0.19	0.96
Saturates (g.100 mL^{-1})	0.1–2.8	3–0	0.16	0.97	0.19	0.95
Fibre (g.100 mL^{-1})	0.0–1.6	3–0	0.15	0.72	0.21	0.47

[a] Root mean squared error (RMSE) values are expressed in the same units as the nutrition labeling parameters

(Table 1). We believe that it is due to a small reference range and low variance in fiber content in the products studied. Future research (e.g., with artificially spiked samples) is needed for improved selection of ranges specific for fiber. On this stage, we judge the NMR procedure as a usable screening procedure to quantitatively control the labeling of the major nutrition parameters and to pre-select suspicious samples for confirmatory analysis by other methods.

3.3 Classification

It was shown that the combination of NMR spectroscopy and PCA can uncover key relationships and find clusters in the complex data. In our example, five classes were identified (Fig. 2). Next, it is interesting to show the predictive power of the chemometric methods by classifying new samples. To do this, different data analysis methods (SIMCA, SVM classification, LDA, and PLS-DA) were evaluated for predicting class membership of milk samples. We used NMR spectra as predictors. The preliminary investigation has shown that the SIMCA method performs better than other techniques applied for the classification of our samples.

The calibration set for SIMCA consists of 72 milk samples; the test set consisted of 12 objects (three for milk and lactose-free milk, two for oat, rice, and soy samples). First, PCA was performed on each class (milk, lactose-free milk, oat, rice, and soy) with the calibration sample set. PCA models with minimum prediction error and optimum number of PCs were used to classify samples from the test set. All samples were correctly recognized, but some samples were classified as belonging to two classes (false positive) at the 5% significance level. It was possible to reduce the number of false positive results by increasing the significance level (from 5% to 25%). In all cases, we did not observe false negative results. All samples were 100% correctly classified (no false positive, no false negative results) at the 25% significance level. Thus, our results have shown that the SIMCA method is a useful tool to provide appropriate classification of five classes of milk and milk substitutes. With this method, one can define the type of milk and milk substitutes with high probability.

3.4 Quantitative NMR method for lactose analysis

While the manufacturers of lactose-free milk typically specify on the labeling that the milk contains "less than 1 $g.L^{-1}$ of lactose" (without specifying the actual content), the German Food Chemical Society recently stated that the claim "lactose-free" would demand <0.1 $g.L^{-1}$ of lactose (Gesellschaft Deutscher Chemiker 2010). Our aim was therefore to provide a detection limit of <0.1 $g.L^{-1}$. Our experimental protocol for the non-targeted analysis (i.e., without any sample preparation) was able to provide sensitivities down to 0.5 $g.L^{-1}$ of lactose but not to the level of 0.1 $g.L^{-1}$. If this kind of sensitivity is necessary, a removal of the fat fraction by acid hydrolysis and centrifugation is necessary, which considerably reduced the matrix and improved the detection limit. The reason for this is the presence of fat, which leads to signal broadening. The emulsion also leads to a non-consistent distribution of the internal standard. After centrifugation, the emulsion is broken, and the solution is nearly fat-free, so that the resolution, signal shape, and distribution of internal standard are adequate for quantification. Furthermore, 2D J-resolved NMR

is necessary to quantify at the low levels found in lactose-free milk because in 1D the low signal/noise ratios lead to problems with integration (see Fig. 3a). Therefore, we used 2D J-resolved NMR for quantification of the lactose-free or lactose-reduced products, as this showed higher resolution and the peaks are easier to integrate (see Fig. 3b). With this protocol, the lowest calibrant at 0.05 g.L^{-1} still shows an NMR signal, which is more than 10× greater than the standard deviation of the noise.

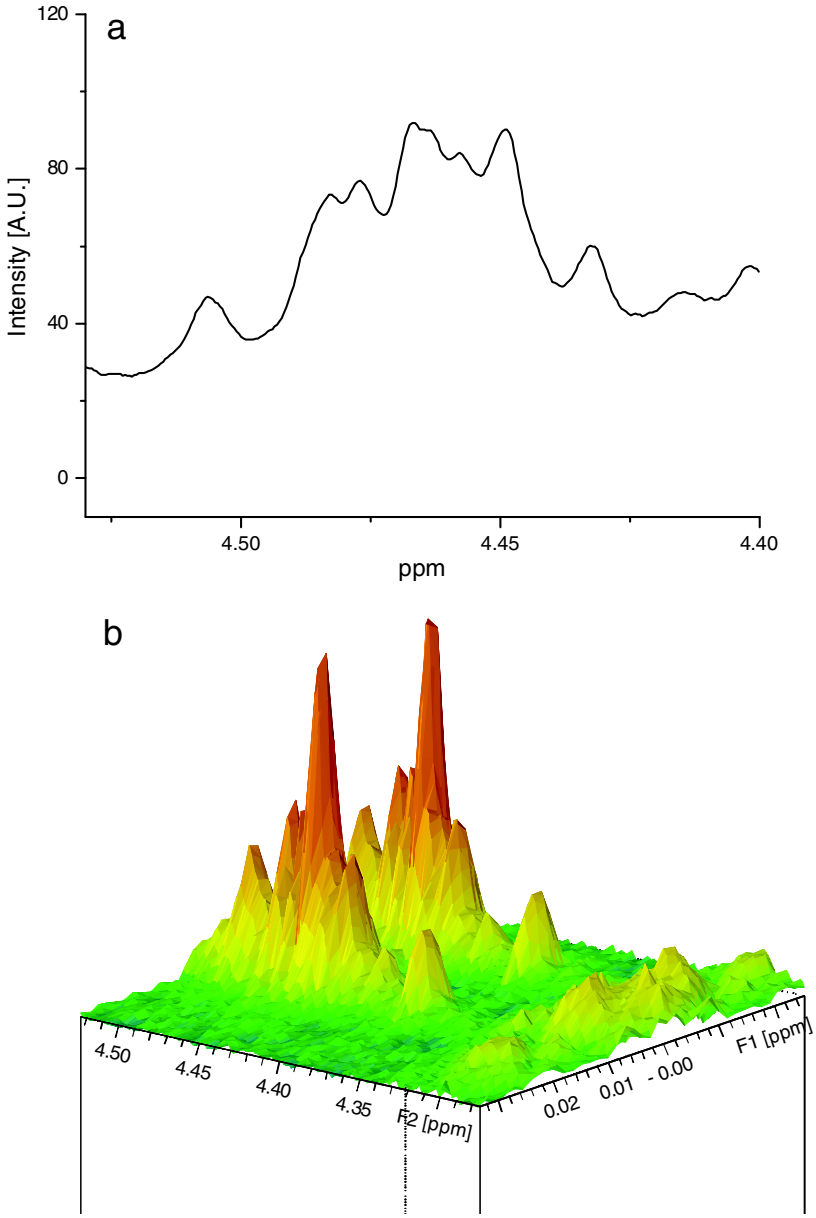

Fig. 3 1D (**a**) and 2D J-resolved (**b**) NMR spectra of lactose-free milk with a residual lactose content of 1 g.L^{-1} (the region of lactose doublet is shown)

To provide an accurate quantification, we evaluated several internal standards (nicotinamide, acetaldehyde, sucrose, and benzaldehyde) and found that nicotinamide suits best for quantification purposes. Nicotinamide is not contained in the milk matrix and leads to better quantitative results (i.e., with lower measurement uncertainty) than if TSP is used for internal standardization. The problem is that the area of TSP is not well correlated to the one of lactose, while nicotinamide is better correlated and shows signals exclusively in the low-field region, which is undisturbed by matrix signals.

A method validation for lactose was conducted. The linearity of the calibration curves was proven between 0.05 and 50 $g.L^{-1}$ (R>0.999), which is enough to provide an accurate quantification of lactose in lactose-free products as well as in ordinary milk samples. When determined according to DIN 32645 (1994), the values of LOD and LOQ are 0.03 and 0.06 $g.L^{-1}$ (using nicotinamide as internal standard), which offers the required sensitivity to control the limit. Validation of the method was further conducted by repeated sample preparation of standard samples and authentic milks (conventional cow's milk and lactose-free milk) (Table 2). For the standard samples and authentic cow's milk, the relative standard deviations (RSD) were always below 3%. For the lactose-free milks, the RSDs were slightly higher but still acceptably below 10%. The recovery rates were also acceptable for reliable lactose quantification. Therefore, NMR spectroscopy is capable of providing accurate lactose determination below the level recommended by the German Food Chemical Society.

The essential drawback of the majority of existing analytical methods for lactose determination in milk was that many of them could not detect low lactose concentration (below 0.5 $g.kg^{-1}$) (Ammam and Fransaer 2010; Sasic and Ozaki 2001) and could only be used for conventional lactose-containing milk samples. An attempt to use a chemometric PLS approach for lactose calibration in the NIR region was unsuccessful due to strong absorbance of water (Sasic and Ozaki 2001). Besides, the limit of detection for lactose in NIR appears to be generally inadequate for products with low lactose content such as lactose-free products or soy substitutes.

NMR techniques were previously used only for quantification of lactose in standard milk. In the paper by Hu et al. (2007), the univariate calibrations of single peaks of fat, tripalmitin, lactose, and other compounds by ^{1}H–^{13}C heteronuclear single quantum coherence 2D-NMR were reported. Klein et al. (2010) used NMR to quantify 23 different milk metabolites including lactose but only for lactose-containing milks in a concentration range between 15 and 60 $g.L^{-1}$. In this paper, we have expanded the scope of quantitative NMR analysis down to the residual contents

Table 2 Precision and accuracy of the NMR method for analyzing lactose in milk

Sample	RSD (%) (n=5)	Recovery (%) (n=5)
Standard solution (1.0 $g.L^{-1}$)	1.1	99
Authentic milk sample (48 $g.L^{-1}$)	2.7	–
Spiked lactose-free milk (1.0 $g.L^{-1}$)	8.6	94

RSD Relative standard deviation

of lactose found in lactose depleted products. So far, NMR is the only screening technique that offers this kind of sensitivity.

We also compared the results of lactose quantification by NMR with the enzymatic reference analysis. To do this, we determined lactose in 15 additional milk samples (both lactose-containing and lactose-free samples). Linear correlation analysis has shown that the results of the two methods are not significantly different (R=0.99, P<0.001).

In this study, we have shown that NMR spectroscopy is a powerful method for determination of small lactose contents and the discrimination of lactose-containing from lactose-free milk and milk substitutes. Therefore, the models build could be used to rapidly check the accuracy of labeling. Soy products should be also carefully labeled and controlled because soy is among the most common food allergens (Savage et al. 2010). SIMCA classification of the mid-field ^{1}H NMR region provides a simple method for such controlling. Thus, the chemometric model for predicting the type of dairy products from NMR spectra can replace time-consuming reference measurements.

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