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ARTICLE

NMR-Spectroscopy for Nontargeted Screening and Simultaneous Quantification of Health-Relevant Compounds in Foods: The Example of Melamine

Dirk W. Lachenmeier,*,† Eberhard Humpfer,‡ Fang Fang,‡ Birk Schütz,‡ Peter Dvortsak,‡ Constanze Sproll,† and Manfred Spraul‡

†Chemisches und Veterinäruntersuchungsamt (CVUA) Karlsruhe, Weissenburger Strasse 3, D-76187 Karlsruhe, Germany, and ‡Bruker BioSpin GmbH, Silberstreifen, D-76287 Rheinstetten, Germany

The recent melamine crisis in China has pointed out a serious deficiency in current food control systems, namely, they specifically focus on selected known compounds. This targeted approach allowed the presence of melamine in milk products to be overlooked for a considerable time. To avoid such crises in the future, we propose that nontargeted screening methods need to be developed and applied. To this end, NMR has an extraordinary potential that just started to be recognized and exploited. Our research shows that, from the very same set of spectra, ^{1}H NMR at 400 MHz can distinguish between melamine-contaminated and melamine-free infant formulas and can provide quantitative information by integration of individual lines after identification. For contaminated Chinese infant formulas or candy, identical results were obtained when comparing NMR with SPE-LC/MS/MS. NMR was found to be suitable for routine nontargeted and targeted analyses of foods, and its use will significantly increase food safety.

KEYWORDS: Nuclear magnetic resonance (NMR) spectroscopy; melamine; nontargeted screening; infant food; infant formula; milk; dairy products

INTRODUCTION

Melamine (2,4,6-triamino-1,3,5-triazine, CAS # 108-78-1) was first prepared and described by Liebig in 1834 (*1*) and has since become an increasingly important chemical commodity. Most of the melamine production is used in the fabrication of melamine–formaldehyde resins (*2*). The first analytical methods related to food were therefore developed to detect melamine migration from resins used in food contact materials (*3*). However, this melamine migration was generally judged to be negligible (*4*, *5*). Recently, melamine has become infamous as being one of the most effective adulterants used to increase the nitrogen content in foods and feeds. Melamine contains about 66.6% nitrogen, and the addition of 1% melamine to protein leads to a false increase in the Kjeldahl protein content by 4.16% (*6*). The first cases of melamine adulteration were detected in fish meals from Italy in the late 1970s (*7*, *8*), and methods to detect melamine in potato proteins were developed in Switzerland in the 1980s (*6*). Since then, melamine adulteration cases have not been reported in the literature until 2004 and 2007, when melamine was found in pet foods, causing renal failure in dogs and cats (*9*, *10*). Nephrotoxicity also appears to be the major toxic effect in humans (*11*, *12*).

With the intentional adulteration of human foods, including baby foods, with melamine, the economically motivated fraud in the food chain has reached a new dimension, for which food control systems were unprepared. The problem first became evident in China as an increase in urinary tract stone formation in infants beginning in the spring of 2008 (*13*). More than 294,000 children have reportedly been affected by adulterated formula, with over 50,000 hospitalized, and at least 6 deaths (*14*). Twenty-two dairy companies were implicated in the melamine fraud, with the Sanlu company being identified as the one that had most seriously violated the law (*13*). Apparently, the adulterations occurred at the raw milk collection stations, for which no systematic state surveillance had been implemented (*13*). The adulterators ostensibly used relatively sophisticated techniques such as special protocols for premixes and were said to have provided training regarding the use of those premixes after diluting the milk with water (*15*). Because of globalization and the worldwide food trade, the melamine contaminated foods were detected in a large number of countries, including the United States (*14*) and the European Union (*16*). Some commentators expect similar cases in the future with even improved techniques (*15*). This crisis has highlighted not only the hazards of an increasingly globalized food chain but also the weaknesses in the control systems (*17*).

We think that in order to improve consumer protection in the future, completely new strategies for food surveillance must be developed. The current food control system is generally target-oriented, meaning that quantitative analyses of certain health-relevant compounds such as heavy metals, pesticides, or carcinogenic contaminants are conducted (*18*). While the current system has not paid great attention to intentional adulterations (*17*), testing laboratories are also faced with the problem of choosing parameters for spot checks, as systematic full analyses are not possible for economical reasons (*19*). Therefore, analysis

*Corresponding author. Tel: +49-721-926-5434. Fax: +49-721-926-5539. E-mail: Lachenmeier@web.de.

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parameters are usually chosen by purely arbitrary choices (*19*) or by a risk-oriented approach (*20*), both of which most certainly would not include novel high-tech adulterants (*15*) chosen specifically with the intent of evading the food control system. For all of these reasons, we propose that future food control must implement what we call a nontargeted approach. This is an approach that must be able to detect deviations in food composition even by compounds that are not specifically being searched out or ones that are unknown to the analyst. This would enable food control to work proactively, rather than being one step behind the adulterators, as is currently the case. The most preferred form of nontargeted analysis would be a screening technique that would allow measurement of a large number of samples in a short time frame.

Nuclear magnetic resonance (NMR) spectroscopy has been proposed as probably the best nontargeted technique for use in screening food extracts (*21*). In a comparison of spectroscopic screening techniques, much richer information was provided using NMR in comparison to near-infrared or Fourier transform infrared spectroscopy, and selective and sensitive qualitative and quantitative information could be gathered from the spectra (*22*). Even though 400 MHz NMR machines are still expensive, compared with the cost of other analytical systems, the cost per sample for NMR can be very low, depending on the turnover to be achieved. The successful application of nontargeted screening approaches for food analysis has been previously demonstrated for a number of food matrices (*21*). However, the focus was always placed on authenticity control and not on the detection of health-relevant compounds. In this study, on the basis of our experience with beer (*23*) and fruit juice screening (*19*, *24*), we demonstrate for the first time that NMR is useful for nontargeted screening of baby food formula for health-relevant compounds such as melamine and also for labeling controls (e.g., lactose-free products). The technique is evaluated using authentic samples from the melamine crisis. Additionally, we provide information that the 400 MHz NMR spectra of liquid sample extracts can also be used for quantification of melamine. Using 700 MHz high-resolution magic angle spinning (HRMAS) NMR, we demonstrate that melamine can be quantitatively detected in complicated food matrices without any sample preparation.

MATERIALS AND METHODS

Samples. Authentic Chinese infant formulas ($n = 9$), including one contaminated Sanlu infant formula, were purchased in Beijing (China) in September 2008 during the melamine crisis. German infant formulas ($n = 13$) were sampled in the context of official food control by governmental food inspectors in the German Federal State Baden-Württemberg in December, 2008. Melamine-positive Chinese candy from the German market was also included in the study.

^{1}H NMR Measurements at 400 MHz. All NMR measurements were performed on a Bruker Avance 400 spectrometer (Bruker Biospin, Rheinstetten, Germany) equipped with a 5-mm BBI inverse probe with Z-gradient coils, using Bruker Automatic Sample Changer (B-ACS 60). All spectra were acquired at 300.0 K. ^{1}H spectra were acquired using one-dimensional (1D) nuclear Overhauser enhancement spectroscopy (NOESY) pulse sequence implemented with a low-power continuous wave presaturation during the relaxation delay and during the mixing time. The relaxation delay and mixing time were set to 4 s and 10 ms, respectively. The data were acquired automatically under the control of ICON-NMR (Bruker Biospin, Rheinstetten, Germany), requiring about 12 min per sample.

^{1}H HRMAS NMR Measurements at 700 MHz. The Proton HRMAS experiments were carried out using a 700 MHz AV III NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany) with a 4 mm HRMAS standard bore probe (^{1}H, ^{13}C, ^{31}P, and ^{2}H-lock) and gradient. The temperature was controlled at 300.0 K using a BCU05 precooling unit for the N_2 bearing gas flow and a temperature control unit (BVT300 MAS). The rotor spin rate was 7000 Hz; therefore, the spectral window was free from spinning side bands. A single pulse proton experiment with a 30° flip angle was executed with 4 s acquisition time, a relaxation delay of 4 s, and a sweep width of 20 ppm. A baseopt filter was applied during acquisition. All parameters have been derived from standard Bruker metabonomics NMR parameters. The number of scans for the standard experiments was ns = 64 (experimental time 9:15 min). For purity tests, up to 1024 scans have been applied. The processing was done with zero-filling to 128k data points and an exponential multiplication with lb = 1.

Chemometrics. The chemometrical analyses of the spectra were performed using MatLab (The Mathworks, Version R2008a) routines developed in-house. For the quantitation of the melamine concentrations, the signal of the NH_2-groups at about 5.93 ppm (**Figure 1**) was

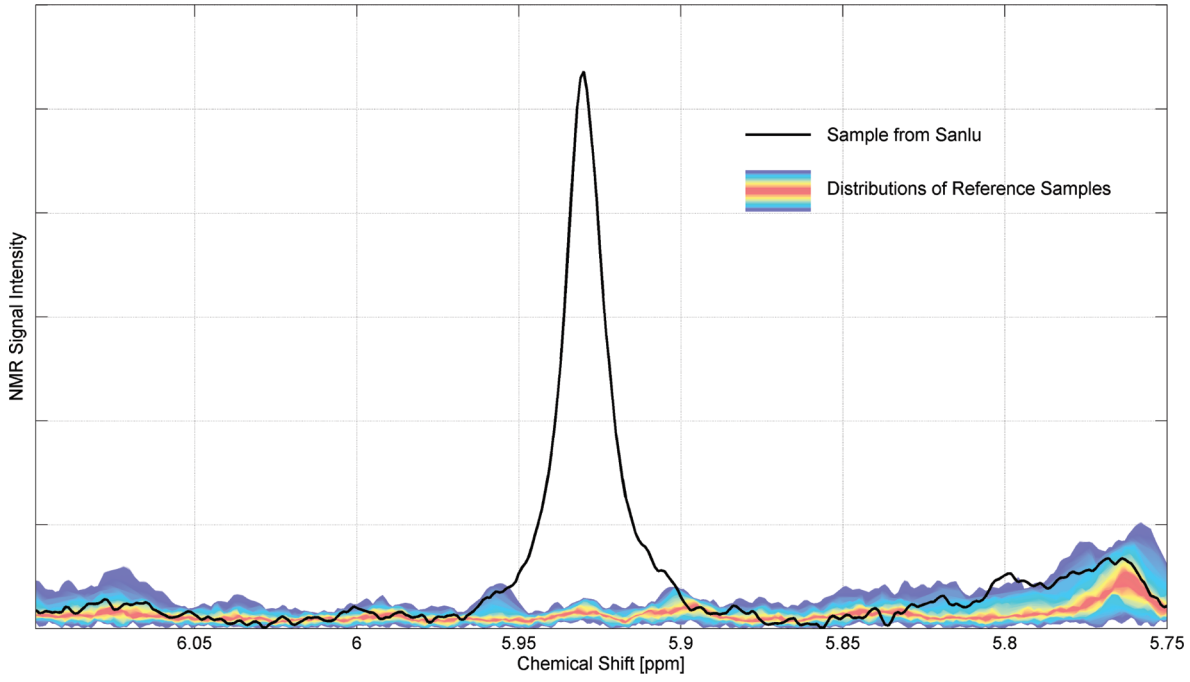

Figure 1. Comparison of melamine-contaminated Sanlu sample with the reference distribution of a collection of infant formulas (measurement in DMSO-d_6 at 400 MHz). The signal at 5.93 ppm derives from the NH_2-groups of melamine.

investigated. Its intensity was derived by fitting a theoretical signal on the spectrum, which minimized the sum of squares of the residues and therefore optimally explained the melamine influence on the NMR spectrum. The integral value of this fitted signal was linearly dependent on the melamine concentration and did not require a calibration for each sample when fixed acquisition and preparation parameters were used. The fitting was done in a fully automated mode using a nonlinear bound optimization algorithm, which optimized parameters such as line shape, position, height, and signal-underground (baseline).

The nontargeted analysis of the data was performed using simple robust techniques to describe the univariate distributions of the NMR spectra at each ppm value. **Figure 1** illustrates this method which uses the quantiles of the distributions of the reference samples as an estimate of the medians and the corresponding variances at each chemical shift. Since this procedure yields a robust description, a few atypical samples in the reference group can even be accepted. The melamine signal of the Sanlu infant formula was easily detected (manually and in automation) since its intensity translates into a z-score larger than 56 ($\text{median}_{\text{reference}} + z \times \text{STD}_{\text{reference}}$; a z-score of about ± 3 would cover 99% of all samples under normal distribution assumption).

This approach emphasizes deviating spectral regions and signals, which can then be investigated with sophisticated NMR experiments or other conventional analyses. Nondeviating samples will pass this screening, and adulterations up to certain limit can be ruled out.

Sample Preparation. In the first stage, for nontargeted analysis, 20 ± 0.4 mg of infant powder was weighed and dissolved in 2 mL of water−buffer solvent (90% distilled water and 10% buffer; 1.5 M KH_2PO_4/D_2O and 0.1% TSP at pH 7.0) to obtain a final concentration of 10 ± 0.2 g/L. This solution was shaken for 1 min on a vortex mixer and 3 min in an ultrasonic homogenizer. Then 600 μL of homogenized solution was transferred to a standard 5 mm NMR tube for analysis. When it became evident that the melamine peak itself could not be measured in water, the sample preparation was repeated in 99.8% DMSO-d_6 (Deutero GmbH), while 20 ± 0.4 mg of infant powder was dissolved in 2 mL of DMSO-d_6. For calibration, melamine standards in DMSO-d_6 at a concentration range between 2 and 200 mg/L were measured. To determine the limit of detection, a separate calibration curve in the range between 0.2 and 2 mg/L was established.

For HRMAS determination, 10 mg of sample was filled into the 50 μL HR-MAS rotor, and 45 μL of DMSO-d_6 was added and stirred. The rotor was prepared with a 50 μL upper spacer placed at a 3.0 mm depth and sealed with a Teflon screw. The material from the White Rabbit candy was scraped off and powdered in a mortar before analysis. For HRMAS calibration, a blank matrix (Nestlé Student Sweetened Milk Powder, Nestlé China, absence of melamine was confirmed by reference analysis) was filled in the rotor, and 45 μL of melamine standard solution in DMSO-d_6 was added. The calibration range was 9 to 900 mg/kg, and the second curve for the determination of limit of detection was in the range between 0.9 and 9 mg/kg.

Reference Method SPE-LC/MS/MS. For infant formula, 1 g of sample was added to a centrifuge tube, and 10 mL of acetonitrile was added. The solution was homogenized for 1 min on a vortex mixer. Ten milliliters of deionized water, a small amount (covering the tip of a spatula) of ammonium acetate, and 0.2 mL of glacial acetic acid were then added to the tube. The solution was again vortexed for 1 min and centrifuged for 10 min at 4000g. A 2.5 mL aliquot of the supernatant was placed into a conditioned solid-phase extraction (SPE) tube (Strata-X-C 200 mg/6 mL tubes, Phenomenex, Aschaffenburg, Germany). For candy, a different sample preparation had to be conducted because they were not soluble in cold solvents. Therefore, 1 g of candy sample was dissolved in 50 mL of hot water after the addition of 1 mL of acetic acid. After cooling, 30 mL of acetonitrile was added, and the solution was ultrasonicated for 15 min. After adjustment with acetonitrile to 100 mL, an aliquot of the extract was centrifuged and transferred to an SPE tube as described above.

The SPE tubes were conditioned using 15 mL of methanol followed by 15 mL of water. Cleanup was conducted using 6 mL of HCl (0.1 M) and 3 mL of methanol. The elution used 3× 5 mL of methanolic ammonia solution (5%). After each elution step, the extract was dried at 50−60 °C using a nitrogen stream. Then, 500 μL of acetonitrile/water (1:1) was added, and the solution was membrane filtered (0.2 μm) and used for chromatography (injection volume 20 μL). The LC/MS/MS system consisted of an Agilent (Waldbronn, Germany) 1100 HPLC system (binary pump, degasser, and autosampler) coupled with a Thermo Fisher Scientific (formerly Thermo Finnigan) (Dreieich, Germany) TSQ 7000 mass spectrometer. LC separation was performed on an Aqua 250 × 2 mm i.d., 3 μm, 125 Å, C18 column (Phenomenex, Aschaffenburg, Germany) at 30 °C, using mobile phase A (acetic acid, 0.1%) and mobile phase B (acetic acid in methanol, 0.1%) in the following gradient program. Flow rate: 0.2 mL/min, 0−5 min, 90% A; 5−5.5 min, 90% A to 20% A; 5.5−7.5 min, 20% A; 7.5−8 min, 20% A to 90%; 8−13 min, 90% A. Atmospheric pressure chemical ionization (APCI) used a capillary temperature of 270 °C, a vaporizer temperature of 400 °C, and a corona discharge current of 2.0 μA. The sheath gas was nitrogen at 50 psi, and argon was used as the collision gas. The collision cell of the triple quadrupole was operated with a collision energy of 35 eV for all transitions. For quantitative analysis, the following fragmentations were monitored in the selected reaction monitoring (SRM) mode: m/z 127 → 85, m/z 127 → 68, and m/z 127 → 43 for melamine. In the calibration range between 5 to 1000 ng/mL, melamine exhibited good linearity, with regression coefficients greater than 0.99. The limit of detection was 10 ng/mL and the limit of quantitation was 30 ng/mL. The recovery was 105 ± 5% in spiked baby formula (n = 8), 81 ± 13% in spiked ready-to-eat baby purees (n = 6), 98 ± 3% in different spiked egg powders (n = 4), or 92 ± 6% in candy (n = 11). The precision (expressed as coefficient of variation) was 7.5% (n = 6, bakery products), 5.1% (n = 8, baby formula), or 6.4% (n = 11, candy).

RESULTS AND DISCUSSION

Quantitative NMR Analysis of Melamine. At this point in time, only one study in the literature has presented ^{1}H NMR data on melamine in the context of a kinetic study (*25*). The signal assignment (DMSO-d_6, 6.02, s, NH_2) reported previously corresponds excellently with the findings in our study. The identification of the singlet peak of NH_2 at 5.93 ppm in DMSO-d_6 for melamine was confirmed using measurements of pure standards as well as standard addition to positive samples. Other studies have used HRMAS NMR to study the structure of a cyanuric acid−melamine system (*26*) or the formation of melamine condensation products (*27*); however, no ^{1}H data were presented in either study that could be used as a comparison with our results.

Table 1 shows a comparison between the sensitivities of the different methodologies under investigation. From the methods we evaluated, the reference LC/MS/MS procedure was the most sensitive, followed by 700 MHz HRMAS, and then 400 MHz liquid NMR. This was not unexpected, on the basis of the

Table 1. Comparison of Method Performance Data between NMR and LC/MS/MS

methodology	calculation basis	limit of detection [a]	limit of quantitation [a]
NMR 400 MHz tube	measuring solution (mg/L) [b]	0.33	1.16
	matrix (mg/kg) [c]	33.26	115.97
NMR 700 MHz HRMAS	matrix (mg/kg) [d]	0.69	2.76
SPE-LC/MS/MS	measuring solution (mg/L) [b]	0.010	0.030
	matrix (mg/kg) [e]	0.005	0.015

[a] Determined according to DIN 32645 using a separate calibration curve in the range of LOD. [b] Measuring solution is the final sample extract filled into the NMR tubes or injected into the LC/MS/MS system. [c] Calculated for a sample weight of 10 mg in 1 mL of DMSO-d_6. [d] Determined using a HRMAS rotor filled with 10 mg of authentic matrix. [e] Calculated for a sample weight of 1 g reconstituted in 500 μL after SPE cleanup.

physical characteristics of the methods. The results clearly show that NMR can be used for screening purposes at levels down to the lower mg/kg range. For example, the WHO melamine recommendations of 1 mg/kg in powdered infant formula and 2.5 mg/kg in other foods (*28*) can be reached by HRMAS. The sensitivity of the 400 MHz procedure could be increased if an optimized sample extraction or sample preconcentration step was included (e.g., a SPE procedure similar to that conducted prior to LC/MS/MS); however, it was our intention to leave the sample preparation as simple as possible. In contrast to the previous belief that NMR is only suitable for structure verification, elucidation, and purity analysis (*24*), our results clearly demonstrate that NMR can be used for quantitative analysis of foods for health-relevant compounds. Of course, the power of NMR resides in the nontargeted approach (see below), but as we also had previously demonstrated in beer and fruit juice analysis (*19*, *23*, *24*), the same spectra can be used to acquire quantitative data along with the results from multivariate analysis.

Regarding the precision of the methods, the 400 MHz method showed a coefficient of variation of 3.2% ($n = 9$; replicated measurement of authentic contaminated Sanlu sample), while the coefficient of variation of the HRMAS 700 MHz method was 2.6% (measurement of spiked infant formula, $n = 11$). The precision of NMR was judged to be sufficient for the purposes of food analysis. The LC/MS/MS procedure, in comparison, also had precisions of this order of magnitude or above (due to the complicated sample preparation procedure). Regarding accuracy, the NMR results were compared to the LC/MS/MS reference procedure. For the contaminated Sanlu formula, the 400 MHz NMR, the 700 MHz NMR, and the LC/MS/MS melamine results were 412 mg/kg, 460 mg/kg, and 470 mg/kg, respectively, and for the contaminated candy, the melamine results were 47 mg/kg, 74 mg/kg, and 68 mg/kg, respectively. The 400 MHz procedure showed lower results than either of the other procedures, which was judged to be due to an incomplete extraction during sample preparation. This exemplifies the advantages of the HRMAS procedure, which gave results very similar to those of the reference procedure. Nevertheless, we also judge the 400 MHz procedure in the current form as suitable for food screening because any amount of melamine is of course not admissible so that even a qualitative result would be sufficient (e.g., in the context of raw product screening in industry).

Our study is the first to apply HRMAS NMR to quantitatively determine melamine in authentic food matrices. HRMAS appears to be eminently suitable for analyzing complicated matrices, such as solid or semisolid foods, that are difficult to extract by conventional means. Many of these foods form emulsions or are not completely soluble in organic solvents, as in the case of the candy. HRMAS is a form of gel-phase NMR, where the sample is mixed with a solvent that swells the matrix. Using rotation of the sample, we can obtain high resolution ^{1}H spectra in a routine manner (*29*). A major application of HRMAS appears to be the detection of tumor tissues (*30*−*33*). So far, relatively few applications in the field of food control have been presented, and these have involved the authentication of cereal foods (*34*, *35*), cheese (*36*−*38*), or beef (*39*). None of these studies included quantitative analysis.

In the previous literature, LC/MS/MS with SPE extraction is clearly the preferred method for melamine quantification in all kinds of foods (*40*−*43*). According to our own experience, SPE cleanup is needed; otherwise, matrix compounds lead to interferences. The advantage of NMR and especially HRMAS is the minimal time for sample preparation compared to that in the tedious LC/MS/MS procedure.

Nontargeted Screening of Baby Foods. Unfortunately, melamine contamination is not the first instance of unsafe milk formula in China. In 2003, 12 children died of malnutrition in Anhui Province as a result of being fed infant formula of poor quality. In this incident, 55 different infant formulas, 40 corporations, and 10 provinces were involved, which exposed key public health gaps in food safety and public protection (*44*). This incident might even have indirectly led to the melamine crisis because government directives against diluted preparations were implemented. The fact that melamine could increase the apparent protein content and, furthermore, could make the product look milky may have been irresistible to those who would adulterate milk formulas (*14*). As we have detailed in Introduction, consumer safety cannot be ensured only by targeted analysis of compounds such as melamine because the adulterators are always one step ahead.

In our research to establish a quantitative method for NMR analysis of melamine, it became quickly evident that even by a simple visual inspection of the NMR spectra, the Sanlu sample showed differences. These were observed in several spectral ranges, even in the analysis of the aqueous solutions, in which the melamine peak at 5.93 ppm is not visible because of rapid proton exchange on the NH_2 groups with the solvent protons. For example, the Sanlu sample showed deviations in the range between 3.6 and 4.2 ppm. A search in our library of NMR spectra of pure compounds showed that this spectral pattern can be assigned to sucrose (**Figure 2**). Sucrose was obviously added to increase the sugar content of the diluted milk.

In the same fashion, we found that one of the German infant formula samples diverged from all other samples. In this case, the sample was missing the NMR signals for lactose. This formula was a special product for lactose-intolerant babies. This finding shows that the NMR method can also allow one to check recipes and labeling claims (such as lactose-free, hypoallergenic, etc.). Confirming these factors is comparably important to ensure the health of those subgroups of consumers with special nutritional needs.

We therefore conclude that the routine application of NMR in the screening of baby food products would provide a considerable improvement in consumer protection. Similar to what we have shown in the screening of fruit juices or beer, our chemometric approach allows the establishment of a standard model of a typical infant formula. This model can then be used without manual intervention to provide a judgment about a sample, regarding whether it does or does not correspond to the typical composition. In the case of noncompliance, the large depth of spectral information of NMR then allows one to make the assignment of compounds using databases (as in the case of lactose or sucrose). In the case of compounds not in databases (e.g., such as melamine when we started our study), further NMR experiments can be conducted for structural elucidation. The option for nontargeted screening, along with the structural information inside the spectra, is certainly the major advantage of NMR over other techniques that have been recently proposed. For example, surface desorption mass spectrometry (*45*−*47*) or enzyme-linked immunosorbent assays (*48*) again have the disadvantage that they are focused on sensitive target analysis. These will certainly detect melamine but will miss novel adulterants that might be used in its place in the future. The only other proposals in the literature that might be usable for a nontargeted approach are several infrared spectroscopy methods (*49*−*51*). Multivariate regression analysis was able to derive models to quantify melamine from Raman, mid, or near-infrared spectra. However, the use for other compounds in a nontargeted fashion was not researched. So far, only NMR can

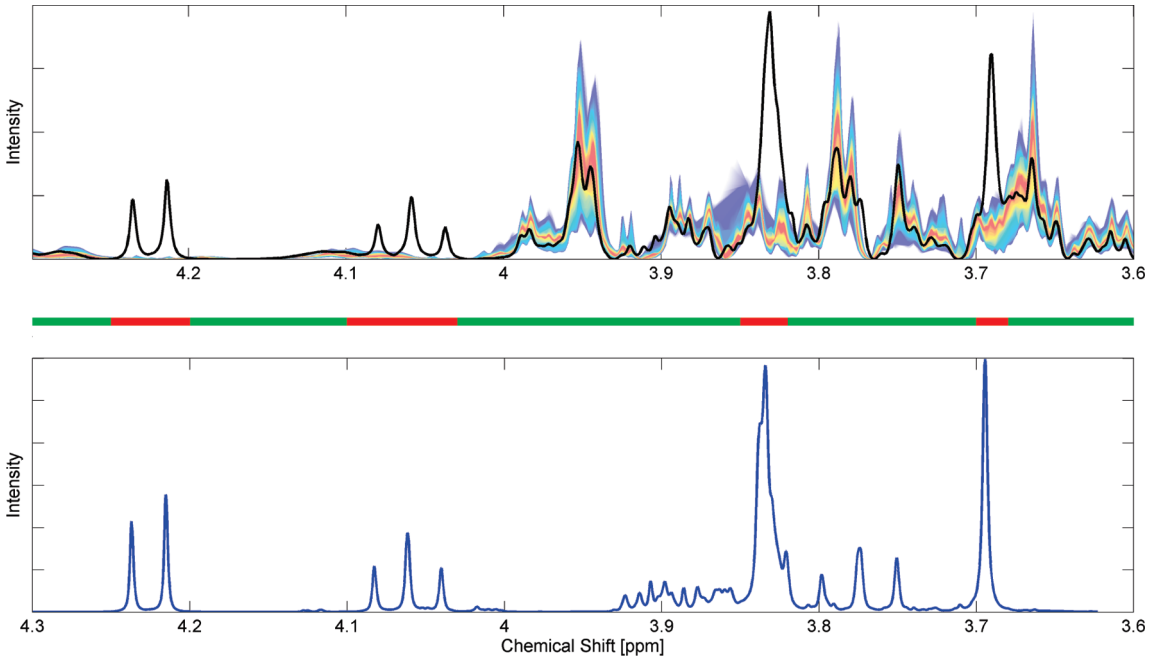

Figure 2. Upper panel: Comparison of melamine-contaminated Sanlu sample with the reference distribution of a collection of infant formulas (measurement in water at 400 MHz). Lower panel: Reference measurement of sucrose from the spectral library.

be seen as a holistic approach for detecting adulteration in infant formula.

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