Analytical Methods

Rapid detection of melamine adulteration in dairy milk by SB-ATR–Fourier transform infrared spectroscopy


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A R T I C L E   I N F O

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A B S T R A C T

Melamine is a nitrogenous chemical substance used principally as a starting material for the manufacture of synthetic resins. Due to its very high proportion of nitrogen melamine has been added illegitimately to foods and feeds to increase the measured protein content, which determines the value of the product. These issues prompted private as well as governmental laboratories to develop methods for the analysis of melamine in a wide variety of food products and ingredients. Owing to this fact present study is aimed to use single bounce attenuated total reflectance (SB-ATR) Fourier transform infrared spectroscopy (FTIR) method as an effective rapid tool for the detection and quantification of melamine in milk (liquid and powder). Partial least-squares (PLS) models were established for correlating spectral data to melamine concentration with R² > 0.99, and RMSEC 0.370. Linear calibration curves were obtained over the calibration range of 25–0.0625%. The LOD and LOQ of the method was 0.00025% (2.5 ppm) and 0.0015% (15 ppm) respectively. Proposed SB-ATR–FTIR method requires little or no sample preparation with an assay time of 1–2 min.

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1. Introduction

Melamine (2,4,6-triamino-1,3,5-triazine) is an inexpensive synthetic molecule commonly used as an industrial chemical in the production of melamine formaldehyde resins for manufacturing laminates, plastics, coatings, commercial filters, adhesives, kitchenware, flame-retardants, fertiliser and other products (Sun et al., 2010). Melamine (MEL) is not approved as an ingredient in food, but some manufacturers illegally used it as an adulterant to increase the apparent protein content. In 2008, a large-scale MEL contamination incident was made public in China and many other countries (Yan, Zhou, Zhu, & Chen, 2009). It is found in a variety of food sources, including raw milk, ammonium bicarbonate, protein powder, and non-dairy creamer (NDC). These food sources had also been used in the production of processed foodstuffs, such as infant formula, milk-containing foods, biscuits, instant beverages, frozen yoghurt, candy, coffee drinks, cereal-based ingredient and soup products. A motivating force for the adulteration of a food product with MEL because of its high nitrogen level (66% by mass) increases the discernible protein content measured by standard protein analysis tests, such as Kjeldahl or Dumas that measure total nitrogen content as an indication of protein levels (Mauer, Chernyshova, Hiatt, Deering, & Davis, 2009). Safety limit for MEL level in milk and milk based products were set by both the US Food and Drug Administration (FDA) and the European Union at 2.5 mg/kg (Vaclavik, Rosmus, Popping, & Hajslova, 2010). WHO/FAO experts stated that a limit of 1 mg/kg in infant formula would provide a sufficient margin of safety for dietary exposure relative to the tolerable daily intake (TDI) (Lutter et al., 2011). Ingestion of MEL at levels above the safety limit may cause kidney failure and even death, particularly for vulnerable individuals such as infants and young children (Ding, Yan, Ren, & Chen, 2010). Moreover reports have shown MEL toxicity resulted in kidney illness of varying degrees affecting about 300,000 babies, 6 of whom died (Elvira, Rodriguez, & Lynnworth, 2009).

Various methods for the analysis of melamine and related compounds in foods for human consumption and animal feeds have been reported, including high-performance liquid chromatography (HPLC) or gas chromatography (GC) combined with a selective detection technique, such as tandem mass spectrometry (MS/MS), single-stage mass spectrometry (MS), diode array detection (DAD) and ultraviolet absorption (UV) (Sun et al., 2010), enzyme-linked immunosorbent assay (ELISA) (Yin et al., 2010), capillary electrophoresis (CE) (Chen & Yan, 2009), spectrophotometric methods (Cheng et al., 2010), chemiluminescence (CL) (Wang, Chen, Gao, & Song, 2009), colorimetric nanoparticles (Ai, Liu, & Lu, 2009), nuclear magnetic resonance (NMR) spectroscopy (Lachenmeier et al., 2009), sweeping-micelles electro kinetic chromatography (SMEC) (Wu, Tsai, Sun, & Kuo, 2011) and molecularly imprinted polymer film (MIP) (Pietrzyk et al., 2009). However, the high cost of...
operation and maintenance of GC/LC–MS systems as well as the lab-
our intensive derivatization that GC–MS requires limits their use in
the milk product factories (Venkatasami & Sowa, 2010). An HPLC
method can be used to quantitatively analyse melamine at ppm le-
vel but it is inadequate for qualitative analysis and trace-level anal-
ysis (Yan et al., 2009). Currently, the FDA uses a liquid 
chromatography-triple-quadrupole tandem mass spectrometry
(LC–MS/MS) method to detect residues of melamine in dry infant
formula. Although this method provides limits of detection as
low as 250 ppb, the sample preparation and cleanup procedures
are time-consuming and labour-intensive. Therefore, the use of
this method as a screening tool for a large number of samples is
not practical or cost-effective. Regardless of the chromatographic
approach used, sample pretreatment is relatively time-demanding:
one or more extraction steps are required to decrease matrix inter-
ference and preconcentration is required to obtain an adequate
detection level. Moreover, most of these procedures require large
volumes of organic solvent, which might be harmful to the envi-
ronment and human health (Chao, Lee, Wei, Kou, & Huang,
2011). Thus, there is still a need for a rapid, high-throughput,
widely available, cost effective method for detecting melamine in
infant formula and dairy milk. Rapid infrared spectroscopy meth-
ods have been successfully used in adulteration detection for a
wide range of complex food products, including oils, carbohydrate
powders, juices, honey, coffee, milk, vinegar, crab meat, and wheat
(Ellis et al., 2012). Mauer et al. (2009) have reported MEL detection
in infant formula powder using near and mid IR regions with PLS
model, however no liquid milk samples were tested. Recently a
method has been reported for MEL detection by mid and near-IR
region in liquid and powder milk samples with glass cell accessory.
In addition authors have also used expensive statistical approaches
and special software complex for spectra computing and pretreat-
ment of non-linear regressions models to achieve 1 ppm detection
limit (Balabin & Smirnov, 2011). However FDA and European Union
have established a threshold of 2.5 ppm. Therefore in present work
we have used simple and more versatile single bounce attenuated
total reflectance (SB-ATR) sample-handling accessory with PLS
model, thereby reducing the cost of implementing FTIR melamine
analysis. Furthermore, a ZnSe SB-ATR accessory provides access to
much more spectral information than the transmission cell em-
ployed in commercial FTIR analyzers owing to its very short path
length, as well as the much lower transmission cutoff of ZnSe com-
pared with CaF2.

2. Materials and Methods

2.1. Reagent and samples

Analytical grade melamine (purity 99%) was procured from Sig-
ma–Aldrich (St. Louis, Missouri, USA). All other reagents were of
high purity available. Raw milk sample from dairy farm was used
as stock milk for dilution of standards. The raw milk samples were
freeze dried through Labcon freeze dryer (Labcon corporation Kan-
sas city, Missouri, USA). The safe temperature for drying of milk
sample was −5 to −40 °C.

2.2. Analytical protocol for standard

A stock powder was prepared by intermingles of a 1:1 w/w ratio
of melamine and the powder/freeze dried milk sample. This mix-
ture was further diluted to 25%, 22%, 18%, 14%, 11%, 8%, 5%, 2%,
1%, 0.5%, 0.25%, 0.125%, and 0.0625% melamine by geometric mix-
ing of equal mass ratios of freeze dried milk sample blend to a final
mass of 2 g. The sample with the lowest concentration of melamine
was prepared by diluting a 0.0625% w/w melamine stock powder
to a melamine level of 0.00025 w/w (2.5 ppm). All samples were
prepared in duplicate and stored in sealed glass vials at room tem-
perature prior to analysis, and all dilutions were analysed on FTIR-
ATR.

2.3. Sample preparation

Five different brand milk samples (2 liquid and 3 in powder form)
were purchased from local retails. The liquid milk samples
were freeze dried with the addition of known amount (2.5–
15 μg mL⁻¹) of melamine. Also the powder milk samples were
mixed with different proportion (2.5–25 μg g⁻¹) of melamine.

2.4. FTIR spectral measurements

Spectra of samples in the mid-infrared region (4000–650 cm⁻¹)
were collected using a Thermo Nicolet Avatar 330 FTIR spectrometer
outfitted with a removable ZnSe crystal, deuterated triglycine sul-
fate (DTGS) detector controlled by OMNIC software (Thermo Nicolet
Analytical Instruments, Madison, WI). The spectrum of all the stand-
ard or sample was proportion touching an open beam fresh back-
ground spectrum recorded from the bare ATR crystal; earlier to
collection of each background spectrum, the ATR crystal was cau-
tiously cleaned with hexane, methanol and acetone to remove any
lingering involvement of the preceding sample, and remaining sol-
vent was then evaporated using a brook of nitrogen gas.

2.5. FTIR calibrations/validation

A series of 14 calibration standards covering the range of
0.0625–25% melamine was analysed by SB-ATR–FTIR instrumental
method. This spectrum along with their respective reference mel-
amine spectra was input into the TQ Analyst program to develop
partial least squares (PLS) calibrations. Correlation and variance
spectra were generated to identify spectral regions that contained
information related to the grouped and ungrouped data and subse-
quently explored for calibration development and refinement.
The performance of the calibrations was assessed by linear regression,
and evaluated by running the samples of known melamine profile.
To evaluate the competence of the models to fit the calibration
data and to calculate the deviation of the models, root mean square error of calibration (RMSEC), root mean square error of cross vali-
dation (RMSECV) and root mean square error of prediction (RMSEP) were used.

2.6. Limit of detection and quantification

The limit of detection and quantification was calculated as de-
scribed previously (Saba Naz, Sherazi, Talpur, & Mahesar, 2011).
Briefly the limit of detection (LOD), described as the minimum con-
centration from which it is possible to deduce the presence of the
analyte with reasonable statistical certainty. To determine the lim-
it of detection (LOD) and limit of quantification (LOQ) of proposed
method, the selected band area was measured at low concentra-
tions of standards, until the melamine related signal disappeared.
The analysis at the lowest amount which produced substantial sig-
nal was repeated eleven times and calculated by the following
formula:

\[
\text{LOD} = 3 \times \text{SD} \times \frac{C}{M}
\]

where SD is the standard deviation of the band area; C is the con-
centration of analyte and M is the mean band area.
While LOQ was determined by the same way with following equation:
\[
\text{LOQ} = 10 \times \text{SD} \times C/M
\]

### 3. Results and discussion

Infrared Spectroscopy is an important tool for the analyst to provide detailed information about structure of organic compound with their specific fingerprint pattern. Fig. 1(a) depicts the ATR-FTIR spectra of melamine, the 3000–3633 cm\(^{-1}\) band is assigned to N-H stretching vibration modes, the 1100–1630 cm\(^{-1}\) band corresponds to the stretching vibrations related to C-N, C=N, and is generally associated with the skeletal stretching vibrations of these aromatic rings. Furthermore the absorption band present at 1630 cm\(^{-1}\) results from the stretching vibrations of >C–N– bonds present in the triazine ring of melamine (Zhao, Yu, Zhou, Tian, & Yanagisawa, 2005). The Fig. 1(b) shows the comparative spectra of raw milk and melamine + milk (1:1 w/w) in the region of 860–760 cm\(^{-1}\) with different concentration of melamine in the selected region of 860–760 cm\(^{-1}\). The spectra show the good linearity of the calibration model in proposed method. The LOD and LOQ were determined as 0.00025% and 0.0015% correspond to 2.5 and 15 ppm respectively.

A threshold of 2.5 ppm for melamine in dairy milk has been set by the FDA.\(^4\) The present method was able to distinguish between unadulterated milk samples and that containing 2.5 ppm MEL, with no misclassifications. The proposed detection limit is more sensitive than some previously published methods that have LOD’s in the range 5–10 ppm, such as using HPLC for the determination of melamine in rice, corn flours (Ehling, Tefera, & Ho, 2007) and HPLC-DAD for plant origin powders (Ding et al., 2008). The assay time for melamine detection for proposed FTIR method is 2 min which is quite faster compared with most of the common methods. The current FDA LC–MS/MS method has a detection limit of 250 ppb in infant formula powder, but the total time to detection is >3 h (Turnipseed, Casey, Nochetto, & Heller, 2008). The obvious advantages revealed in the established ATR-FTIR method, such as easy sample preparation without solid phase extraction, short analysis time and low cost etc., could facilitate future development of rapid screening of melamine residues in food.

For validation of the method, two standards from the calibration set were selected automatically and used as validation standards to check the accuracy of the models. Interestingly, it was observed that both PLS calibration models have a \(R^2\) in the range of 0.99893–0.99996 and were considered as an excellent regressions. The PLS model in which calibration was done without baseline showed very low RMSEC and RMSECV in comparison to the PLS model, in which calibration done with baseline, except the RMSEP for the former model was comparatively higher shown in Table 2. For that reason, the PLS model without baseline was selected as optimum for the determination of MEL in milk samples.

The quantitative results and the recoveries of the method, which were determined by adding different amounts of MEL in
raw and powder milk are listed in Table 3. It is shown that the average total recoveries of MEL ranged from 95.44% to 102.07%. In addition, no melamine was found within the LOD of proposed method in branded milk available locally, indicating that the proposed FTIR-ATR method has promising feasibility for rapid detection of melamine in milk.

4. Conclusions

Here we have investigated accurate, reproducible and sensitive analytical method for the determination of melamine in dairy milk. The results indicated that SB-ATR FTIR and PLS calibration method could be applied for rapid and precise assessment of melamine.
present in dairy milk. Furthermore proposed method is very simple, environmental friendly and devoid of any sample pretreatment. For food labelling point of view the proposed method could be easily applied on dairy milk for fast analysis of melamine alternative technique to commonly used methods.

References

Table 2
Prediction capabilities of PLS-FT-IR of the two measurement model for the determination of melamine.

<table>
<thead>
<tr>
<th>Spectral region (cm (^{-1}))</th>
<th>840.78–726.09 (cm (^{-1}))</th>
<th>840.78–726.09 (cm (^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>None</td>
<td>Two points 840.78–726.09 (cm (^{-1}))</td>
</tr>
<tr>
<td>Factors</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>Validation standard</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>RMSEC</td>
<td>0.370</td>
<td>1.55</td>
</tr>
<tr>
<td>RMSECV</td>
<td>5.75</td>
<td>6.68</td>
</tr>
<tr>
<td>RMSEP</td>
<td>1.55</td>
<td>3.48</td>
</tr>
</tbody>
</table>

Table 3
Result of the determination of melamine in raw and powder milk.

<table>
<thead>
<tr>
<th>Milk sample</th>
<th>Original amount</th>
<th>Added ((\mu)g mL(^{-1}) or g(^{-1}))</th>
<th>Found ((\mu)g mL(^{-1}) or g(^{-1}))</th>
<th>Recovery (%)</th>
<th>RSD %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw milk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>–</td>
<td>2.5</td>
<td>2.39</td>
<td>95.44</td>
<td>2.14</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>5</td>
<td>5.10</td>
<td>102.00</td>
<td>1.62</td>
</tr>
<tr>
<td>M2</td>
<td>–</td>
<td>10</td>
<td>10.12</td>
<td>101.20</td>
<td>2.69</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>15</td>
<td>15.31</td>
<td>102.07</td>
<td>2.76</td>
</tr>
<tr>
<td>Powder milk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1</td>
<td>–</td>
<td>2.5</td>
<td>2.44</td>
<td>97.60</td>
<td>2.63</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>5</td>
<td>5.01</td>
<td>100.24</td>
<td>3.44</td>
</tr>
<tr>
<td>P2</td>
<td>–</td>
<td>10</td>
<td>10.12</td>
<td>101.20</td>
<td>3.14</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>15</td>
<td>15.22</td>
<td>101.47</td>
<td>1.31</td>
</tr>
<tr>
<td>P3</td>
<td>–</td>
<td>20</td>
<td>20.01</td>
<td>100.06</td>
<td>2.26</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>25</td>
<td>25.14</td>
<td>100.56</td>
<td>2.00</td>
</tr>
</tbody>
</table>

\(^{a}\) \(\mu\)g mL\(^{-1}\) for raw milk and \(\mu\)g g\(^{-1}\) for powder milk sample.