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Analytical Methods

Rapid and sensitive detection of melamine in milk with gold nanoparticles by Surface Enhanced Raman Scattering

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ABSTRACT

A rapid and sensitive method to detect melamine in liquid milk based on Surface Enhanced Raman Scattering (SERS) spectroscopy is presented, exploiting the selective binding of gold nanoparticles (AuNPs) with this analyte. This interaction promotes the aggregation of the AuNPs inducing a huge enhancement of the melamine signals in the Raman spectrum due to the formation of SERS "hot spots". An external standard calibration method was employed for quantitative analysis and the method was validated for linearity, sensitivity, repeatability and recovery. A good linearity (R^2 = 0.99) was found in the concentration range of 0.31–5.0 mg l^{-1} in milk with a limit of detection of 0.17 mg l^{-1}. This method does not require a long extraction procedure (total analysis time can be lower than 30 min) and can be reliably used for melamine detection in milk matrix in accordance with the European law limits.

1. Introduction

Melamine is an important industrial material that is mainly used for resin production, for thermosetting plastic and for polymer manufacturing in general (Sugita, Ishiwata, & Yoshihira, 1990). Its fame, unfortunately, came out recently because it was used as a food adulterant in milk, pet and animal feed (Brown et al., 2007; Burns, 2007). As a high rich-nitrogen molecule, melamine was intentionally added into food ingredients to produce an incorrectly high reading in the measurement of the protein content based on total nitrogen. The main concern on melamine, as a food additive, is the ability of combining with its analogues, such as cyanuric acid, leading to the formation of insoluble crystals which were responsible for kidney failures and even death in infants in China (Dobson et al., 2008; Hau, Kwan, & Li, 2009; Lam et al., 2009). Considering its potential toxicity, the Codex Alimentarius Commission has set a limit of 1 mg l^{-1} for powder infant formula and 2.5 mg l^{-1} for other foods and animal feed (Report on the Thirty-Third Session of the Joint FAO/WHO Food Standards Programme, 2008). Currently, gas chromatography (GC) or liquid chromatography (HPLC) coupled with mass spectrometry (MS) (Squadrone et al., 2010; Tyan, Yang, Jong, Wang, & Shiea, 2009), matrix-assisted laser desorption/ionisation MS (Tang et al., 2009), ELISA (Zhou et al., 2012) and IR spectroscopy (Mauer, Chernyshova, Hiatt, Deering, & Davis, 2009), represent the major categories of techniques for melamine detection. However, these methodologies usually require expensive instrumentations and long sample preparation procedures are needed mainly due to analyte extraction steps. Recently, several methods to detect melamine based on gold nanoparticles (AuNPs) have been developed (Ai, Liu, & Lu, 2009; Li, Li, Cheng, & Mao, 2010; Wei et al., 2010). Some of these methods were based on a colorimetric visual inspection of the nanoparticles solution colour change upon melamine interaction. Melamine interaction with modified or unmodified gold nanoparticles decreases the stability of the AuNPs provoking the formation of aggregates and inducing a shift of the surface Plasmon resonance with a consequent variation of the colour solution from red to blue, that can be easily monitored by UV/Vis absorption measurements. However, in the presence of interference substances in milk, such as other organic molecules or even positively charged ions and whose competing with melamine for AuNPs binding, a change in the AuNPs aggregation state can be seen, even in absence of the analyte and thus leading to a false positive response. In order to avoid these problems, Raman spectroscopy was used since it can provide a fingerprint of the melamine molecule in the Raman spectrum. Raman spectroscopy together with the help of gold or silver nanoparticles offers a very high sensitivity due to the Surface En-

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hanced Raman Scattering (SERS) effect that occurs when a molecule is adsorbed or grafted on a rough metallic surface. The Raman signal of the molecule can be enhanced theoretically up to a 10^{13} factor for a potential single molecule detection. Different methodologies for the detection of melamine based on the SERS effect were developed. Most of them were based on the fabrication of SERS substrates (Betz, Cheng, & Rubloff, 2012; Cheng & Dong, 2011; Kim, Barcelo, Williams, & Li, 2012), usually prepared by metallic nanoparticles deposition on silicon or glass or by photolithography techniques. SERS substrates demonstrated to achieve a very high sensitivity (detection limit in the $\mu g\,l^{-1}$ range) but they usually suffered of lack of reproducibility and homogeneity of the molecule distribution on the SERS substrate, leading to problems in the quantification. Other SERS analysis were developed in liquid, mainly based on silver nanoparticles, achieving very good results for melamine detection in milk (Zhang et al., 2010). As for gold nanoparticles, instead, only few works have been published. Lou et al. (2011) developed a very sensitive indirect method (LOD 0.1 $\mu g\,l^{-1}$) to detect melamine in milk by SERS using 4-mercaptopyridine-modified AuNPs. However, the linearity response of this method is between 0.5 and 100 $\mu g\,l^{-1}$ which might affect the practical application of this assay in routine analysis. Moreover, the melamine quantification is done by using a Raman reporter and not by the melamine itself. Another interesting work was proposed by Yazgan, Boyaci, Topcu, & Tamer (2012) who developed a rapid and sensitive method to detect melamine in milk by using spherical magnetic-core gold-shell nanoparticles and rod-shaped gold nanoparticles labelled with a Raman-active compound. They reached the limits of detection (LOD) and quantification (LOQ) of 0.38 and 1.27 $mg\,l^{-1}$, respectively.

In this work we propose a simpler method (based on the use of only one type of spherical AuNPs), which guarantees high sensitivity and gives a linear response in a range of concentrations useful for practical applications. We selectively tested spherical AuNPs with different dimensions in order to have the highest SERS effect. Moreover, we tuned the AuNPs concentration to reach the linearity in the selected melamine range. For the calibration of the Raman spectrometer we used the acetonitrile (ACN) Raman band to normalise the Raman intensity of melamine, minimizing possible variations due to laser power, focal distance and environmental parameters (temperature, humidity). The method developed proved to be simple and not requiring a long extraction procedure. The total analysis time can be lower down than 30 min.

2. Material and methods

2.1. Reagents and materials

Hydrogen tetrachloroaurate trihydrate ($HAuCl_4\ 3H_2O \geqslant 99\%$), trisodium citrate dihydrate ($\geqslant 99\%$), melamine (99%) and 10 nm Gold nanoparticles stabilized suspension in citrate buffer were purchased from Sigma–Aldrich (Milan, Italy). Sodium hydroxyde (NaOH 97%), Hydrochloric acid (HCl 37%), Nitric acid (HNO_3 68%), absolute ethanol (99,99%) and acetonitrile (>99.5%) were obtained by Carlo Erba Reagents (Rodano, Italy). All solutions were prepared with Milli-Q quality water (18 MΩcm). Liquid semi-skimmed milk used for the assays was purchased in a local supermarket in Torino, Italy.

2.2. Gold nanoparticles preparation

All glassware used in the experiment was soaked in aqua regia ($HCl:HNO_3$ 3:1) and rinsed thoroughly in water and dried with nitrogen prior to use. AuNPs were synthesized according to Frens, 1973. For the preparation of 40 nm AuNPs, 5 ml of a 1% aqueous solution of trisodium citrate was rapidly injected into 500 ml boiling solution of $HAuCl_4$ (0.01% v/v). The mixture was further refluxed for 10 min and then cooled to room temperature under continuous stirring and a wine-red colour solution of AuNPs was obtained. For the preparation of 80 nm AuNPs, 2.5 ml of trisodium citrate (1% water solution) was injected into 500 ml boiling solution of $HAuCl_4$ (0.01% v/v). The above mentioned procedure was then applied. All AuNPs solutions were stored at 4 °C before use.

2.3. Gold nanoparticles characterization

AuNPs characterization was done by UV–Vis absorption measurements and by Scanning Electron Microscopy (SEM) imaging. UV–Vis absorption spectra were collected with the Evolution 60s spectrophotometer (Thermo Scientific). The surface plasma resonance peaks of AuNPs solutions were measured to be 518, 530 and 554 nm for AuNPs dimensions of 10, 40 and 80 nm, respectively. SEM characterization was carried out using a SEM FEI Quanta 3D or Inspect F in UHV mode with the SE detector. Typical settings for the imaging are: 10 kV accelerating voltage, 2.5 spot (18 pA) or 3.5 spot (30 pA), 10 mm WD.

2.4. Melamine standard solutions

Melamine stock standard solution was prepared by accurately dissolving 50 mg of standard in 50 ml of Ethanol/H_2O 50:50 (v/v), to reach a concentration of 1000 $mg\,l^{-1}$. Melamine standard solutions were prepared by subsequent dilutions from the stock solution in water to reach the following concentrations: 100, 20, 10, 5, 1, 0.5, 0.2, 0.1 $mg\,l^{-1}$. These pure melamine standards were used to set up the analytical procedure. Aliquots of the melamine standards were mixed in a 1:1 ratio with AuNPs stock solutions, mixed with vortex for 3 s and subsequently analysed by UV–Vis and the Raman spectrophotometer.

Melamine standard solutions in negative matrix pool were also prepared for the external calibration of the Raman spectrometer, as explained in the paragraph 2.7. Consecutive dilutions were made starting from 10 $mg\,l^{-1}$ to reach the following concentrations in matrix: 1, 0.50, 0.25, 0.125, 0.063 $mg\,l^{-1}$. These solutions were mixed with AuNPs (1:1) and analysed by Raman spectroscopy to build the calibration curve.

2.5. Detection of melamine in liquid milk by SERS

Aliquots of the 100 $mg\,l^{-1}$ melamine stock solution were added to milk to obtain concentrations of 0.5, 1, 3, 5 and 10 $mg\,l^{-1}$. Melamine-free milk was processed as the spiked milk and used to prepare blank samples. The extraction procedure was carried out by first adding 200 µl of 1 M HCl to 4 ml of spiked milk and vigorously mixing by vortex for 10 s. The samples were then transferred into 1.5 ml centrifuge tubes and centrifugated for 30 min at 14000 rpm. Supernatants from the same sample were collected and filtered with a 0.22 µm PTFE filter. The pH of the filtered solution was adjusted at 4.7 by adding 60 µl of 1 M NaOH. 10 ml of pure ACN were then added inducing the precipitation of most of the proteins in solution. A final centrifugation step was carried out at 14000 rpm for 30 min in order to remove any aggregates. 250 µl of the resulting supernatant was mixed in a 1:1 ratio with a 10-fold concentrated 40 nm AuNPs solution and immediately analysed by Raman spectroscopy. The 10-fold concentrated 40 nm AuNPs solution was obtained by centrifugating the AuNPs stock solution at 4000 rpm for 30 min and subsequently resuspending in a proper amount of water solution.