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# Development of an Optical Biosensor Based Immunoassay to Screen Infant Formula Milk Samples for Adulteration with Melamine

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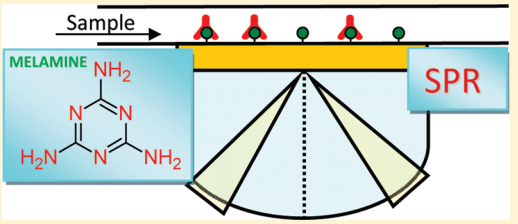
**ABSTRACT:** The illegal adulteration of milk with melamine in 2008 in China led to adverse kidney and urinary tract effects in hundreds of thousands of children and the reported deaths of six. The milk had been deliberately adulterated to elevate the apparent protein content, and subsequently melamine was detected in many milk-related products which had been exported. This led to the banning of imports of milk and milk products from China intended for the nutritional use of children and to the implementation of analytical methods to test products containing milk products. An optical biosensor inhibition immunoassay has been developed as a rapid and robust method for the analysis of infant formula and infant liquid milk samples. A compound with a chemical structure similar to that of melamine was employed as a hapten to raise a polyclonal antibody and as the immobilized antigen on the surface of a biosensor chip. The sensitivity of the assay, given as an $IC_{50}$, was calculated to be 67.9 ng $mL^{-1}$ in buffer. The antibody did not cross-react with any of the byproducts of melamine manufacture; however, significant cross-reactivity was observed with the insecticide cyromazine of which melamine is a metabolite. When sample matrix was applied to the assay, a limit of detection of <0.5 $\mu$g $mL^{-1}$ was determined in both infant formula and infant liquid milk. The development of the immunoassay and validation data for the detection of melamine is presented together with the results obtained following the analysis of melamine-contaminated milk powder.




Melamine ($C_3H_6N_6$), 1,3,5-triazine-2,4,6-triamine, is a trimer of cyanamide ($CH_2N_2$); its chemical structure is shown in Figure 1. The three amine groups facilitate polymerization through reaction with formaldehyde to form a heat-tolerant, fire-resistant resin used in the manufacture of flame retardant materials; it is a highly stable structure that makes it a suitably durable material for the manufacture of laminated surfaces such as Formica, whiteboards, and flooring as well as kitchen utensils.

Melamine can be formed through dealkylation of the pesticide cyromazine (see Figure 1), which is used to control insect growth. The pesticide, which may be used to protect plant material[1] and as a pour-on application for sheep,[2] can be metabolized to melamine. Cyromazine is not authorized for use in the EU as a treatment for animals producing milk for human consumption, and the European Medicines Agency (EMA) established a maximum residue level (MRL) of 300 $\mu$g/kg for the pesticide in sheep muscle, fat, liver, and kidney in 2001.[3] The EMA found that the risk from melamine, as the major metabolite (less than 15%) of the pesticide, to be insignificant although investigations regarding the carcinogenicity of melamine did show that at a high dose in male rats there was an abnormal proliferation of cells in the bladder, accompanied by the discovery of bladder stones, consisting mainly of melamine. It was, however, concluded that melamine should not be regarded as a carcinogenic compound as the stones occurred in the bladder only at high melamine doses and it is the stones, not melamine, that promote the growth of tumors.

Two separate outbreaks of pet food associated renal failure in 2004 and 2007 were attributed to kidney stones formed through reaction of melamine and cyanuric acid, a structural analogue (see Figure 1).[4] The two compounds reacted to form melamine cyanurate, which was deposited as insoluble crystals in the kidney tubules. The contaminated lots of pet food had been prepared with wheat gluten originating from China.[5] The gluten was found to contain melamine and cyanuric acid and to a lesser degree ammeline and ammelide, which are two hydrolysis products of melamine and are also triazines (see Figure 1). Intentional adulteration with melamine can falsely increase the apparent protein content of a food product because standard protein determination assays (Kjeldahl) cannot differentiate between protein nitrogen and nonprotein nitrogen. While melamine and cyanuric acid have been shown to have no effect on renal function individually in cats,[6] the combination of the two was reported to have proved fatal for hundreds of cats and dogs during the contaminated pet food incident. In 2008 in China, the illegal adulteration of milk with melamine led to kidney and urinary tract effects in 294 000 children with 6 reported deaths.[7] The deliberate corruption of

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the milk was intended to elevate the apparent protein content, and consequently melamine was detected in many milk-related products which had been exported. Many countries took action to prevent any potentially severe adverse health effects. The European Commission (EC) prohibited imports of milk and milk products from China intended for the nutritional use of infants and young children and implemented testing of composite foodstuffs (chocolate and biscuits) that contained at least 15% of milk product originating from China.[8] Those found to contain melamine in excess of 2.5 mg $kg^{-1}$ were to be destroyed.

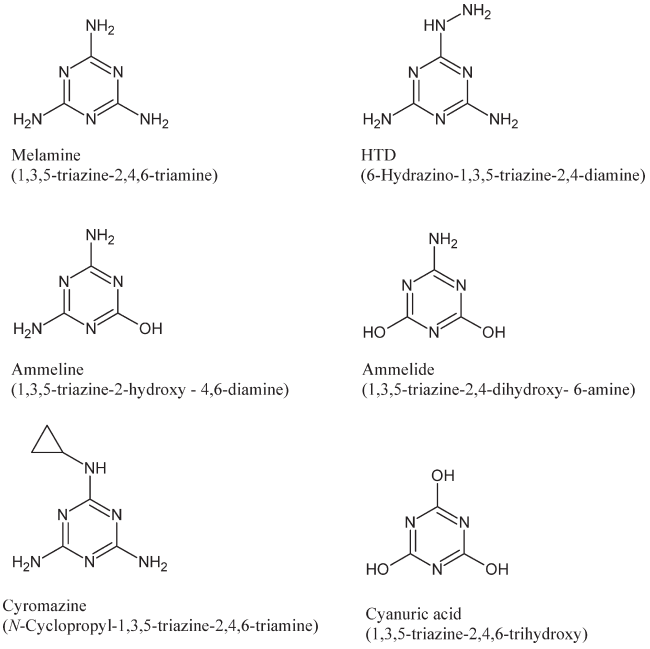


**Figure 1.** Chemical structures of melamine, HTD (the hapten), ammeline and ammelide (metabolites of melamine), and other related analogues.

Difficulties in determining the milk product content of composite foodstuffs led to Commission Decision 2008/798/EC[9] (repealing 2008/757/EC) which implemented testing of all goods for which the milk product content could not be established. The subsequent discovery of melamine by Member States in soya, soya products, and ammonium bicarbonate led to the further inclusion of these commodities in the testing requirements as declared in Decision 2008/921/EC,[10] amending 2008/798/EC. Analytical methods had been published before the contaminated pet food and milk product incidents, largely for the detection of the pesticide cyromazine and melamine as its metabolite. The contamination incidents led to the development of a number of detection techniques which are summarized in Table 1.

The use of immunochemical techniques for the detection of melamine rely on the production of a specific antibody by introducing the antigen (or an analogue of it) to the immune system of a host animal and collecting the resultant antiserum. As a result of its low molecular weight, melamine must be coupled to a large carrier protein so that the conjugate will illicit an immune response in the host animal. Preparation of such a conjugate is complicated by the small chemical structure of melamine and the three amine groups that are available for reaction with a protein. Lei et al. overcame this problem by derivatizing a chlorinated analogue of melamine (2-chloro-4,6-diamine-1,3,5-triazine) with

**Table 1. Analytical Techniques Developed to Detect Melamine (and Related Compounds)**

| analytical technique | analytes | sample type | limits of detection | ref |
|---|---|---|---|---|
| HPLC | cyromazine<br>melamine | poultry meat<br>eggs | 0.02 ppm | Chou et al.[11] |
| LC−MS | cyromazine<br>melamine | chard | <0.01 mg $kg^{-1}$ | Sancho et al.[12] |
| ELISA | melamine | pet food | 0.02 $\mu$g $mL^{-1}$ | Kim et al.[13] |
| HPLC | | | 0.1 $\mu$g $mL^{-1}$ | |
| GC/MS | melamine, ammelide, ammeline, cyanuric acid | milk powder | 0.002 mg $kg^{-1}$ | Miao et al.[14] |
| HPLC | melamine | infant formula | 0.1 $\mu$g $mL^{-1}$ | Venkatasami and Sowa[15] |
| TOF-MS | melamine<br>cyanuric acid | milk powder | 170 $\mu$g $kg^{-1}$<br>450 $\mu$g $kg^{-1}$ | Vaclavik et al.[16] |
| micellar electrokinetic chromatography | melamine, ammelide, ammeline, cyanuric acid | tableware<br>flour | 2.4−5.0 $\mu$g $L^{-1}$ | Hsu et al.[17] |
| surface enhance Raman spectroscopy | melamine | NA | 0.1 mg $L^{-1}$ | Du et al.[18] |
| colorimetric biosensor | melamine | NA | $8 \times 10^{-10}$ M | Cao et al.[19] |
| electrochemical biosensor | melamine | milk products | 0.25 ppm | Zhu et al.[20] |
| ELISA | melamine | dog food | 1 $\mu$g mL | Garber[21] |
| ELISA | melamine | infant formula | <1 $\mu$g $mL^{-1}$ | Garber and Brewer[22] |
| | | wheat products | <2.5 $\mu$g $g^{-1}$ | |
| ELISA | melamine | NA | 2.6 ng $mL^{-1}$ | Lei et al.[23] |
| ELISA | melamine | milk | 0.1 mg $L^{-1}$ | Yin et al.[24] |
| | | milk powder | 0.2 mg $kg^{-1}$ | |
| | | feeds | 0.5 mg $L^{-1}$ | |

both 6-aminocaproic acid and 3-mercaptopropanoic acid to produce two haptens that could be easily coupled to proteins via carbodiimide reaction leaving the two amine groups as epitopes for antibody production.[23] The approach of Yin et al. was to derivatize melamine itself using *tert*-butyl bromoacetate followed by acid hydrolysis to create a hapten that was also coupled to protein via the carbodiimide reaction.[24]

In this study, a polyclonal antibody to melamine was produced using a structural mimic, 6-hydrazino-1,3,5-triazine-2,4-diamine (HTD; see Figure 1) which negated the need for hapten derivatization. Instead a suitable bifunctional cross-linker was attached to the carrier protein before reaction with the hapten. The resulting antibody was then incorporated into a surface plasmon resonance (SPR) optical biosensor immunoassay which, as far as we know, is the first of its kind. Milk powder contaminated with melamine was obtained from the European Commission and analyzed to ascertain if the developed procedure could be successfully applied to the analysis of this type of deliberately corrupted material.

## MATERIALS AND METHODS

**Reagents and Chemicals.** Melamine (2,4,6-triamino-1,3,5-triazine) (M2659), ammeline (4,6-diamino-2-hydroxyl-1,3,5-triazine) (45613), cyanuric acid (1,2,5-triazine-2,4,6-triol) (16614), cyromazine (*N*-cyclopropyl-1,3,5-triazin-2,4,6-triamine) (45414), 2-(*N*-morpholino) ethanesulfonic acid (MES; M3671), and bovine thyroglobulin (BTG; T1001) were obtained from Sigma-Aldrich (Poole, Dorset, U.K.). Ammelide (6-amino-2,4-dihydroxy-1,3,5-triazine) (A0645) was obtained from TCI Europe (Zwijndrecht, Belgium). 6-Hydrazino-1,3,5-triazine-2,4-diamine (HTD) was purchased from Specs (Delft, The Netherlands), and succinimidyl 4-formylbenzoate (SFB; PN22419) was purchased from Fisher Scientific (Loughborough, U.K.). PD-10 desalting columns containing Sephadex G25 (17-0851-01), sensor chip CM5 research grade (BR-1000-14), HBS-EP buffer (BR-1001-88), NHS coupling solution (22-0526-53), and EDC coupling solution (22-0526-54) were obtained from GE Healthcare (Little Chalfont, U.K.). Montanide ISA 50 V adjuvant was obtained from Seppic (Paris, France). Melamine-contaminated milk powder was kindly supplied by the Institute for Reference Materials and Measurements (Geel, Belgium).

**Instrumentation.** An optical biosensor, employing surface plasmon resonance, was used as the platform to develop the immunoassay. The Biacore Q was obtained from GE Healthcare (Little Chalfont, U.K.)

**Preparation of Melamine Immunogen Using HTD Hapten.** BTG (50 mg) was dissolved in 5 mL of phosphate buffered saline (PBS) pH 7.2. The cross-linker SFB (2 mg) was dissolved in 100 $\mu$L of dimethyl formamide (DMF), and 20 $\mu$L of this (0.4 mg) were added to the protein solution slowly with stirring. The mixture was allowed to incubate for 2 h at room temperature, and the reaction was stopped by removing any remaining SFB by gel filtration (PD-10 desalting column) against 0.05 M MES buffer containing 0.15 M sodium chloride pH 4.7. The structural mimic of melamine, HTD (1 mg), was dissolved in 1 mL of hot water (approximately 80 °C), and 200 $\mu$L (0.2 mg) were added to the modified BTG. The mixture was allowed to react for 2.5 h at room temperature before purification by dialysis against normal saline.

**Immunization and Blood Sampling Protocol for Antibody Production.** The prepared immunogen (0.5 mg of coupled protein in 1 mL of saline) was mixed with 1 mL of Montanide ISA 50v adjuvant to create an emulsion. A New Zealand White rabbit was immunized every 4 weeks with the immunogenic emulsion at four injection sites. Blood samples were collected 10 days after each immunization and assessed for specific antibody titer by measuring the extent of binding to HTD immobilized on a biosensor chip in the presence of melamine as a competitor. When a satisfactory titer was obtained, the antibody was harvested from the rabbit. All procedures were performed in accordance with The Animals (Scientific Procedures) Act 1986, U.K.

**Immobilization of HTD onto Sensor Chip.** A CM5 sensor chip was immobilized with the melamine mimic HTD. Briefly, the carboxymethyl dextran surface was activated by contact with a 1/1 mixture of 0.2 M EDC/0.05 M NHS (50 $\mu$L) for 30 min. Reactants were removed and HTD (0.7 mg) in sodium acetate buffer pH 4.5 (350 $\mu$L) immediately applied to the chip surface (50 $\mu$L). This was allowed to react overnight at room temperature. Any unreacted sites were deactivated by incubating with 1 M ethanolamine pH 8.5 (50 $\mu$L) for 20 min. The chip surface was then washed with deionized water and dried with nitrogen gas. The immobilized sensor chip was stored refrigerated in the presence of a desiccant when not in use.

**Protocol for Biosensor Assay to Analyze Milk Samples.** Infant formula known to be free from melamine contamination was prepared according to the manufacturer's instructions. Typically, infant formula (4.6 g) was weighed into a polycon and dissolved in deionized water (30 mL) by stirring for 15 min. Calibrants were prepared by adding working standards (containing 0 and 20 $\mu$g mL$^{-1}$ melamine) (50 $\mu$L) to reconstituted infant formula (2 mL) or infant liquid milk (2 mL) to produce calibration points equivalent to 0 and 0.5 $\mu$g mL$^{-1}$ liquid samples. Biosensor buffer (50 $\mu$L) was added to all test samples (2 mL). All calibrants and samples were allowed to stand for 10 min at room temperature, transferred (1 mL) to microcentrifuge tubes, and centrifuged at 13 000 rpm for 10 min. Supernatants (100 $\mu$L) were transferred to plastic bijous and diluted (1/20, v/v) by the addition of biosensor buffer (1900 $\mu$L). The diluted extracts were applied to a 96 well microtiter plate and mixed with antibody (1/1, v/v) prior to injection over the chip surface at a flow rate of 20 $\mu$L min$^{-1}$ (2 min). Regeneration of the chip surface was achieved by injection of 100 mM sodium hydroxide (20 $\mu$L) at a flow rate of 20 $\mu$L min$^{-1}$.

**Assay Validation.** A range of commercially purchased infant formula and liquid infant milk products known to be free from melamine contamination were extracted and analyzed, as described above, both as blanks ($n$ = 21) and fortified with melamine at a concentration of 0.5 $\mu$g mL$^{-1}$ ($n$ = 21). Seven blank and seven fortified samples were extracted and analyzed in a single run, and this was repeated on 3 successive days to determine the detection capability (CC$\beta$) of the method for melamine. The assay was further validated by construction of a calibration curve at concentrations of 0, 0.125, 0.25, 0.5, 1, 5, and 10 $\mu$g mL$^{-1}$ (as previously described) to determine the inter-assay ($n$ = 3) and intra-assay ($n$ = 10) coefficients of variation following fortification of infant formula with melamine at concentrations of 0.5 and 1 $\mu$g mL$^{-1}$

**Melamine-Contaminated Milk Powder Samples.** Following the health scares in China concerning melamine-corrupted milk powder, the European Commission's Directorate General for Health and Consumer Protection (DG SANCO) conducted a

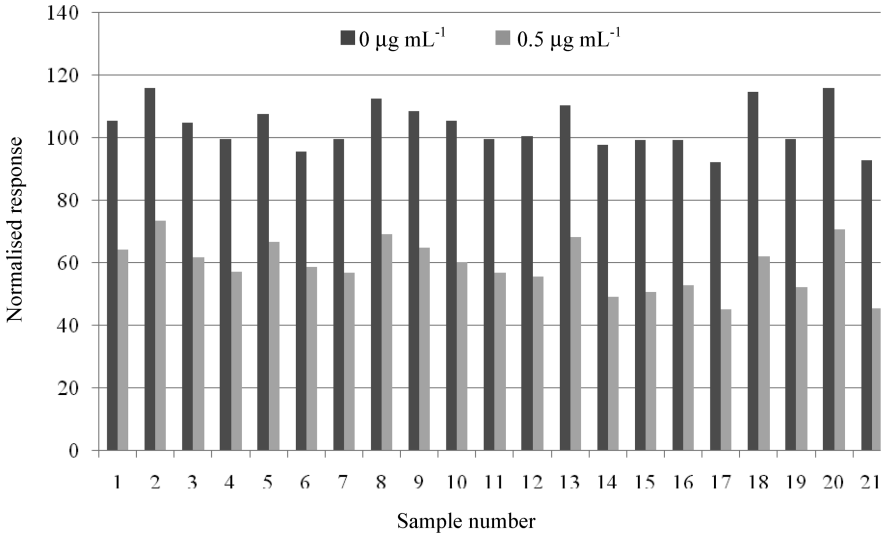


**Figure 2.** The range of responses obtained from 21 blank and fortified (0.5 $\mu$g mL$^{-1}$) samples used to determine the detection capability of the assay.

proficiency test in 2009 to assess the capabilities of laboratories to determine melamine in milk powder.[25] To this end, milk powder contaminated with melamine at a concentration of 10 ± 0.6 mg kg$^{-1}$ was produced and distributed to 31 laboratories including 21 of the 27 European Member States. Samples of this melamine contaminated milk powder were subsequently obtained by this laboratory and analyzed to assess the ability of the developed biosensor immunoassay to detect melamine at this concentration in the tainted material.

## RESULTS AND DISCUSSION

A structural mimic (HTD) for melamine was used as a hapten for antibody production, removing the need for chemical manipulation of melamine itself. A commercially available cross-linker (SFB) was used to convert primary amines on the carrier protein to benzaldehyde moieties to allow direct reaction with the hydrazide group of the hapten.

The rabbit polyclonal antibody produced was assessed for specificity in both assay buffer and sample matrix by using melamine as the reference compound (100%) to compare with binding profiles obtained from other compounds (ammeline, ammelide, cyromazine, and cyanuric acid) which are structurally related to melamine (see Figure 1). While no cross-reactivity of the antibody with ammeline, ammelide, or cyanuric acid was observed, a degree of binding to ammeline could have been predicted according to structural similarities. Therefore it is reasonable to suggest that differences in electronic configuration caused by the extra lone pair of electrons from the hydroxyl group in ammeline were enough to inhibit binding with the prepared antibody. This is supported by the strong binding obtained with cyromazine (243% and 224% in buffer and infant formula milk powder, respectively), which exhibits a more similar electronic configuration to melamine (and the hapten) with respect to the triazine ring structure. The high cross-reactivity with cyromazine means that if a sample is found to be positive by the assay, it is not conclusive evidence of the adulteration of infant formula or infant liquid milk with melamine. Therefore, samples screened as positive should be analyzed for confirmation of the presence of melamine (or cyromazine) by a physicochemical technique. However the detection of cyromazine should not be seen as a detrimental feature of the assay as this compound is not authorized by the European Commission for use as a pesticide in animals that produce milk for human consumption. Indeed melamine is the major metabolite of the pesticide, and so it could be assumed that a sample contaminated with cyromazine will also contain an amount of melamine. In any case, a melamine specific antibody will not be able to differentiate between the metabolic product of cyromazine and melamine which has been added fraudulently to samples.

**Table 2. Concentrations Obtained in a Single Biosensor Immunoassay for Samples Fortified at 0.5 and 1 $\mu$g mL$^{-1}$ for the Determination of Intra-Assay Variation ($n$ = 10)**

| | concentration obtained from fortified samples ($\mu$g mL$^{-1}$) | |
|---|---|---|
| sample number | fortified at 0.5 $\mu$g mL$^{-1}$ | fortified at 1 $\mu$g mL$^{-1}$ |
| 1 | 0.499 | 1.10 |
| 2 | 0.502 | 1.02 |
| 3 | 0.539 | 1.07 |
| 4 | 0.485 | 1.04 |
| 5 | 0.509 | 1.00 |
| 6 | 0.495 | 0.99 |
| 7 | 0.518 | 1.07 |
| 8 | 0.481 | 1.11 |
| 9 | 0.492 | 1.07 |
| 10 | 0.523 | 1.04 |
| mean | 0.504 | 1.051 |
| standard deviation | 0.018 | 0.040 |
| coefficient of variation | 3.6% | 3.8% |

The developed immunobiosensor assay was validated in accordance with Commission Decision 2002/657/EC.[26] In buffer, the sensitivity of the assay (given as an $IC_{50}$) was calculated to be 67.9 ng mL$^{-1}$. Analysis of infant formula and liquid infant milk products known to be free from melamine contamination ($n$ = 21) and again after fortification with melamine at a concentration of 0.5 $\mu$g mL$^{-1}$ ($n$ = 21) showed that the method


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**Table 3. Concentrations Obtained from Three Biosensor Immunoassays for Samples Fortified at 0.5 and 1 $\mu$g mL$^{-1}$ for the Determination of Interassay Variation ($n$ = 3)**

| | concentration obtained from fortified samples ($\mu$g mL$^{-1}$) | |
|---|---|---|
| sample number | fortified at 0.5 $\mu$g mL$^{-1}$ | fortified at 1 $\mu$g mL$^{-1}$ |
| 1 | 0.499 | 1.10 |
| 2 | 0.510 | 0.99 |
| 3 | 0.545 | 0.90 |
| mean | 0.518 | 0.997 |
| standard deviation | 0.024 | 0.100 |
| coefficient of variation | 4.6% | 10.0% |

had a detection capability (CC$\beta$) of <0.5 $\mu$g mL$^{-1}$ for melamine in infant formula and infant liquid milk. The range of normalized responses obtained for the blank and fortified samples is shown in Figure 2, which demonstrates the clear differential between the two values. The detection capability is a factor of 5 lower than the level set by the European Commission required for detection of melamine in food products.

The individual values obtained for intra- and interassay variation are given in Tables 2 and 3, respectively. Intra-assay variations were found to be 3.6% and 3.8% at concentrations of 0.5 and 1.0 $\mu$g mL$^{-1}$ melamine, respectively, while interassay variations were 4.6% and 10.0% at the same concentrations, respectively.

Three samples produced for the DG SANCO 2009 proficiency test for determination of melamine in milk powder[25] were analyzed by the developed method. The milk powder samples had been contaminated with melamine at a concentration of 10 ± 0.6 mg kg$^{-1}$. The screening results obtained showed melamine concentrations of 10.45, 10.82, and 9.85 mg kg$^{-1}$ for the three samples analyzed and compare very favorably with the results achieved during the proficiency test.

## CONCLUSIONS

As shown previously for other food sample matrixes and related contaminants,[27−29] this biosensor immunoassay provides a sensitive, reproducible, and accurate analytical method for the detection of melamine in infant formula and liquid milk. Biosensor technology provides an automated platform with which to process an immunoassay and so is suitable for the analysis of large numbers of samples, a requirement of increasing importance for the food producer and regulator alike.

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