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Synthesis and characterization of hapten-quantum dots bioconjugates: Application to development of a melamine fluorescent immunoassay

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ABSTRACT

A general and universal analytical strategy for characterization of hapten–BSA conjugates based on complementary optical spectroscopy and molecular mass spectrometry techniques is here described. The proposed procedure provides highly-valuable information about the molecular weight of the conjugate, its stoichiometry and the concentration of the precursors (hapten and BSA) in the conjugate; such information is of great analytical interest for further development of novel quantitative immunoassays.

Further, due to great demand of new, simple and robust methodologies for the melamine analysis in milk infant formula, a new immunoprobe melamine-bovine serum albumin-quantum dot was synthetized, characterized and successfully applied in a competitive fluorescent quantum dot-based immunoassay. It should be highlighted that the limit of detection achieved without any sample pretreatment, 0.15 mg kg^{-1} for melamine in milk infant formula, is one order of magnitude lower than the maximum concentration level allowed by international legislation in such type of samples. Finally, this simple approach was validated by the use of an alternative technique (HPLC–UV) for the analysis of melamine in contaminated milk infant formula, showing a good agreement between the results obtained by using both analytical methodologies.

1. Introduction

In 2008 dramatic events that occurred in China, related to the contamination of infant formula milk with melamine, along with the knowledge of other episodes of these adulterations in some other countries have given rise to a clear food-safety alarm activation. As a result, a strong demand on research and development of new methodologies for the detection, screening and determination of melamine at trace levels in food and particularly in infant formula milks [1–3] has arisen nowadays. Melamine structure (MEL, IUPAC name: 1,3,5-triazine–2,4,6-triamine) has a high nitrogen content (around 66%), favouring its fraudulent use as food adulterant to increase the apparent protein content [1]. Furthermore, toxicological studies show that ingestion of melamine in large quantities may lead to reproductive damage or even bladder cancer, due to the formation of bladder/kidney stones [4]. Such evidences explain that maximum levels of melamine in dairy products are regulated by many International Agencies (2.5 mg mL^{-1} for food and feed in general and 1 mg mL^{-1} in infant formula milk) [5,6].

Current analytical methods for melamine detection in different matrixes (milk, infant formula, eggs, fish, urine, serum, kidney tissue, muscle, soil, etc.) involve typically the use of separation techniques, including capillary electrophoresis [7], high-performance liquid chromatography (HPLC) with spectrophotometric detectors [3,8], and liquid or gas chromatography–mass spectrometry [9,10]. Most of such already described methods offer low detection limits in the order of μg kg^{-1} but often present some important disadvantages, including complex and time-consuming sample pretreatment, expensive instrumentation, long analysis times, etc. Thus, research aimed to develop simpler, faster and lower cost analysis methods suitable for daily routine screening of melamine in food is required.

Up to now, some commercial ELISA test kits for melamine routine control in food samples [3,11,12] are already available. A sensitive fluorescence polarization immunoassay for the analysis of melamine in milk and milk powder samples was recently developed by Wang et al. [13] with a promising detection limit around 9 ng mL^{-1}. Additionally, quite recently, different analytical methods for melamine control have been proposed based on direct measurement of chemiluminescence [14,15], on changes on the VIS–UV spectra employing gold and silver nanoparticles as probes [16–18], or on the quenching of photoluminescent from water-soluble quantum dots (QDs) [19,20].

In this context, QDs-based methodologies developed up to date for melamine detection consist on the direct interaction of the analyte with the QD surface producing a quenching or

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enhancement of the QD signal. This strategy is rather unspecific because many other molecules or ions present in the sample can interact with the QDs in the same way. Thus, a previous rigorous step for analyte isolation is mandatory. In the present work, we get around this problem by combining the specific capability of an appropriate antibody (Ab) as selective recognition element and the QDs only as the sensitive fluorescent label of the sensor system, due to QDs exhibit interesting properties as luminophores, such as high quantum yields, narrow emission spectra with a great separation from the excitation one, and high resistance to photo degradation processes.

Thus, the synthesis, characterization and application of melamine-BSA-QDs conjugates to the determination of melamine in infant formula milk are here described. A special effort has been done first in proposing a rather simple, universal and comprehensive methodology to characterize in depth both conjugates, the melamine-BSA and the fluorescent immunoprobe melamine-BSA-QD as well as the luminescent immunoassay. The advantages of an ELISA immunoassay (simple sample preparation and high throughput) are combined in this way with the unique properties of fluorescent QDs to develop a convenient quantitative analysis of melamine in infant formula milk.

2. Experimental

2.1. Reagents, materials and instrumentation

All reagents were of analytical grade and used as received without further purification. Deionised ultrapure water (resistivity 18.2 $M\Omega\ cm^{-1}$) was used throughout the work. A description of the reagents used, as well as the different instruments employed, can be found in the Suplementary information.

3. Protocols

3.1. Preparation of melamine-BSA conjugates

A commercial kit was used for conjugation of melamine to BSA based on a covalent amide binding between the amino groups from melamine and the carboxylic groups from BSA, following the carbodiimide chemistry [21]. The ratio of the precursors Mel:BSA:EDC used was 525:1:1700. A great excess of hapten is used to ensure that all binding sites from BSA are covered and to be sure that the melamine only uses one of its NH_2 terminal groups in the conjugation, leaving free the other two NH_2 terminal groups that conform the epitope (i.e., that part of an antigen that is recognized by the immune system, typically the antibody). Briefly, the protocol for the conjugation kit is as follows: 2 mg of hapten (melamine) were dissolved in 450 μL of Imject® EDC conjugation buffer. This solution is added to 200 μL of 10 $mg\ mL^{-1}$ carrier protein (BSA) solution and, after well mixing, this carrier–hapten mixture was poured into a vial that contents 10 mg of EDC and dissolve by gentle mixing. The reaction is incubated for 2 h at room temperature with stirring. The conjugate is purified by desalting. The columns were first conditioned with 1 mL of purification buffer and centrifuged at 1000*g* for 2 min three times. Then, the sample is slowly applied to the center of the compact resin bed and again is centrifuged at same conditions. The liquid collected corresponds to the Mel-BSA conjugate, being retained in the column the excess of hapten and other by-products of the reaction. The purified conjugate is split in aliquots and stored in the freezer at −18 °C.

3.2. Synthesis and solubilization of CdSe/ZnS quantum dots

The CdSe/ZnS QDs used as fluorescent labels in the immunoassay were synthetized in our laboratory via organometallic route and water-solubilized by coating them with an amphiphilic polymer as previously described [22]. The stock solution of water-solubilized QDs in 50 mM SBB pH 12 solution buffer was stored at room temperature (in darkness).

3.3. Synthesis and characterization of the immunoprobe: Mel-BSA-QDs

The carbodiimide chemistry was used to synthesize the immunoprobe Mel-BSA-QD forming a chemical bond between the carboxylic groups from the polymeric coating of the QDs and the amino groups from the BSA. The optimized molar ratios used for the bioconjugation reaction were 2:1:1500 (QDs:Mel-BSA:EDC). The reaction was performed at room temperature for 2 h with constant stirring. A purification step is required in order to isolate the immunoprobe from the excess of reagents and the by-products. For such purpose and considering the differences in the molecular weights of the compounds to separate (Mel-BSA 71 kDa, Mel-BSA-QD > 150 kDa), ultrafiltration (UHF) was the technique selected. Experimental conditions used for UHF were 5000 rpm, 5 min × 6 cycles (three washes) and 4 °C using a 100 kDa membrane filter. The concentration of the melamine in the immunoprobe was estimated following the previously reported methodology of Trapiella-Alfonso et al. [22].

3.4. Matrix assisted laser desorption/ionization-mass spectrometry

To characterize the synthetized Mel-BSA conjugate, MALDI-MS was selected to obtain molecular weight and stoichiometry information, using sinapinic acid as the matrix. A solution of 5 $mg\ mL^{-1}$ of the matrix in 30% of acetonitrile and containing 0.1% of TFA was prepared. An amount of 1–10 pmol of protein in the sample was needed (in our experiments we have ensured a concentration of protein of 15 $nmol\ mL^{-1}$). As the sample has to be ideally clean of salts and the Mel-BSA conjugate was stored in a 28 mM Na_3PO_4, 300 mM NaCl and 33 mM sorbitol buffer, a previous clean-up step was carried out (sample desalting by an Amicon membrane filter of 10 kDa, where the sample was washed with Milli-Q water three times and centrifuged at 14,000*g* during 3 min each time). A solution of 1 $mg\ mL^{-1}$ of BSA standard in water was used as reference. The MALDI plate was loaded with standards, matrix and/or samples, and when the spots are dried (at least after 30 min) the MALDI-MS analysis was performed.

3.5. Fluorescent competitive immunoassay format

Most of the immunoassays developed using nanoparticles as tags make use of nanoparticle-labelled antibodies. However, during the process of labelling, recognition capabilities of the antibody are many often altered. Alternatively, in this paper QDs were conjugated to the antigen and a competitive immunoassay format was selected for determination of melamine, where the hapten and the immunoprobe (Mel-BSA-QD) compete for the limited binding sites of the immobilized antibody. An immunoassay scheme rather similar to that described in a previous paper from our research group for progesterone sensing [22] is here proposed, after concentrations and ratios of the reagents were optimized trying to obtain the maximum sensitivity on the detection into the concentration range required for the particular analysis of melamine.

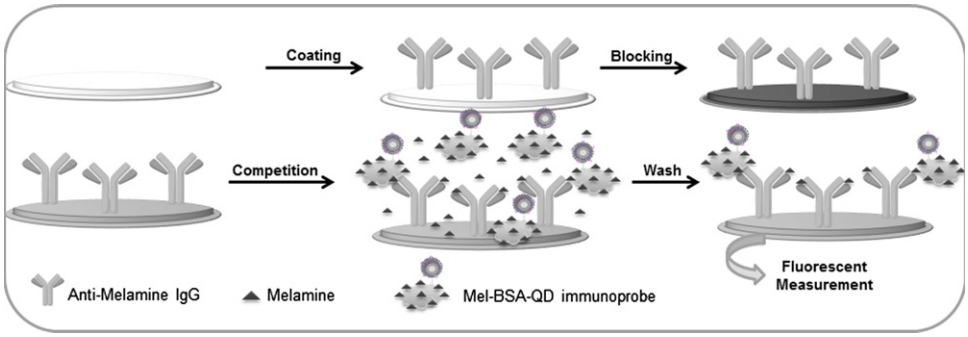

Fig. 1. Schematic representation of the QD-based fluorescent competitive immunoassay.

The procedure for the immunoassay is schematically represented in Fig. 1. First, the microtiter plate is coated with the solution of the antibody and is incubated for 2 h at 37 °C. After that, the solution of antibody is removed and the blocking step is done by adding the 3% casein solution in water in order to avoid further unspecific binding. The plate is incubated overnight at 4 °C. The next step consists on washing the plate three times with the washing solution (10 mM PBS pH 7.4+0.05% Tween 20) to remove the excess of reagents. Then, the competition is established by the addition of a mixture of the standard (or sample) and a known amount of the immunoprobe (Mel-BSA-QD). The reaction is incubated at 37 °C for 2 h. After the necessary washing step, the fluorescence emission of the photoactivated QDs from the Mel-BSA-QD recognised by the antibody is measured as the analytical signal of this competitive immunoassay.

3.5.1. Milk analysis

Fresh bovine whole milk was used to carry out the optimization of the immunoassay and to perform the calibration curves. This milk was selected because is free of melamine, constituting a perfect blank of the matrix where the analysis will be done.

Three different brands of powdered milk infant formula (named M1, M2 and M3) were selected as samples under study in order to assess the applicability of the developed immunoassay. Two of them were milk for newborns (M1 and M3) and the other one was milk for infants over 6 months (M2). It should be noted that it was not necessary to perform any sample pretreatment of the milk samples, except for a 10-fold milk dilution with Milli-Q water, prior to the melamine determination.

4. Results and discussion

4.1. Characterization of Mel-BSA conjugates

Mel-BSA conjugates, used as precursors to label further the melamine with water-soluble QDs, are not commercially available. Thus, first of all, here we had performed their synthesis, purification and characterization. The molecular weight and the stoichiometry of the synthesized conjugate were investigated by MALDI-MS analysis (see procedures in Section 2). Results from MALDI experiments (see Fig. 2) were used to calculate the molecular weight of the conjugate, being 71.0 ± 0.3 kDa (n=8). As can be seen in Fig. 2, comparing the mass spectra of the BSA standard with the Mel-BSA conjugate mass spectra, there is a mass shift that can be attributed to the incorporation of the melamine in the BSA structure. This increase in the mass (ΔM) can be used for the estimation of the stoichiometry of the precursors in the conjugate, taking into account that the molecular weight of the melamine is 126.12 g mol^{-1}. Thus, dividing the ΔM observed by the molecular weight of melamine the

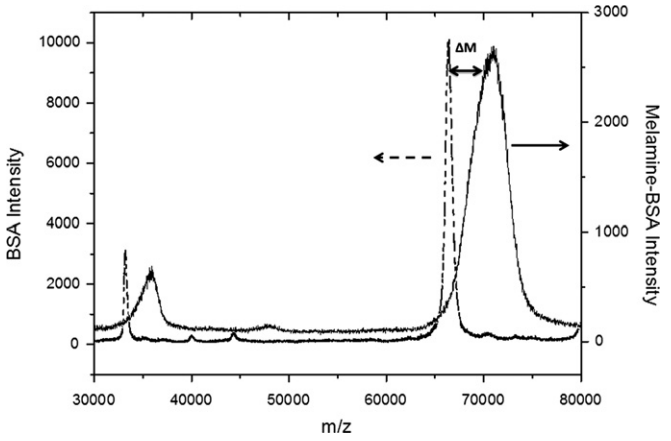

Fig. 2. MALDI-MS characterization of the synthesized Melamine-BSA conjugate. The mass spectra were obtained using sinapic acid as matrix. Mass spectra of BSA standard (dash line) and of the Mel-BSA conjugate (black line) are represented, showing clearly the mass shift between them.

number of Mel per BSA can be obtained. In our case the found value was 37 ± 2 (n=8) Mel per BSA, leading to a ratio of Mel:BSA of 37:1.

Once the melamine:BSA ratio was obtained, a Bradford test was used to estimate the BSA concentration and the concentration of such species in the final conjugate, using Eq.(1):

$$C_{Mel} = 37 C_{BSA}(MW_{Mel}/MW_{BSA}) \quad (1)$$

where C_{Mel} is the concentration of melamine in the conjugate, 37 is the ratio Mel:BSA, C_{BSA} is the BSA concentration in the conjugate obtained by the Bradford test, MW_{Mel} is the molecular weight of the melamine and MW_{BSA} is the molecular weight of the BSA.

Results obtained in this way showed that the concentrations of BSA and melamine in the final conjugate were 2.1 ± 0.1 and 0.147 ± 0.008 mg mL^{-1} (n=3), respectively. Such information is of great value to further optimize the conjugation Mel-BSA to the QDs.

4.2. Synthesis and characterization of Mel-BSA-QD immunoprobe

The strategy selected to synthesize the proposed fluorescent immunoprobe has been described above (Section 2.2.3) and corresponds to the activation of the carboxylic groups present in the amphiphilic polymer coated QDs via the EDC chemistry and the subsequent attachment to the amino groups of the BSA from Mel-BSA conjugate.

After appropriate purification, mandatory characterization of the QD-based immunoprobe was performed. In this context, it was first investigated the possible effect of both the melamine

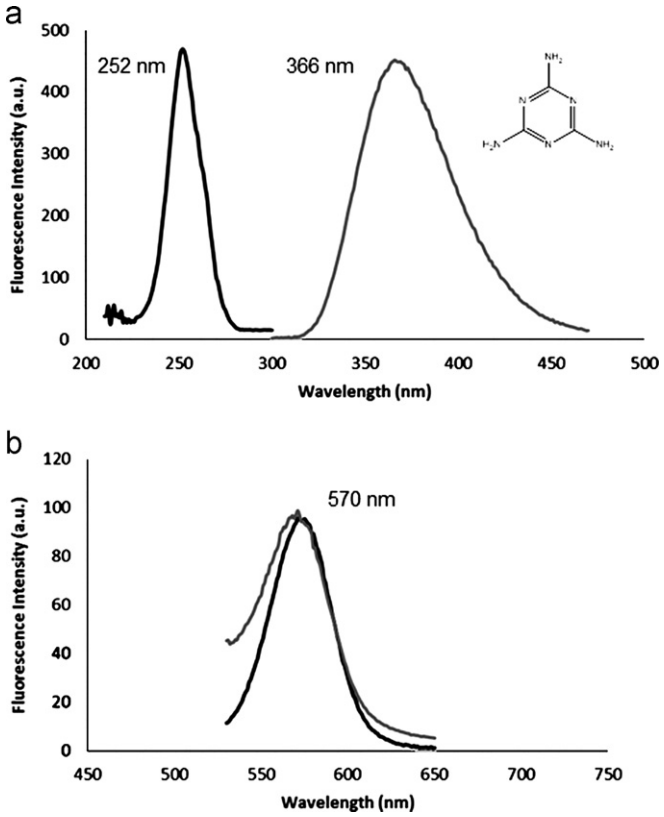

Fig. 3. (a) Excitation–Emission luminescence spectra of melamine. Inset in the graph is the chemical structure of Melamine showing its high nitrogen content. (b) Fluorescent emission spectra of QDs (Black), and the emission from the immunoprobe Mel-BSA-QDs (Grey).

itself and the bioconjugation process on the luminescent emission of the QDs labelled to the Mel-BSA. Fig. 3a shows the excitation–emission luminescent spectra of the melamine. As can be seen the luminescence emission from melamine appears in a spectral region well separated from the QDs emission. Therefore, there is not a risk of overlapping between the fluorescence emission from the immunoprobe and from free melamine that could interfere the final analyte quantification. Moreover, in Fig. 3b it was observed that the bioconjugation process does not affect the emission from the QDs used as labels and purified Mel-BSA-QD bioconjugate still emits an intense fluorescence at the same wavelength maximum characterizing free colloidal CdSe/ZnS QDs.

Next, the efficiency of the bioconjugation reaction and the concentration of melamine in the final conjugate were evaluated, following a previously described procedure [22]. Such procedure is based on a modified Bradford test that correlates absorbance measurements with the concentration of the hapten in the immunoprobe, using the following equations:

$$[BSA]_{Bioc} = [BSA]_{Standard} - [BSA]_{UHF} \quad (2)$$

$$[Mel]_{Bioc} = [BSA]_{Bioc}(37\ M_{Mel}/M_{BSA}) \quad (3)$$

$$Yield = ((mol_{BSA^0} - mol_{BSA^{UHF}})/mol_{BSA^0})100 \quad (4)$$

where $[BSA]_{Bioc}$ is the concentration of the BSA in the purified immunoprobe, $[BSA]_{UHF}$ is the concentration of BSA in the conjugate not labelled to QD (present in the filtered fraction), $[Mel]_{Bioc}$ is the concentration of progesterone in the purified immunoprobe, M_{Mel} is the molecular weight of melamine and M_{BSA} is the molecular weight of BSA. Finally, mol_{BSA0} is the BSA initial moles existing in the Mel-BSA conjugate and $mol_{BSA\ UHF}$ are the BSA moles of the Mel-BSA conjugate not bioconjugated to QDs.

Using these equations the concentration of the melamine in the immunoprobe could be estimated as 0.139 ± 0.007 mg mL^{-1}. The yield obtained for the bioconjugation reaction turned out to be $95 \pm 3\%$.

Finally, a spectrophotometric immunoassay with a secondary Ab tagged with horseradish peroxidase (HRP) was performed in order to evaluate the capability of the anti-melamine Ab to recognize both the antigen and the labelled antigen (immunoprobe). Thus, the same range of concentrations of the free and labelled antigen were assayed in the ELISA plate and after the two steps of immunoreaction (one with anti-melamine Ab and the other one with the secondary Ab-HRP) the plate was measured. Not significant differences in the signal were found, showing that the Ab is able to recognize the immunoprobe with almost the same sensitivity than the antigen.

4.2.1. *Fluorescent competitive immunoassay of melamine*

The concentration of antibody used for coating and immunoprobe concentration added in the competition step were optimised for the development of the immunoassay. Best results (searching for a low detection limit) were obtained with a 3 μg mL^{-1} of Ab solution and 10 μg mL^{-1} of the immunoprobe (Mel-BSA-QDs) solution (keeping the ratio in volume 1:3 between the sample and the immunoprobe added to establish the competition).

To assess the analytical performance of the proposed fluorescent immunoassay, a series of standard solutions with different melamine concentrations were prepared in diluted fresh bovine milk and the analysis was performed under the optimized experimental conditions. The inhibition curve obtained is shown in Fig. 4. This inhibition curve was fitted using a four-parameter equation with "SOFmax Pro software" and the analytical parameters were obtained from this mathematical application. It should be noted that the points that better fit the curve are obtained from the average of three replicates, and all of them present a precision better than 5% (estimated as the RSD of the signals obtained). A linear relationship in the concentration, ranging from 0.30 to 3.50 mg kg^{-1} (covering the limits established by the legislation), was achieved enabling melamine trace determinations in milk. These linearity limits are defined by the inhibitory concentration (IC) values IC_{20} and IC_{80}. The sensitivity of the assay, determined by the concentration of the analyte which inhibits the measured analytical signal by a factor of 50% (IC_{50}), was 1.04 mg kg^{-1} of melamine in milk. The detection limit (DL), calculated as IC_{10} in the inhibition curve, was 0.15 mg kg^{-1} of melamine, referred to a non-diluted whole milk matrix. It is

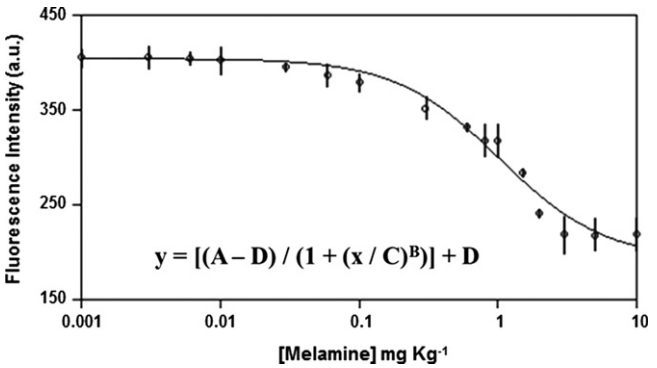

Fig. 4. Fluorescent inhibition curve obtained for the analysis of Melamine in milk infant formula using melamine labelled with QDs as tracer in the competitive immunoassay. Each point of the curve is the average of three independent measurements and the standard deviation is represented for each point being in all cases less than 5%. Inset the graph is the four-parameter equation.

Table 1
Analysis of three milk infant formula brands.

Sample	Melamine in non-spiked sample (mg kg^{-1})[a]	Internal validation[a]			External validation[b] (mg kg^{-1}) Melamine in non-spiked sample
		Spiked melamine (mg kg^{-1})	Melamine in the spiked sample (mg kg^{-1})	Recovery (%)	
M1	0.16 ± 0.06	0.3	0.42 ± 0.09	98 ± 9	0.14 ± 0.03
		1	1.11 ± 0.07	97 ± 4	
M2	0.21 ± 0.03				
M3	0.29 ± 0.04				

[a] Sample analysis by competitive fluorescent immunoassay.
[b] Sample analysis by HPLC–UV (Anal. Chim. Acta 665, 2010, 227–230).

important to highlight that this detection limit is about one order of magnitude lower than the maximum level of melamine allowed in infant formula (1 mg kg^{-1}) by the legislation [6]. The inter-assay reproducibility of the proposed immunoassay for melamine determination in milk was evaluated and estimated as the RSD of the signals obtained for three independent immunoassays done in three different days. The inter-assay reproducibility achieved was 5 ± 2%, showing the robustness of the methodology. In addition, what is very interesting, the here proposed methodology has the benefit of avoiding any sample pretreatment except for 10-fold dilution with Milli-Q water.

4.2.2. Analysis of milk infant formula samples and validation of the methodology

The applicability of the proposed competitive fluorescent immunoassay based on QDs for the determination of melamine in milk infant formula was assessed by analysing three infant formula brands.

In order to perform an internal validation of the proposed methodology one of the samples under scrutiny, M1, was further spiked with 0.3 and 1 mg kg^{-1} of melamine standards and analysed following the optimized assay. As can be seen in Table 1, the results obtained fully agree with those achieved by direct analysis (sample without fortification) showing good recoveries (> 95%). Besides the internal validation and in order to certify that the developed fluorescent immunoassay is effective in the determination of melamine in milk infant formula, the same sample M1 was analysed by HPLC–UV following the procedure developed in a previous work by Venkatasami et al. [8]. In this case a simple sample pretreatment was done consisting of sample dissolution in 50% methanol aqueous solution by sonication and centrifugation in order to separate the extract from the residual milk. This extract was further purified by filtration through a 0.45 μm membrane filter. Finally, the filtrate was injected in the chromatographic system and its analysis was carried out. The results obtained are collected in Table 1 and again show a complete agreement with those found by the immunoassay here proposed. Once the proposed methodology was validated, two more samples, M2 and M3, were analysed obtaining in all cases melamine concentrations lower than 1 mg kg^{-1}, which is the maximum limit allowed by the legislation.

5. Conclusions

A simple and general approach for complete characterization of hapten-BSA conjugates is here proposed based on the combination of optical spectroscopy and molecular mass spectrometry techniques (Bradford test+MALDI-MS). Combination of such complementary techniques offers highly valuable information in terms of molecular weights, stoichiometry of the conjugate and concentration of the precursors in the conjugate. This is fundamental information to develop novel quantitative bioassays.

Further, synthesis and characterization of an immunoprobe (Mel-BSA-QD) was performed following a general strategy recently developed in our group [22]. The applicability of this new immunoprobe was tested by developing a competitive fluorescent QD-based immunoassay for the determination of melamine in infant formula milk. The proposed new methodology for the direct analysis of this adulterant gives rise, without any cumbersome sample pre-treatment step, to a detection limit one order lower than the limit established by the legislation. Finally, this methodology was internally (spiking the sample) and externally (using a chromatographic technique for the analysis) validated, showing in both cases a full agreement with the results obtained with the fluorescent immunoassay.

Finally, it should be stated that the carboxylic functionalization of the nanoparticles makes the process of protein labelling easy and universal. In fact, once we have proven that the system is valid for milk samples analysis, the methodology here proposed could be simply extended for further bioconjugations to different molecules of interest using QDs of different sizes (different emissions) allowing further developments of multiplexing assays.

Acknowledgements

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.01.027.

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