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Determination of monensin in milk samples by front-surface long-wavelength fluoroimmunoassay using nile blue-doped silica nanoparticles as labels

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ABSTRACT

A heterogeneous immunoassay for monensin determination in milk samples using a tracer formed by anti-monensin antibodies bound to nile blue (NB)-doped silica nanoparticles (NPs), 96-well microplates as solid supports and long-wavelength fluorescence measurements is described for the first time. The assay relies on the competition of the monensin present in the samples with a monensin–bovine serum albumin conjugate, which was immobilized onto the well surface, for the active sites of anti-monensin antibodies. After subsequent incubation and washing steps, the fluorescence of the bound tracer fraction is measured onto the dry surface of the well. An antigen capture format was also assayed by immobilizing anti-sheep IgG previously to the incubation of sheep anti-monensin antibodies and using a tracer formed by monensin bound to nile blue-doped silica NPs, which competes with the analyte for binding the immobilized antibody. Although the fluorescence signal obtained in both formats can be correlated to the analyte concentration, better results were obtained using the antibody capture format. After the optimization of the system using this format, the method features a detection limit of 0.015 $\mu g\,L^{-1}$ and a dynamic range from 0.05 to 5 $\mu g\,L^{-1}$. The precision, assayed at two different analyte concentrations, 0.2 and 1 $\mu g\,L^{-1}$, and expressed as relative standard deviation, gave values of 5.9% and 4.0%, respectively. The method was satisfactorily applied to the analysis of milk samples, which only required a simple extraction step in order to remove the proteins from samples, giving recoveries in the range 83.3–107.5%.

1. Introduction

Monensin is a veterinary drug polyether ionophore produced by *Streptomyces cinnamonensis* that exhibits both antibacterial and anticoccidial activities and has been traditionally used to prevent coccidiosis in poultry [1]. This antibiotic has been considered as a growth-promoter, since this disease prevents the growth of poultry owing to the bloody diarrhea and weight loses associated. Although the use of many veterinary antibiotics as growth promoters has been banned since 2006, they can be still administered to some species according to the EU Regulation 1831/2003/EC [2]. Also, the treatment with monensin has been extended to calves for which the EU has set maximum residue limits (MRL) of 2 $\mu g\,kg^{-1}$ in bovine muscle, kidney and milk, 10 $\mu g\,kg^{-1}$ in fat and 30 $\mu g\,kg^{-1}$ in liver, the residues being determined in all the samples as unchanged monensin, as stated in the 37/2010/EC regulation [3].

The concern on food safety has led to the search for reliable analytical methods to screen and confirm the presence of antibiotic residues in foodstuffs to reduce the increasing bacterial resistance to antibiotics. The determination of polyether ionophore antibiotics has been traditionally performed using liquid chromatographic methods with UV and fluorometric detection after their derivatization [4,5]. This step is required owing to the lack of suitable chromophore and/or fluorophore groups in their structure, except for lasalocid, which has been determined by measuring its intrinsic fluorescence [4]. Nowadays, several LC–MS/MS methods have been developed for the determination of coccidiostats [6–10] and for multiclass residue analysis including the ionophore class [11–13]. These confirmatory methods require the availability of a sophisticated and expensive technique and, sometimes, are time-consuming owing to the extraction and further clean-up procedures involved. For this reason, the use of screening methods plays two essential roles: in one hand, the number of samples subjected to confirmatory analysis is lower and, on the other, the cost of the analysis is reduced. Immunoassays, whenever used in a quantitative or semi-quantitative way, have shown their usefulness as screening methods for antimicrobial residues in foodstuffs [14–21]. Several commercial and non-commercial enzyme-linked immunosorbent assays (ELISAs) have been described for the determination of monensin in biological [14–18] and environmental [19] samples. Most of these methods involve the use of photometric measurements, reaching detection limits close to 1 $ng\,mL^{-1}$, but

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a lower detection limit (0.06 ng mL^{-1}) has been reported using chemiluminescence detection [18]. Also, time-resolved fluoroimmunoassays involving the use of an europium(III) chelate as label have been described for monensin determination [20,21], but the detection limit reached is very similar to those obtained using ELISA with photometric detection.

The use of functionalized inorganic matrix nanoparticles (NPs) as alternative labels in immunoassay is a relatively new trend justified by their versatile physicochemical properties which allow the improvement of the features of these assays [22–25]. Among these NPs, doped silica NPs are a useful option owing to their chemical and thermal stability, fine dispersion in aqueous solution, transparency to visible light, capability to encapsulate a wide variety of compounds and relatively inert environmental behavior, in addition to their large surface area and easy surface functionalization [25]. These properties have given rise to a great variety of immunoassay methods, which have been mainly devoted to the determination of macromolecules, such as tumor markers [23,24].

The immunoassay reported here shows the usefulness of nile blue (NB)-doped silica NPs as labels for the determination of monensin in milk samples using long-wavelength fluorescence measurements, which provide the spectral selectivity required to avoid interferences from the sample matrix. The synthesis and optical properties of these NPs have been recently reported [26]. Although a limitation of dye-doped silica NPs is the potential dye leakage, owing to the porosity of silica material, NB-doped silica NPs show an excellent stability, which is ascribed to the interaction of the cationic dye with the negative silanol groups from silica matrix. These NPs have been used as labels in a heterogeneous immunoassay for soy protein determination in several food samples [27], reaching a detection limit about 10 times lower than the afforded by a commercial ELISA kit available for the determination of these proteins.

To the best of our knowledge, the method proposed here is the first immunoassay for monensin determination using NPs as labels, which shows their capability to improve the analytical features, such as the detection limit, of the immunoassays previously described for the determination of this drug [14–18,20,21]. Although most of these assays are applied to the analysis of plasma and liver samples, milk samples have been chosen in this instance because they are easily obtained and allow a non-invasive detection of monensin for its safe use in milk producing animals. The sample treatment is quite simple as it only requires a deproteinization step and a suitable dilution.

2. Experimental

2.1. Instrumentation

A 1420 Multilabel counter Victor ^{3}V microplate reader (Perkin Elmer and Analytical Sciences, WallacOy, Turku, Finland) was used to perform fluorescence measurements. Two filters (nominal wavelength/passband) were used to select the excitation (620/8 nm) and emission (680/10 nm) wavelengths of the doped NPs. An additional filter (531/25 nm) was used to perform absorbance measurements using the Komarowsky reaction in order to calculate monensin concentrations. Fluorescence measurements were performed using the constant voltage mode of the instrument with an integration time of 1 s. NP size characterization was performed by TEM as described elsewhere [26,27]. Black and shallow 100-μL well proxy-plate 96-well microplates (Perkin Elmer) were assayed as support for the development of the heterogeneous immunoassay method.

2.2. Reagents and solutions

All the reagents were of analytical grade and were used as supplied by the manufacturer. Triton X-100, NB chloride, sodium acetate, sodium chloride, sodium borohydride, monensin sodium salt, anhydrous N-dimethylformamide (DMF), N-hydroxysulfosuccinimide sodium salt (sulfo-NHS), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDAC), bovine serum albumin (BSA) and sodium carbonate were obtained from Sigma–Aldrich (USA). Tetraethyl orthosilicate (TEOS), (3-aminopropyl)-triethoxysilane (APS), 3-(trihydroxysilyl)propylmethylphosphonate monosodium salt (THPMP) and vanillin were supplied by Aldrich (Germany). 1-Hexanol and di-potassium hydrogen phosphate were acquired from Merck (Germany). Acetone, absolute ethanol, sodium tetraborate, sulfuric acid and methanol were obtained from Panreac (Spain), sodium metaperiodate from Scharlau (Spain) and sheep polyclonal antibody to monensin was supplied by Abcam (United Kingdom).

NB solution was prepared in distilled water according to the aforementioned procedures [26,27]. A 1.0 g L^{-1} stock solution of monensin was prepared by dissolving the appropriate amount of monensin sodium salt in ethanol. Intermediate and working monensin solutions were prepared by further dilution in phosphate buffer solution (0.015 M, pH 8.0). Phosphate (0.015 M, pH 8.0), acetate (0.1 M, pH 5.5 and pH 4.5), and carbonate (0.05 M, pH 9.0) buffer solutions were prepared by dissolving the appropriate amount of these salts and adjusting the pH with either hydrochloric acid or sodium hydroxide when appropriate. The Komarowsky reagent solution [5,28] was prepared by dissolving vanillin (4 g) in methanol, adding 2 mL of concentrated sulfuric acid to adjust the pH and raising the mixture to 100 mL with methanol. This solution was freshly prepared whenever used.

2.3. Procedures

2.3.1. Synthesis and functionalization of NPs

The procedure used to synthesize NB-doped silica NPs is similar to a reverse-micelle microemulsion method previously reported [26,27]. Briefly: an amount of Triton X-100 (510–530 mg or 0.79–0.82 mmol) was dissolved in 9.6 mL (0.53 mol) of distilled water by stirring vigorously this mixture for 5 min. Then, a volume of 100 μL (0.44 mmol) of TEOS was added and the solution was stirred for 5 min. A volume (1.8 mL) of 10^{-3} M (1.8 μmol) NB was added and the mixture stirred again for 5 min. Afterwards, 3 mL (0.024 mol) of hexanol was added and the microemulsion formed was stirred for 15 min. Concentrated ammonium hydroxide (70 μL, 0.9 mmol) was then added and the mixture stirred for 5 min to start the TEOS hydrolysis and condensation reactions. The mixture was then placed in a thermostated tank at 25 °C for 24 h in the dark and, afterwards, it was centrifuged for 5 min at 537 × g to separate the phases involved. The upper phase (about 3 mL) was transferred to a 10-mL beaker and 40 μL of APS and 80 μL of THPMP were added to introduce amino groups onto NP surface. This mixture was magnetically stirred during 1.5 h in a water bath at 25 °C. Afterwards, the microemulsion was broken by adding 10 mL of acetone and stirring for 5 min. The supernatant was discarded and NPs were recovered from the bottom with 8–10 mL of distilled water. The mixture was centrifuged for 5 min at 9300 × g to separate the NPs from unreacted reagents. Then, the NP precipitate was washed with ethanol and water until the fluorescence intensity of supernatants was similar to the blank and, finally, the NPs were re-dispersed in 1 mL of phosphate buffer solution.

2.3.2. Preparation of tracers using nile blue-doped silica NPs

NB-doped silica NPs were coupled to either anti-monensin antibodies or monensin to obtain the tracers required to study the potential determination of this drug using antibody or antigen capture format, respectively.

The synthesis of the NPs–anti-monensin antibodies tracer involves an oxidation step of carbohydrate moieties of the antibody molecule to give rise to aldehyde groups capable of reacting to amino groups of the NP surface. Firstly, 200 μL of 1.2 μM anti-monensin antibody solution prepared in 0.1 M phosphate buffer solution at pH 7.5, were diluted with 0.1 M sodium acetate buffer solution (pH 5.5) until a volume of 2 mL, and 200 μL of 0.1 M sodium metaperiodate solution were added to this volume. The mixture was let to stand for 20 min at 0 °C and then, layered on a HiTrap desalting column (GE Healthcare) equilibrated with 25 mL acetate buffer solution (pH 4.5), at a 5 mL/min flow-rate, and 400-μL fractions were taken. Their absorbance was monitored at 280 nm and the anti-monensin protein was recovered from fractions 7 to 11, accounting for about 2 mL of solution. This volume was added to the amino-modified NP-doped silica NPs, which had been previously centrifuged to remove phosphate buffer solution. The mixture was incubated for 5 min at room temperature and then, 50 μL of 0.66 M $NaBH_4$ solution were added to the dispersion and allowed to react for 20 h at 4 °C. Afterwards, the reaction mixture was centrifuged and the NP–anti-monensin protein conjugate obtained was washed with phosphate buffer solution (0.015 M, pH 8.0). Finally, the tracer was re-dispersed in 1 mL of the same phosphate molar concentration solution and stored at 4 °C for further use.

The synthesis of the NPs–monensin tracer was performed using a carbodiimide reaction in which 200 μL of a 2 g L^{-1} monensin solution in DMF were mixed with the same volume of a 0.2 M EDAC solution prepared in 0.05 M 2-(N-morpholino)ethanesulfonic acid (MES) at pH 6.2 and 21.6 mg of sulfo-NHS were then added, being the mixture raised to 2 mL with the same MES buffer solution and incubated at room temperature for 15 min. Then, 40 μL of 1 M 2-mercaptoethanol were added and the mixture was left to stand for 10 min. An appropriate volume (50 μL) of this mixture was mixed together with the amino-functionalized NB-doped silica NPs and 950 μL of phosphate saline buffer were added and the resulting mixture was incubated for 2 h at room temperature. After this time, the synthesized tracer was washed using absolute ethanol and phosphate buffer solution, reconstituted in 1 mL of the same phosphate molar concentration solution and stored at 4 °C until use.

2.3.3. Synthesis of BSA–monensin conjugate

The use of a BSA–monensin conjugate was required to immobilize monensin onto the wells. The coupling of monensin to BSA molecule was performed via a carbodiimide reaction [29] using the following procedure: an amount of monensin (0.2 mmol) was dissolved in 1 mL of dry DMF, then sulfo-NHS (23.0 mg, 0.1 mmol) and EDAC (41.2 mg, 0.2 mmol) were added in order to activate the carboxylic acid group of monensin. The mixture was stirred at room temperature for 4 h, and then was centrifuged to remove the precipitate from the acylisourea derivative formed. An aliquot (250 μL) of the activated hapten solution was added dropwise to a stirred BSA solution prepared by dissolving BSA (50 mg) in 5 mL of 0.05 M borate buffer (pH 7.8) and DMF (1.05 mL). The synthesized conjugate was purified using a HiTrap desalting column (GE Healthcare) by a similar procedure to that described above for the synthesis of NP-anti monensin conjugate.

The concentration of monensin in the conjugate was determined using the Komarowsky reaction [5,28] by reacting 500 μL aliquots of monensin standard or BSA–monensin conjugate dilutions prepared in methanol to 1 mL of Komarowsky reagent in glass test tubes. These tubes were sealed and placed in a water bath at 80 °C for 5 min. The red–purple color indicative of monensin presence was measured at 520 nm immediately after cooling the tubes to room temperature. The BSA concentration in the synthesized conjugate was calculated from the absorbance of the protein measured at 280 nm. As a convention, the concentration of the conjugate will be given in terms of the monensin concentration found by using the abovementioned Komarowsky reaction.

2.3.4. Determination of monensin

The competitive heterogeneous immunoassay with antibody capture involves the previous immobilization of the BSA–monensin conjugate in the well, which was performed by adding 60 μL of a 10 mg L^{-1} BSA–monensin conjugate prepared in carbonate buffer solution (0.05 M, pH 9.0) to each well, and incubating the microplates overnight at 4 °C. Afterwards, wells were washed three times with phosphate solution (0.015 M, pH 8.0) and the plates were stored at 4 °C until use.

To perform the immunoassay, a volume (60 μL) of a pre-incubated mixture, prepared by mixing 75 μL of NB-doped silica NPs-labeled anti-monensin antibody (10 nM) in phosphate molar concentration solution (0.015 M, pH 8.0), and 225 μL of monensin antibiotic standard or sample solution (0.05–5 μg L^{-1}) were added to each well. The microplate was incubated for 1.5 h at 37 °C, the wells were washed for three times with the same phosphate buffer solution, and dried for 10 min at room temperature. Then, fluorescence measurements were directly performed at λ_{ex} 620 and λ_{em} 680 nm onto the solid surface of the well.

2.3.5. Analysis of milk samples

The sample preparation involved the deproteinization of food samples using a slight modification of a previously described procedure [21]: 0.4 g of milk sample was acidified with 0.1 mL of 0.1 M hydrochloric acid and mixed with 1.6 mL of pure acetonitrile in order to quantitatively precipitate all proteins. Then, the mixture was vigorously mixed during 30 min and centrifuged (2722 × *g*, 10 min, 10 °C). The supernatant was collected in an Eppendorf tube® and was evaporated to dryness under a gentle stream of nitrogen at 65 °C in order to achieve a medium compatible with the immunoassay. Then, the samples were reconstituted in 3 mL of phosphate buffer solution 0.015 M pH 8.0.

Spiked samples were prepared by adding suitable amounts of monensin standard to 10 g of skimmed milk, semi skimmed milk and whole milk and allowing sample matrix and standards to equilibrate for 1.5 h. Aliquots of 0.4 g were treated as described above for non-spiked samples.

3. Results and discussion

3.1. Choice and optimization of the immunoassay system

Two heterogeneous competitive immunoassays, involving antibody or antigen capture format, were assayed to choose the best option for monensin determination using NB-doped silica NPs as labels. The first format was based on the immobilization of a BSA–monensin conjugate and the use of an anti-monensin antibody–NP tracer, which was synthesized by a similar procedure to that described in an immunoassay for soy protein determination [27]. The second assay needed a monensin–NP tracer, synthesized via a carbodiimide reaction using EDAC, and the immobilization of anti-monensin antibodies by coating the wells with anti-sheep IgG. Preliminary assays performed using both formats showed that the difference in the fluorescence signals obtained for 0.0 and 0.2 μg L^{-1} monensin standard using the antibody-capture format was about 3-fold higher than that obtained using the analyte-capture assay, so the first format was chosen to develop the new immunoassay for monensin determination.

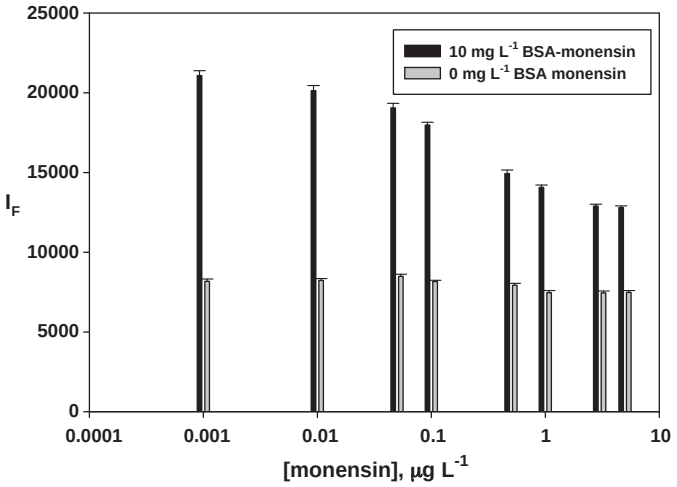

Fig. 1. Fluorescence intensity signals obtained using 0 and 10 mg L^{-1} BSA–monensin conjugate at different monensin concentrations. [tracer] = 10 nM; [phosphate] = 0.015 M; pH 7.5.

Since monensin is a hapten, its immobilization required the use of a protein–hapten conjugate to avoid potential steric hindrance that would prevent its reaction with the tracer. BSA was chosen to produce the conjugate via a carbodiimide reaction with the carboxylic acid of monensin, which also allows the simple immobilization of monensin by passive adsorption onto the well surface. It was found that under the reaction conditions, one molecule of BSA was coupled to approximately one molecule of monensin, by calculating the BSA concentration by measuring the absorbance at 280 nm and the monensin concentration by the Komarowsky reaction [5,28]. A study of the potential non-specific adsorption of the tracer using this format was performed by assaying the system in the absence and presence of BSA–monensin at different monensin concentrations (Fig. 1). In the presence of BSA–monensin conjugate, the signal can be correlated to monensin concentration, as a result of the competition between monensin and BSA–monensin conjugate. The results obtained in the absence of BSA–monensin conjugate showed that the signal obtained was almost constant at all the monensin concentrations assayed and lower than the signal obtained at the highest analyte concentration assayed. These results show that the tracer concentration being attached to the well in the absence of BSA–monensin conjugate is almost negligible and, thus, non-specific adsorption does not occur.

The optimization of the system was carried out by modifying the experimental conditions at different monensin concentrations. The concentration of the immobilized BSA–monensin conjugate on the well surface is an important variable on the system, which influences the sensitivity of the assay, since immobilized and in solution monensin compete for the active sites of the tracer. The influence of this variable was assayed in the range of 0–60 mg L^{-1} (expressed as monensin concentration) finding that 10 mg L^{-1} provided the best results as the dynamic range obtained for the calibration curve was wider and the sensitivity obtained was also better than those obtained at concentrations below and above (Fig. 2A). Another important variable that influences the sensitivity and working range of the assay is the tracer concentration (Fig. 2B), which was assayed in the range from 5 to 12.5 nM, finding that a 10 nM concentration provided the best sensitivity for the assay.

The influence of pH and phosphate molar concentration on the system was also studied to optimize the reaction conditions. The pH was investigated in the range of 6.0–8.5, finding that there was an increase in the signal at pH values above 7.0, being the best results obtained at pH 8.0, from which a slight decrease in the fluorescence signal was observed. The study of buffer concentration showed that the assay sensitivity remained independent on this variable in the range of 0.01–0.03 M, being a 0.015 M concentration chosen as the optimum value. The incubation temperature and time were studied in the ranges 25–40 °C and 30 min–2 h, respectively, choosing 37 °C and 1.5 h as optimum values.

3.2. Analytical features

Fig. 3 shows the calibration curve obtained for monensin under optimal experimental conditions and measuring the fluorescence signals at λ_{ex} 620 and λ_{em} 680 nm. The method presents a dynamic range of 0.05–5 μg L^{-1}. The calibration curve was adjusted to a 4-parameter logistic curve using the Sigma Plot 2001 software, which equation is: $y = y_0 + \left(\frac{a}{1+(x/x_0)b}\right)$, where y is the

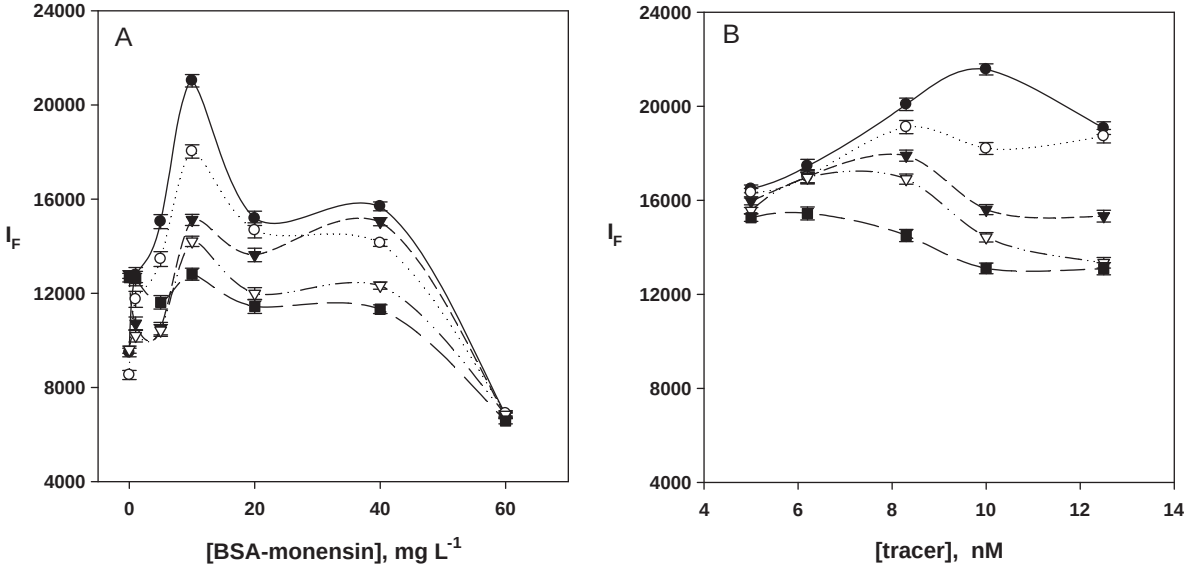

Fig. 2. Influence of BSA–monensin (A) and tracer (B) concentration on the assay in the presence of different monensin concentrations (μg L^{-1}): (1) 0.0; (2) 0.1; (3) 0.5; (4) 1.0; (5) 5.0. [tracer] = 10 nM in figure A. [BSA–monensin] = 10 mg L^{-1} in figure B. Other experimental conditions in both figures are: [phosphate] = 0.015 M, pH 7.5, assay incubation time = 1.5 h, temperature = 37 °C.

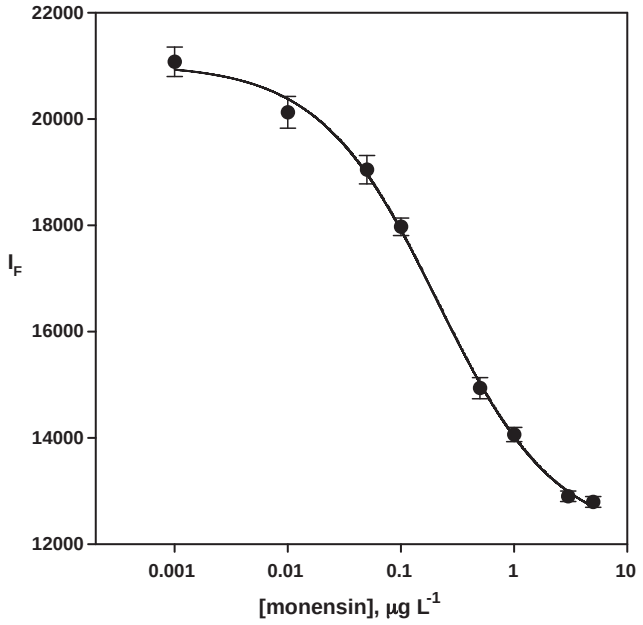

Fig. 3. Calibration curve obtained under optimum conditions.

fluorescence intensity and x is the concentration of monensin expressed in $\mu g\,L^{-1}$. The values of the regression parameters a, b, x_0 and y_0 were $(8.9 \pm 0.4) \times 10^3$, 0.83 ± 0.08, 0.21 ± 0.03, and $(1.21 \pm 0.03) \times 10^4$ respectively, and the regression coefficient was 0.9992. The detection and quantification limits, calculated according to IUPAC recommendations [30], were 0.015 and 0.05 $\mu g\,L^{-1}$, respectively, which would correspond to 0.12 and 0.40 $\mu g\,kg^{-1}$ in the milk samples. The latter value is about 5 times lower than the MRL set by the 37/2010/EC Commission Regulation [3] for monensin in milk samples. Also, the detection limit is four times lower than that reported using ELISA with chemiluminescence detection [18]. The precision, expressed as the percentage of relative standard deviation and assayed at two different monensin antibiotic concentrations, 0.2 and 1 $\mu g\,L^{-1}$, gave values of 5.8 and 4.0% respectively.

The selectivity of the system was studied by assaying different antibiotics of veterinary use belonging to the ionophore group, such as lasalocid, or to other antibiotic groups such as aminoglycosides, tetracyclines and fluoroquinolones, which can be used for therapeutic purposes in animals. Neomycin, oxytetracycline and enrofloxacin were chosen as representative components of these groups. A compound was considered not to interfere at a given concentration when the analytical signal obtained in the presence of this substance was within one standard deviation of the value obtained in its absence. The maximum concentration tested for all these compounds was 200 $\mu g\,L^{-1}$ using a 0.2 $\mu g\,L^{-1}$ monensin concentration, finding that all of them were tolerated at this 1000:1 concentration ratio. This result agrees with the results already reported for other monensin immunoassays [15–17,20,21], which shows the good selectivity of anti-monensin antibodies.

3.3. Applications

The method was applied to the analysis of skimmed, semi skimmed and whole milk samples. The sample treatment was quite simple and consisted in a deproteinization step after which the solvent was evaporated owing to the lack of compatibility of the organic solvent with the immunoassay performance. This compatibility was studied using methanol and ethanol and they proved to decrease the fluorescence signal at percentages above 3% of each solvent. These extraction and evaporation steps are common to those required by immunoassays for monensin determination in sample extracts of feedstuff [15,16] and foodstuff [21] samples. The monensin concentration in the milk samples was determined according to the procedure above described. A recovery study was also carried out to validate the method (Table 1), obtaining values in the range of 83.3– 107.5%.

Table 1
Recoveries obtained for monensin added to milk samples.

Sample	Monensin		
	Added ($\mu g\,kg^{-1}$)	Found[a] ($\mu g\,kg^{-1}$)	Recovery (%)
Skimmed milk	1.5	1.6 ± 0.1	106.7
	2	1.96 ± 0.09	98.0
	4	4.1 ± 0.2	102.5
Semi-skimmed milk	1.5	1.5 ± 0.1	100.0
	2	2.0 ± 0.1	100.0
	4	3.4 ± 0.3	85.0
Whole milk	1.5	1.25 ± 0.07	83.3
	2	1.7 ± 0.1	85.0
	4	4.3 ± 0.3	107.5

[a] Mean ± SD (n = 3)

4. Conclusions

The results obtained have shown the usefulness of NB-doped silica NPs as labels for the determination of monensin using immunoassay, reporting a lower detection limit than those previously reported using other labels [14–18,20,21]. Also, the number of steps of the assay is lower than those required using an enzyme as label. The development of the assay in 96-well microplates, using 100-μL proxy-plate, allows the automation of the method together with a relatively high sample throughput, and reduces the sample and tracer volumes required in comparison with the conventional 300-μL wells, which contribute to minimize the costs associated with the immunoassay.

The low quantification limit of this method allows the determination of monensin at levels below its MRL for milk samples. The use of these samples to control the potential presence of monensin in foodstuffs is a useful alternative to the analysis of other biological samples, since they can be easily obtained.

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