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Development of an automatic high-throughput assay for tetracycline determination by using Eu_2O_3 nanoparticles and dry-reagent technology

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ABSTRACT

The usefulness of europium oxide nanoparticles (Eu_2O_3 NPs) as analytical reagent for the direct determination of organic compounds is described for the first time. Tetracycline, which forms a luminescent chelate with europium, has been chosen as a model analyte. Dry reagent chemistry is used in a 96-well format, which considerably speeds up the determination and contributes to its automation. The NPs are immobilized onto polystyrene wells by adding a volume of a Eu_2O_3 NP dispersion in 2-propanol to each well and drying in an oven until they dry completely. At the moment of analysis, a standard or sample volume (200 μL) in the appropriate medium is added, and the mixture shaken for 15 min at 37 °C. The method allows the determination of tetracycline in the range 20–1000 ng mL^{-1}, with a detection limit of 8 ng mL^{-1}. The inter-assay and intra-assay precision, which were assayed at two different tetracycline concentrations and expressed as relative standard deviation, were in the ranges of 6.5–8.2% and 9.2–12.7%, respectively. The study of the selectivity of the system showed that the method is adequate for tetracycline determination in agri-food samples, since most of antibiotics assayed did not interfere the determination. Only other tetracycline antibiotics provided luminescent signal when reacting to Eu_2O_3 NPs. The method has been applied to the determination of tetracycline in calf urine and in honey samples obtaining recovery values in the ranges of 85.0–110.0% and 99.7–116.7%, respectively.

1. Introduction

Lanthanide-sensitized luminescence is a special type of sensitized phosphorescence of some lanthanide chelates, which relies on the energy transfer from an organic ligand in the triplet excited state to the resonance levels of the lanthanide that emits an intense luminescent signal [1,2]. Lanthanide-sensitized luminescence has been traditionally achieved using lanthanide ions in solution, mainly Eu(III) and Tb(III) ions, which are prepared from high purity salts or by dissolving lanthanide oxides in acidic solutions. The typical ligands for these chelates when used for direct determinations or as labels in bioassays have been β-diketone and other compounds containing aromatic amine and carboxylic acid groups [1,2].

Europium-sensitized luminescence has been described as detection system to determine tetracycline in several kinds of samples, such as biological fluids [3], food [4,5] and water [6,7] samples, using Eu(III) as reagent. As known, tetracycline is a broad spectrum antibiotic administered to treat infections caused by both Gram positive and Gram negative bacteria and it is mostly excreted in urine and faeces as either unchanged drug or microbiologically inactive form [8]. The Eu(III)–tetracycline chelate formation happens by the interaction of tetracycline through its β-diketone group (Fig. 1) to form six-membered rings with Eu (III) ions.

Although most methods for tetracycline determination using Eu(III) as reagent have been developed in solution, the usefulness of dry reagent technology has been also described by immobilizing this lanthanide ion onto solid supports [3,5–7]. Chromatographic paper treated with sucrose has been used for the simultaneous determination of ciprofloxacin and tetracycline in biological fluids by immobilizing Tb(III) and Eu(III) ions onto this support [3]. Other supports described for Eu(III) immobilization have been C_{18}-impregnated silica layers [5,7] and silicate fibers [6], which have been used for tetracycline determination in milk and serum samples, respectively. Although the detection limits reached in these methods are very low, a limitation is the sequential introduction of the sensing probes into the instrument, which decreases the sampling rate desirable for screening purposes.

The combined use of europium-sensitized luminescence and nanotechnology has been described using polymeric [9–11] or silica [12–15] nanoparticles (NPs) doped with a luminescent Eu(III)

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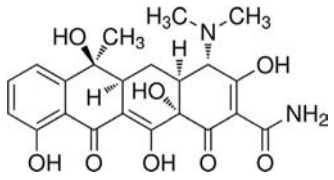

Fig. 1. Chemical structure of tetracycline.

chelate. These articles report the synthesis and luminescent properties of these NPs [10,12–14] and their use as labels in immunoassays [9] and hybridization assays [15]. However, the use of Eu_2O_3 NPs has been scarcely described for analytical purposes. They have been used as labels in an immunoassay for atrazine determination after surface functionalization with silane reagents bearing amine groups [16,17]. Also, Eu_2O_3 NPs has been used for the direct determination of trace amounts of mercury in hair, shellfish, fish and waste water samples [18]. The method relies on the fluorescence quenching of Eu_2O_3 NPs capped with thenoyltrifluoroacetone by Hg(II) ions. However, to the best of our knowledge, no direct determination of organic compounds by sensitizing Eu_2O_3 NPs has been described up to date.

The method presented here shows the first analytical application of commercial Eu_2O_3 NPs as reagents for the direct determination of organic species, using tetracycline as model analyte. Also, the novelty of this study includes the use of these NPs, which show a high stability owing to their inorganic nature, as dry reagents in a 96-well microtiter plate format. This approach allows the development of an automatic high-throughput assay for the simultaneous analysis of a large number of samples in few minutes, improving the sampling rate of the methods based on dry-reagent technology above described that operate sequentially and in a manual way. Also, as described below, the detection limit obtained is similar to those reported using other dry reagent formats [3,4,6,7]. The method only requires the immobilization of Eu_2O_3 NPs onto 300-μL polystyrene wells together with sucrose by adding a volume of NPs dispersion onto each well and letting them to dry in an oven. At the moment of the analysis, just the addition of the buffered standard or sample and a further incubation step are needed.

The practical usefulness of the new method to the analysis of samples related to the direct follow up of tetracycline administration for veterinary purposes has been demonstrated by its application to the analysis of calf urine and honey samples. The monitoring of tetracycline in urine samples is required for the control of the therapeutic use of tetracycline. The drug concentrations found after a dose are quite high and the samples can be easily obtained before sacrificing the animal. This antibiotic can also be used for the treatment of any food producing species, as for instance for beekeeping, so their presence in honey has been evaluated [19].

2. Experimental

2.1. Instrumentation

A 1420 Multilabel counter Victor ^{3}V microplate reader (Perkin-Elmer and Analytical Sciences, Wallac Oy, Turku, Finland) was used to perform fluorescence and time-resolved luminescence measurements. The luminescence intensity was measured using the time-resolved europium protocol, involving the use of filters with nominal excitation and emission wavelength/bandpass with of 340/50 and 615/8.5 nm, respectively. The time-resolved parameters were set to the values defined by the manufacturer, thus being $t_{delay}=400$ μs and the window time or $t_{gate}=400$ μs, with a cycle time of 1000 μs. DELFIA Nunc polystyrene (8 × 12) microplates were used as solid support to develop the analytical reagent and to perform time-resolved luminescence measurements. A SLM Aminco Bowman AB2 series spectrometer fitted with a 7 W pulsed xenon lamp to operate in the time-resolved mode, an R928 photomultiplier tube and a conventional 1-cm path-length quartz cell were used to obtain the excitation and emission spectra of the system.

2.2. Reagents

All reagents were of analytical grade and used as purchased. A stock solution of tetracycline (1000 mg L^{-1}) was prepared after dissolving the appropriate amount of tetracycline hydrochloride from Sigma (St. Louis, MO, USA) in distilled water. Eu_2O_3 nanopowder (< 150 nm, TEM, 99.5%) was obtained from Aldrich (St. Louis, MO, USA) and a 3.1×10^{-3} M Eu_2O_3 (equivalent to 0.06 M in europium) stock solution was prepared in 2-propanol. A 5×10^{-4} M 2-thenoyltrifluoroacetone (TTA) stock solution was prepared by dissolving the appropriate amount of TTA (Aldrich, St. Louis Mo, USA) in ethanol and then filling up to mark with water. Stock solutions of the surfactants cetyltrimethylammonium bromide (CTAB) (10^{-2} M), sodium dodecylsulfate (SDS) (0.1 M) and Triton X-100 (0.1% w/v), (Fluka, Buchs, Switzerland) were prepared in distilled water. Tris(hydroxymethylamino)methane (TRIS) and ammonium chloride (Merck, Schuhard, Germany), hexamethylenetetramine (hexamine, Merck, Schuhard, Germany) were also used. Stock buffer solutions: Tris (0.1 M, pH 7–8), hexamine (0.1 M, pH 6–7.5) and ammonium chloride (0.1 M, pH 7.0–8.0) solutions were prepared by dissolving the appropriate amount of the compound in water and adjusting the pH with either hydrochloric acid or sodium hydroxide. HPLC grade 2-propanol and ethanol (Panreac, Castellar del Vallès, Spain) were also used. Nitrocellulose acetate membranes OPTITRAN BA-S85 (Sigma, St. Louis, MO, USA) were used as the solid substrate for the development of planar dry-reagent assays. Calf urine samples were kindly donated by the Cooperativa Andaluza Ganadera del Valle de los Pedroches (COVAP) and the honey sample was purchased in a local supermarket.

2.3. Procedures

2.3.1. Preparation of dry-reagent wells using Eu_2O_3 NPs

A volume (200 μL) of a dispersion containing 4×10^{-4} M Eu_2O_3 NPs, 5×10^{-6} M TTA and 0.05 M sucrose in 2-propanol was dispensed onto each well and placed in an oven for 2.5 h at 60 °C. Afterwards, the plates were let to cool in a desiccator for 20 min, and once at room temperature, they were sealed and stored at 4 °C in the fridge. These plates were stable for at least three months.

2.3.2. High-throughput determination of tetracycline using Eu_2O_3 NPs as dry-reagent

A volume (200 μL) of tetracycline standard or sample in 0.01 M hexamine buffer pH 7.2 was added to the reagent wells. The mixture was shaken for 15 min at 37 °C using the plate shaker of the instrument. The luminescence intensity was obtained using the europium protocol under the conditions described in the Section 2.1. All the samples and standards were assayed in triplicate.

2.3.3. Determination of tetracycline in calf urine and honey samples

A volume (1.0 mL) of calf urine sample was centrifuged and the supernatant was taken and diluted to 10 mL with deionized water. An appropriate volume of the sample was diluted again with the

appropriate volume of stock solution of hexamine buffer and deionized water to a final volume of 10 mL to obtain a final concentration of tetracycline matching the dynamic range of the calibration curve.

A honey sample was heated for 30 min at 37 °C for melting and 2.5 g was weighed and diluted to 10 mL with the appropriate volume of stock solution of hexamine buffer and deionized water.

In both instances, a volume (200 µL) of diluted sample was treated following the above mentioned procedure for tetracycline determination.

3. Results and discussion

3.1. Selection of the dry-reagent format

As mentioned above, the methods based on dry-reagent chemical technology and lanthanide-sensitized luminescence rely on the use of planar supports to hold the reagent [3,4,7]. Thus, nitrocellulose acetate membranes, chromatographic paper and 96-well microplates were assayed to study the usefulness of Eu_2O_3 NPs as dry reagents. Of the two planar supports, nitrocellulose membranes, although reinforced, featured the problem of low mechanical and thermal resistance. Also, the speed of the drying process was rather slow since the use of relatively high temperatures led to membrane shrinking and hence, poor precision values were obtained. Chromatographic paper was far more resistant than nitrocellulose membranes, but it provided relatively high background signals, which would be ascribed to strong interactions with hydroxyl groups of cellulose.

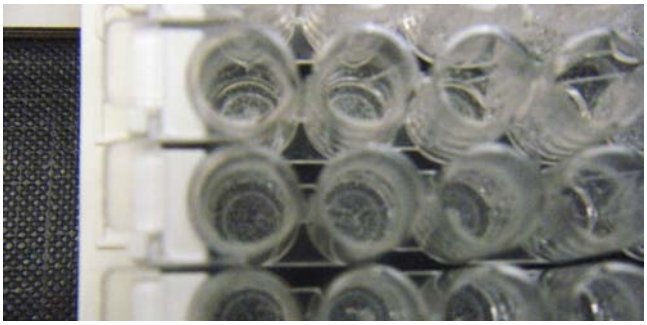

Fig. 2. Top view of dry-reagent wells containing Eu_2O_3 NPs–TTA in 0.05 M sucrose.

The use of well microplates was also evaluated because it can be quite advantageous compared to planar supports, owing to the fact that luminescence measurements are obtained automatically and almost simultaneously, so the sample throughput is highly increased compared to the previously described methods on planar supports [3,4,7]. The influence of sucrose on the assay was evaluated, finding that it helps to improve precision compared to the precision values obtained in its absence. Thus, sucrose was used to reinforce the NPs immobilization onto the well and to avoid the potential negative effects of environmental factors. Its use led to the formation of a shell, which can be easily dispersed in the aqueous medium used to dissolve tetracycline or to dilute the analyzed samples. Fig. 2 depicts a picture of the reagent plates prepared by using this procedure.

3.2. Study and optimization of the system

The development of the dry reagent assay involved the investigation of the required conditions to achieve an intense luminescent signal with adequate precision values. An additional desirable feature is the long-term stability of the dry reagent, which is a significant benefit compared to the use of reagents in solution. The results have shown that these microplates can be stable for at least three months.

The spectral features of the Eu_2O_3 NPs–tetracycline system were investigated in solution using experimental conditions similar to those in the well. This study was also carried out in the presence of TTA because it leads to a luminescence enhancement of the system owing to the formation of a ternary complex. This positive effect can be seen in Fig. 3, which shows the excitation and emission spectra of both luminescent systems. The emission spectra exhibit the typical narrow peak centered at 615 nm, ascribed to the $^5D_0 \rightarrow {}^7F_2$ transition between resonance lines of the lanthanide ion [20], but the presence of TTA increases the luminescence intensity about three-times. Both excitation spectra show a band at 395 nm, which corresponds to the absorption of tetracycline, but another band at 340 nm can be seen in the spectrum obtained in the presence of TTA. As similar luminescence intensity values were obtained using both excitation wavelengths, excitation at 340 nm was chosen because it is actually the nominal wavelength for the excitation filter provided by the manufacturer of the microplate reader used to perform time-resolved measurements.

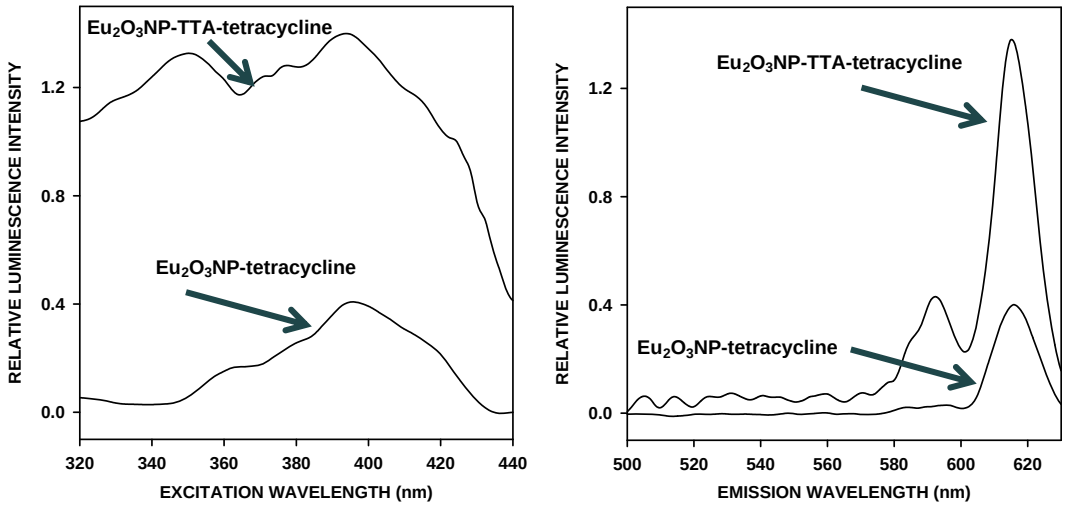

Fig. 3. Excitation (1,1′) and emission (2,2′) spectra obtained using Eu_2O_3 NPs in the absence and presence of TTA, respectively. [Eu_2O_3 NPs] = 4×10^{-4} M, [TTA] = 5×10^{-6} M, [tetracycline] = 200 ng mL^{-1}, hexamine buffer 0.01 M, pH 7.2, [sucrose] = 0.05 M.

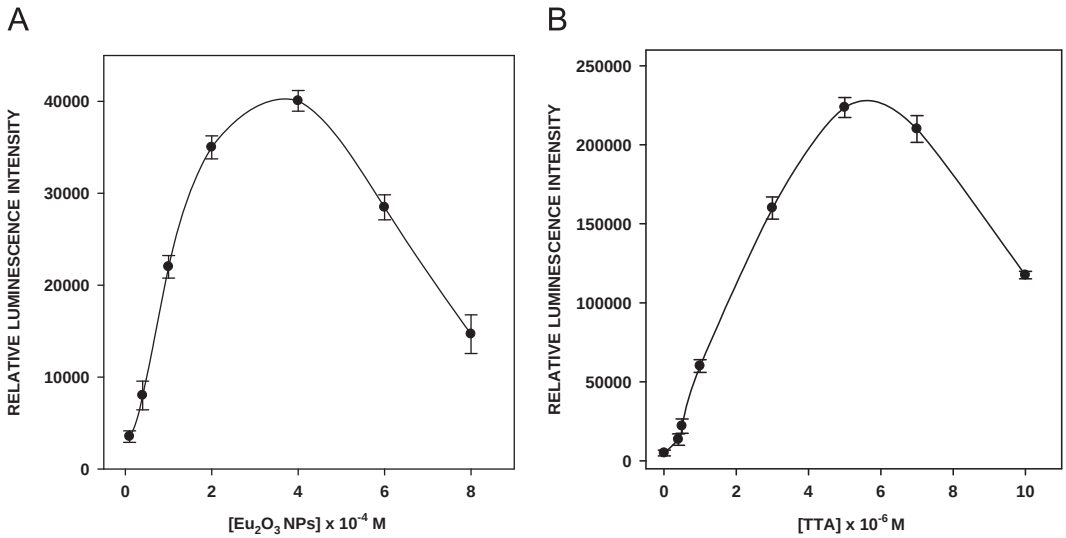

Fig. 4. Influence of Eu_2O_3 NPs (A) and TTA (B) concentration on the luminescence signal of the system. Experimental conditions are: [tetracycline]=200 ng mL^{-1}; hexamine (0.01 M, pH 7.2), [sucrose]=0.05 M, temperature of instrument: 37 °C. In (A) [TTA]=1×10^{-7} M, in (B) [Eu_2O_3 NPs]=4×10^{-4} M.

Although the use of a synergetic reagent such as tri-n-octylphosphine oxide improves the intensity of some europium luminescent systems [9,20], the luminescence of the Eu_2O_3 NPs–tetracycline–TTA system was not modified in the presence of this compound. The optimum pH of the system was in the range of 7.0–7.5. Different buffer solutions, such as TRIS, ammonium acetate and hexamine, were assayed, finding the best results in the presence of hexamine when its concentration was in the range of 0.008–.015 M, thus selecting 0.01 M concentration. The influence of Eu_2O_3 NPs and TTA concentrations on the luminescence of the system is shown in Fig. 4, from which can be seen that the optimum values are 4×10^{-4} M and 5×10^{-6} M, respectively.

The study of the reaction time required to obtain a stable luminescent signal was studied in the range of 1–40 min. A time of 15 min was enough to ensure that the signal remained constant up to the longer time assayed. The effect of the temperature within the instrument was studied in the range of 28–40 °C, obtaining an almost constant signal from 32 to 38 °C and choosing 37 °C for the method development.

The influence of surfactants on the system was also checked since they can protect the luminescence of the chelates from non-radiative quenching processes [2,20,21]. The luminescence of the system was studied in the presence of SDS, CTAB and Triton X-100, chosen as representative anionic, cationic and non-ionic surfactants, assaying concentrations below and above their critical micellar concentration (cmc). However, the use of surfactants was discarded because SDS and CTAB did not have any effect on the luminescence intensity, and only a very slight increase was obtained in the presence of Triton X-100. This behavior could be ascribed to the protection that the oxide structure would have on the luminescence of the system.

3.3. Analytical features

The analytical features of the system under study were obtained by measuring the luminescence intensity at the excitation and emission wavelengths included in the Section 2.3. The method presents a linear range of 20–1000 ng mL^{-1} tetracycline concentration. The values for the slope and *y*-intercept were $(1.05\pm0.09)\times10^3$ and $(7.7\pm0.5)\times10^3$, respectively. The slope features a relatively low error, which is indicative of a good fit of the experimental data to the regression curve together with the regression coefficient (0.997), quite close to the unit. The LOD, which was calculated according to IUPAC recommendations [22] was 8 ng mL^{-1}. This value is similar to those afforded using other dry-reagent formats above described [3,5–7]. The intra-assay (n=10) and inter-assay (n=8) precision values were assayed at two different analyte concentrations (20 and 200 ng mL^{-1}), and were in the ranges of 6.5–8.2% and 9.2–12.7%, respectively.

Table 1
Influence of other antimicrobial agents on the 20 ng mL^{-1} tetracycline determination by using Eu_2O_3 NPs.

Compound	Maximum tolerated interferent:analyte ratio[a]
Tylosin	100
Gentamycin	50
Monensin	50
Neomycin	50
Erythromycin	5
Streptomycin	5

[a] Maximum tolerated ratio assayed was 100-fold of analyte concentration.

The selectivity of the Eu_2O_3 NPs–tetracycline–TTA system was studied by assaying different antibiotics of veterinary use, such as ionophores, aminoglycosides, sulfonamides and macrolides (Table 1). The maximum concentration tested for all these compounds was 2 μg mL^{-1}, using a 20 ng mL^{-1} tetracycline concentration. 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. All the compounds assayed were tolerated at higher concentration levels than that of tetracycline and they did not interfere tetracycline determination at the permitted levels for these compounds in veterinary samples. Erythromycin and streptomycin concentrations higher than 5-times the tetracycline concentration decreased the luminescence of the system, which can be ascribed to their macrolide structure and the relatively high number of hydroxyl groups that would compete with tetracycline for the available coordination sites of Eu_2O_3 NPs. The presence of other tetracycline antibiotics, such as oxytetracycline and chlortetracycline, causes a positive interference on the system as they have a luminescent behavior similar to that of tetracycline [23,24].

3.4. Applications

The method for tetracycline determination using Eu_2O_3 NPs was applied to the analysis of calf urine and honey samples. Tetracycline can be used for veterinary purposes to prevent infections in animals

Table 2
Recovery of tetracyclines added to urine and honey samples.

Sample	Recovery study		
	Added[a]	Found[a,b]	Recovery (%)
Urine 1	20	22 ± 2	110.0
	40	41 ± 4	102.5
	60	55 ± 3	91.7
Urine 2	20	18 ± 1	90.0
	40	34 ± 2	85.0
	60	56 ± 5	93.3
Honey	80	84 ± 6	105.0
	120	140 ± 10	116.7
	160	159 ± 9	99.4

[a] Concentration units: urine, μg mL^{-1}; honey, μg kg^{-1}.
[b] Mean ± SD (n=3).

intended for human consumption, the marker residue being the sum of the parent drug and its 4-epimer. The luminescent behavior of both forms with Eu(III) has been reported to be the same for oxytetracycline [25], so the total determination of both compounds using Eu(III)-sensitized luminescence is feasible. Plasma and urine samples can be used to control the presence of antibiotic residues in animals after their therapeutic use [26] and before the animal sacrifice. Also, the detection of the potential presence of antibiotics in honey is of interest due to the treatment of hives with these antibiotics, which is banned in the European Union (EU). The analysis of both urine and honey samples was quite simple, just their dilution with deionized water and hexamine buffer to match the linear range of the calibration graph. The standard addition method was applied in all instances, since some matrix effect was observed. It was found out a decrease by ca. 12% in the slope of the calibration curve obtained in the presence of 0.125% (v/v) of urine sample, compared with that obtained for aqueous standards, which provided a corresponding LOQ of 18.2 μg mL^{-1} in the original urine sample. For honey samples, although a decrease by ca. 20% was observed in the slope of the diluted sample when compared to aqueous standards, slightly better precision values were obtained in the presence of the same amount of sample matrix, so that the corresponding LOQ in the honey sample was 75 μg kg^{-1}. Tetracycline was not detected in any of the analyzed samples. A recovery study was carried out by adding three different amounts of tetracycline to each sample. Table 2 shows the results obtained for two calf urine samples, which were in the range of 85.0–110.0%, with a mean value of 95.4%, and for a honey sample, with recoveries in the range of 99.4–116.7% and a mean value of 107.0%.

4. Conclusions

The present work has shown the first application of Eu_2O_3 NPs as analytical reagents for the development of an automatic high throughput method for the direct determination of tetracycline, using dry-reagent technology. Although this technology has been previously described for similar purposes using Eu(III) ions and other supports such as chromatographic paper [3], silica layers [5,7] or optical fibers [6], the new method provides several advantages: (1) the preparation of the polystyrene wells with the immobilized reagent is simpler, (2) the use of a microplate reader simplifies the detection step, as it allows the almost simultaneous and automatic measurement of all the wells, unlike other previously described dry reagent methods that require the manual and sequential introduction of each strip or fiber in the instrument, and (3) the cost of the Eu_2O_3 NPs, which are commercially available, is lower than that of the europium salt from the same supplier.

The analytical features of the method using NPs are similar to those described for the methods developed with Eu(III) ions, which shows the capability of tetracycline to access the coordination sites of europium in the NPs. Finally, the analytical applicability of the method has been demonstrated by the results obtained in the analyzed samples, which enables their use to control the treatment of calves or bees with tetracycline.

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