JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Development of a Novel and Automated Fluorescent Immunoassay for the Analysis of β-Lactam Antibiotics

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An automated immunosensor for the rapid and sensitive analysis of penicillin type β-lactam antibiotics has been developed and optimized. An immunogen was prepared by coupling the common structure of the penicillanic β-lactam antibiotics, i.e., 6-aminopenicillanic acid to keyhole limpet hemocyanin. Polyclonal antibodies raised in rabbits after immunization with this conjugate have been applied for the development of a competitive fluoroimmunoassay (FIA), using a novel fluorescent penicillin {[2*S*,5*R*,6*R*]-3,3-dimethyl-7-oxo-6-[(pyren-1ylacetyl)amino]-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxilic acid, PAAP} as the tracer and penicillin G as the reference antibiotic. Protein A/G covalently bound to an azlactone-activated polymeric support was used for the orientated capture of the antibody−antigen immunocomplexes. Upon desorption from the immunosupport, the emission signal generated by the PAAP−Ab complexes is related to the antibiotic concentration in the sample. The 50% binding inhibition concentration of penicillin G standard curves was at 30 ng mL^{-1} with a detection limit (10% binding inhibition) of 2.4 ng mL^{-1} and a dynamic range from 6.0 to 191 ng mL^{-1} (20−80% binding inhibition) penicillin G. The generic nature of the antiserum was shown by good relative cross-reactivities with penicillin type β-lactam antibiotics such as amoxicillin (50%), ampicillin (47%), and penicillin V (145%) and a lower response to the isoxazolyl penicillins such as oxacillin, cloxacillin, and dicloxacillin. No cross-reactivity was obtained for cephalosporin type β-lactam antibiotics (cephapirin), cloramphenicol, or fluoroquinolones (enrofloxacin and ciprofloxacin). The total analysis time was 23 min per determination, and the immunoreactor could be reused for more than 200 cycles without significant loss of activity. The immunosensor has been successfully applied to the direct analysis of penicillin G and amoxicillin in spiked influent and effluent sewage treatment plant water samples with excellent recoveries (mean values for penicillin G and amoxicillin, 99 and 105%, respectively). Results displayed by comparative analysis of the immunosensor with a chromatographic procedure for penicillins showed excellent agreement between both methods.

KEYWORDS: β-Lactam antibiotics; penicillins (PENs); fluorescent immunoassay; environmental analysis, wastewater analysis

INTRODUCTION

The occurrence of antibiotics applied for human or veterinary use in lakes and streams throughout the world has raised an increasing concern due to the toxicity of these drugs for aquatic organisms and the emergence of resistant bacterial strains (*1*−*3*). Important routes of entry into the environment are the direct discharge of aquaculture products, the excretion in urine and feces, and the wash off of topical treatments of livestock animals. Antibiotics concentrations in the aquatic environment, sewage treatment plants (STP), surface, ground, and drinking water are in most cases in the ng L^{-1} range (*4*), although none of them have been included in the list of priority and hazardous substances of the water framework directive of the European Union (*5*, *6*). In consequence, there is an increasing demand for sensitive and reliable methods for the analysis of antimicrobials at low concentrations in ground and surface waters.

β-Lactam antibiotics (BLAs) (**Figure 1**) such as penicillins and cephalosporins are the most frequently used antibiotics for the prophylaxis and therapy of infectious diseases in veterinary

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10.1021/jf0511502 CCC: $30.25
Published on Web 07/23/2005

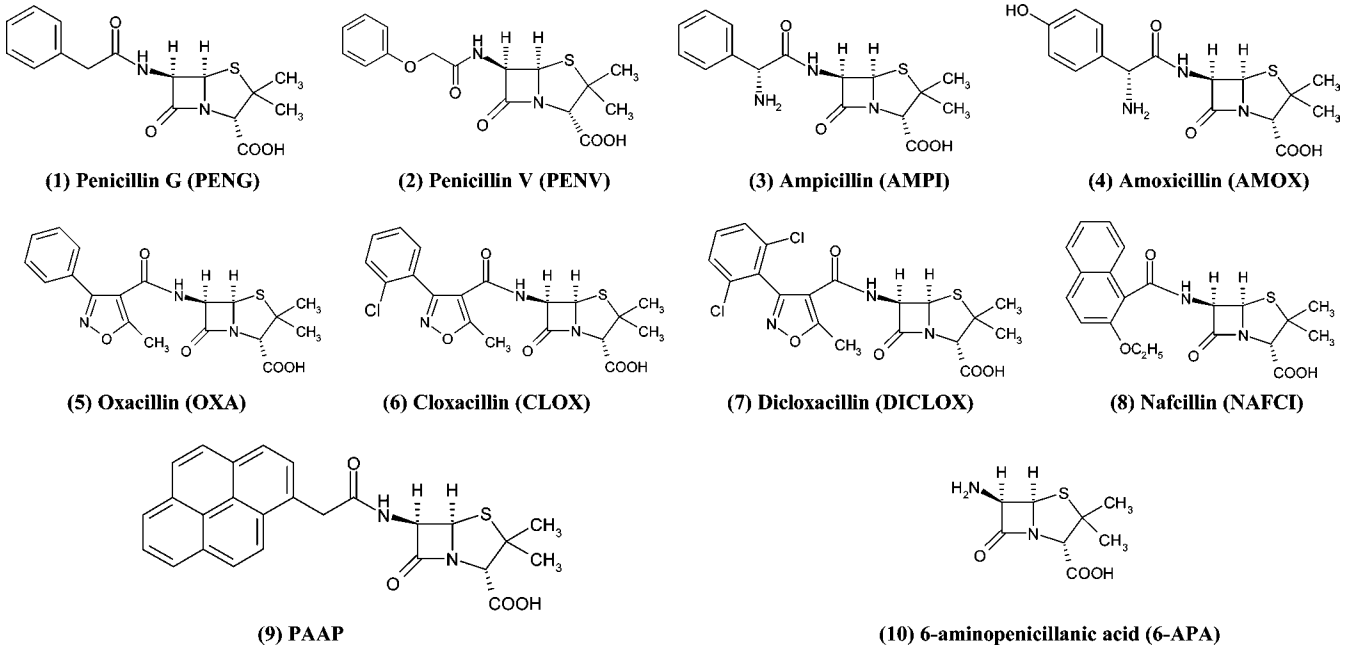

Figure 1. Chemical structure and acronyms of the investigated penicillins (1–8), PAAP (9), and 6-APA acid (10).

practice and are the major contaminants in ground and surface waters. The common structure of natural and synthetic penicillins is a β-lactam ring fused to a thiazolidine ring that binds to transpeptidase and inhibits peptidoglycan synthesis in bacterial cell walls.

Current methods applied for the determination of these antibiotics in water and manure samples are mostly based on high-performance liquid chromatography (HPLC) tandem mass spectrometry (MS) detection (*7*, *8*). These methods, although robust and well-established, are time consuming and expensive and require specialized instrumentation. Moreover, in some cases, the chemicals and toxic solvents required for the analysis may be even more contaminating than the antibiotics themselves (*9*). Additionally, chromatographic methods usually require extensive sample preparation and cleanup and should be restricted to confirmatory purposes only (*10*).

Microbial inhibitor tests, enzymatic assays, enzyme immunoassays, and receptor assays are the basis of several commercial screening tests used for the analysis of these antimicrobials in food samples (*11*, *12*), but their application to environmental samples has been very limited (*11*, *13*−*15*). The advantages of immunochemical techniques include their reliability, low cost, speed of analysis, ease of use, portability, selectivity, and sensitivity (*16*). Moreover, they usually require little sample preparation.

A broad spectrum of commercial polyclonal and monoclonal antibodies against antibiotics has been developed in the past years, but in the case of penicillins, the production of suitable, group specific, and sensitive antibodies has appeared to be difficult (*17*). One of the major problems is associated to the chemical reactivity of the β-lactam ring toward nucleophiles that makes it difficult to obtain antisera against the active form of the antibiotics. With some exceptions (*18*), the optimized immunoassays are either highly specific, present a low sensitivity, or low cross-reactivities (*19*−*24*). We have chosen to prepare an immunogen by coupling 6-aminopenicillanic acid (6-APA), the structure common to the penicillanic BLAs, to the carrier protein keyhole limpet hemocyanin (KLH). The aim was to induce a generic antiserum recognizing several members of the penicillanic BLAs.

The instability of the β-lactam ring is also a major drawback in the preparation of fluorescent penicillin analogues. These fluorescent derivatives have been described in the literature for the study of penicillin-binding proteins to monitor the enzymatic activity of β-lactamases or for chromatographic analysis. However, in most cases, these compounds are difficult to synthesize and purify, are expensive, and/or present short Stokes' shifts (*25*−*27*). To our knowledge, up to now, fluorescent penicillins have not been used in immunosensing.

Heterogeneous phase immunosensors in combination with flow techniques (*28*, *29*) are useful tools for automation and combine the sensitivity and selectivity of immunoassays with the accuracy and simplicity of the flow methods (*30*−*32*). Direct flow-through solid phase fluorescence immunosensors can be carried out in different formats (*33*). In one approach, the analyte and a constant amount of labeled derivative compete in solution for the antibody binding sites. After competition, the immunocomplexes are selectively retained on a polymer sorbent covalently bound to protein A/G and packed into a reactor, and after the application of a desorbent solution, the fluorescence of labeled immunocomplexes eluted from the sorbent is measured and related to the analyte concentration in the sample. The application of the desorbent solution allows the regeneration of the support generally without affecting its binding characteristics. This format has advantages in comparison with other sensing ones. Mainly, it is possible to use the same reagents employed in microtiter plate formats avoiding the synthesis of special reagents, the desorption of the captured immunocomplexes is very easy and effective, and the active working life of the immunosupport reaches a reusability of hundred of cycles. A similar flow injection immunoassay, using amperiometric detection, was described by Meyer et al. (*34*) and Zhi et al. (*35*) for the detection of cephalexin, one of the cephalosporanic BLAs.

This paper describes the development of a flow-through fluoroimmunosensor for the determination of penicillin G in wastewater samples. The immunosensor is based on the competition of the antibiotic with a novel labeled fluorescent analogue, [2*S*,5*R*,6*R*]-3,3-dimethyl-7-oxo-6-[(pyren-1ylacetyl)-amino]-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxilic acid, ab-

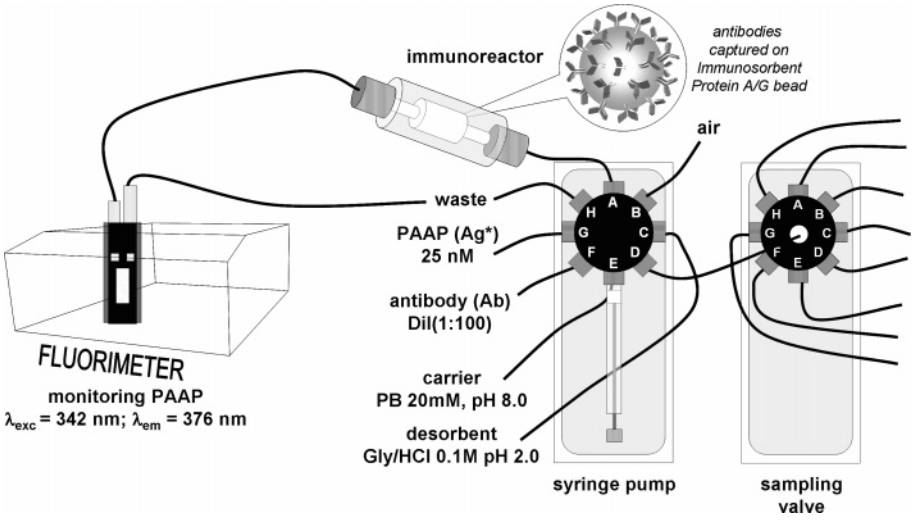

Figure 2. Scheme of the automated flow injection set up used for the measurements.

breviated as PAAP (**Figure 1**) (*36*), for the binding sites of a polyclonal antibody raised against 6-APA. Several parameters affecting the immunosensor performance have been optimized such as the assay pH, the size of the reactor, the concentration of antibody and tracer, the flow rate, the nature of the immunosupport, the temperature, and the incubation time for the immunological reaction and the concentration of the enzyme substrates. The immunosensor has been applied to the analysis of penicillin G and amoxicillin in wastewater samples and validated by a chromatography method optimized previously (*37*).

EXPERIMENTAL PROCEDURES

Reagents and Materials. Penicillin G potassium salt (PENG) (95.7%), amoxicillin anhydrous (AMOX) (87%), ampicillin trihydrate sodium salt (AMPI) (86.6%), penicillin V potassium salt (PENV) (93.7%), oxacillin sodium salt monohydrate (OXA) (92.8%), cloxacillin sodium salt monohydrate (CLOX) (91.8%), dicloxacillin sodium salt monohydrate (DICLOX) (92.2%), nafcillin sodium salt monohydrate (NAFCI) (87.9%), chloramphenicol sodium salt (CLOR), cephapirin sodium salt (CEPH), and 6-APA (96%) standards were purchased from Sigma (St. Louis, Mo). Enrofloxacin (ENRO) (99.7%) and ciprofloxacin (CIPRO) were from Bayer AG (Leverkusen, Germany). Stock solutions for penicillins were prepared in ultrapure water (0.8 g L^{-1}) and stored at 4 °C no longer than 2 weeks. PENG standard solutions for immunoassay calibration purposes were prepared daily upon dilution of the stock solutions in phosphate buffer (20 mmol L^{-1}, pH 8.0).

Acetonitrile (ACN) and methanol (MeOH) (HPLC grade) were provided by SDS (Peypin, France), and trifluoroacetic acid (TFA) (HPLC grade, 99%) was from Fluka (Buchs, Switzerland). Water was purified with a Milli-Q system (Millipore, Bedford, MA). All other chemicals used were of analytical reagent grade. All solutions prepared for HPLC were passed through a 0.45 μm nylon filter before use.

Immunoreagents. *Pyrene-Labeled BLA Analogue (PAAP).* The fluorescent antigen was prepared from 6-APA and the succinimidyl ester of pyreneacetic acid in acetone/water/$NaHCO_3$. After the mixture was stirred overnight, the acetone was removed under reduced pressure and the aqueous mixture was subjected to repeated extraction with diethyl ether in order to remove the unreacted succinimidyl ester. Then, the aqueous phase was brought to pH 2.5 with 10% phosphoric acid and extracted several times with diethyl ether. The combined extracts were dried over anhydrous magnesium sulfate. After the drying agent was removed, the organic solvent was removed by rotavaporation to yield the target compound. The purity and structure of the labeled substrate were confirmed by elemental analysis, Fourier transform infrared analysis. ^1H NMR and ^{13}C NMR spectroscopy, as well as by mass spectrometry with electrospray ionization, are reported elsewhere (*36*).

Polyclonal Antibodies (PAbs) against 6-APA (Lot R543). The synthesis of the immunogen was done by covalently linking 6-APA to KLH through an activated carbodiimide coupling. Thereto, the carrier protein was preactivated with succinic anhydride in order to introduce more potential coupling sites and reduce the change of creating protein cross-links. The actual conjugation reaction between the protein carboxy group and the amino group of the β-lactam was then performed using the water soluble carbodiimide reagent EDC. To preactivate the carrier protein, 5 g of N-hydroxy succinimide (43 mmol) and 4.35 g of succinic anhydride (1 equiv) were dissolved in 30 mL of dichloromethane. A 7.2 mL amount of triethylamine (1.2 equiv) was added, and the reaction mixture was stirred for 3 h at room temperature. It was diluted with 50 mL of ethyl acetate and transferred to a separator funnel. The organic phase was washed twice with 25 mL of ice-cold 1 N HCl, dried over sodium sulfate, and concentrated under vacuum. A 1 g amount of an off-white solid was obtained that was used without further purification. A 200 mL amount of a 10 mg/mL stock solution of KLH in PBS (pH 7) was transferred to an eppendorf vial, and 800 mL of PBS was added to give a final volume of 1 mL. NHS-succinate was dissolved in dimethyl formamide prior to the conjugation to give a 10 mg/mL solution, and 60 mL was added to the protein solution. The reaction mixture was vortexed and left at room temperature for 45 min. Another 60 mL of the same NHS-succinate stock was added, and the reaction mixture was vortexed and left for another 45 min at room temperature. The solution was transferred to a Biomax concentrator (Millipore, cutoff 30000) and concentrated by centrifugation (30 min, 3000*g*, 4 °C). A 1 mL amount of a 5 mM MES buffer (pH 5) was added, and the solution was centrifuged once more. This was repeated twice to afford complete buffer exchange. The protein solution was taken up in 1 mL of MES buffer for the conjugation reaction. The aminoglycoside was dissolved in the same buffer to give a 10 mg/mL stock solution, and 200 mL was added to the protein solution. EDC was dissolved in the same buffer to give a 10 mg/mL stock solution (freshly!), and 200 mL was added to a solution containing both protein and aminoglycoside. The reaction mixture was vortexed and kept at room temperature for 2 h. It was transferred to a Biomax concentrator and concentrated by centrifugation (30 min, 3000*g*, 4 °C). A 1 mL amount of PBS (pH 7) was added, and the solution was centrifuged once more. This was repeated twice to afford complete buffer exchange. The protein solution was diluted with PBS to give a 1 mg/mL solution that was used as such for immunization and booster injections. The resulting antiserum R543 was used without further purification.

The immunosorbent Ultralink immobilized protein A Plus (PA) and Ultralink immobilized protein A/G Plus (PAG) were purchased from Pierce (Madrid, Spain). Prosep-A Media (CPGA) was from Millipore. These supports were able to specifically and reversibly bind all IgG subclasses of rabbit immunoglobulins through their Fc domains.

Measuring System. The automated flow injection manifold is depicted in **Figure 2** (*28*, *29*). An eight-way distribution valve (Kloehn,

Las Vegas, NV) equipped with a 2.5 mL syringe pump was connected to another eight-way distribution valve. The whole system was controlled with the Winpump software provided by Kloehn. The output of the pump was connected to a homemade poly(methyl methacrylate) reactor (10 mm × 4 mm, length × diameter), thermostatized at 20 °C, and packed with Ultralink immobilized protein A/G Plus for the capture of the tracer and analyte−antibody immunocomplexes. The output flow was driven to a flow-through cell (model 176.050, Hellma, Germany) placed in the fluorometer sample holder. Fluorescence intensity measurements (λ_{exc} = 342 nm; λ_{em} = 376 nm) were carried out in a LS-50B (Perkin-Elmer, Sussex, United Kingdom), and the instrumental parameters and data processing were controlled with the original software (FL WinLab version 4.00.02). All of the solutions were thermostatized at 20 °C using a water bath, Polyscience 9015 (Illinois).

Assay Protocol. The measuring protocol was based on the principles of a competitive direct fluoroimmunoassay. The unlabeled analyte (Ag: PENG) competed with a labeled derivate (Ag*: PAAP) for a limited number of receptor binding sites (Ab: anti-6APA IgG). All solutions were prepared in phosphate buffer (PB) 20 mmol L^{-1}, pH 8.0, except the desorbent solution. In a first step, the sample (0.7 mL) was mixed in the syringe with a constant amount (0.2 mL) of labeled antigen (PAAP) and antibody (0.2 mL). After 200 s, the solution (1 mL) was injected into the immunoreactor at a flow rate of 0.25 mL min^{-1} to allow the binding and retention of the immunocomplexes onto the immunosorbent. The reactor was washed four times with 1 mL of PB to remove all unbound complexes. The analytical signal was generated upon dissociation of the antibody−PAAP complexes retained on the immunosupport after competition using 2.5 mL of 0.1 mol L^{-1} glycine/HCl, pH 2.0, at a 0.5 mL min^{-1} flow rate. Preceding a new measurement, the reactor was washed with PB (3 × 1 mL of phosphate buffer, 0.5 mL min^{-1}). A complete cycle for the whole automated assay procedure required approximately 23 min including regeneration. The immunosorbent (PAG) showed a great stability, and the system could be used for more than 200 measurements. Occasionally, the immunoreactor had to be back-flushed with washing buffer to maintain a constant flow rate. The reactor was stored at 4 °C when not in use.

Experimental signals were normalized using the following expression:

$$\text{normalized response} = (B - B_\infty)/(B_0 - B_\infty)$$

where B is the signal (intensity of fluorescence) measured in the presence of the increasing analyte concentrations, B_∞ is the background fluorescence obtained in the presence of an excess of PENG, and B_0 is the signal in absence of antibiotic. The normalized response was plotted as a function of the analyte concentration (in logarithmic scale), and the experimental data were fitted to a four-parameter logistic equation (sigmoidal):

$$\text{normalized signal} = \frac{A_{max} - A_{min}}{1 + \left(\frac{[\text{analyte}]}{IC_{50}}\right)^b} + A_{min}$$

where A_{max} is the asymptotic maximum (maximum emission in absence of analyte), b represents the curve slope at the inflection point, IC_{50} is the concentration of analyte at the inflection point (concentration giving 50% inhibition of A_{max}), and A_{min} is the asymptotic minimum.

The detection limit (LOD) was calculated as the analyte concentration for which the tracer binding to the antibody was inhibited by 10%, and the dynamic range (DR) of the method was evaluated as the analyte concentrations that produced a normalized signal in the 20−80% range.

Selectivity Studies. Cross-reactivity studies were carried out by measuring the competitive curves for other chemically related and nonrelated antibiotics under the optimized conditions. Cross-reactivity (CR) was calculated as the percentage between the IC_{50} value for PENG and the IC_{50} for the interfering compound according to the following equation:

$$\%CR = \frac{IC_{50}\,(\text{PENG})}{IC_{50}\,(\text{cross-reacting compound})} \times 100$$

Sample Analysis and Validation. Water samples were collected in washed 2.5 L amber glass bottles at the influent and effluent streams of a STP in Madrid (Spain). The conductivity values (influent STP, 849 μS cm^{-1}; effluent STP, 569 μS cm^{-1}) and the pH of the samples (influent STP, 7.0; effluent STP, 7.7) were measured before storage at −20 °C. The samples were filtered through a 0.45 μm filter (Whatman, Maidstone, United Kingdom) to remove suspended matter as a unique pretreatment. The samples were fortified with PENG and AMOX at three concentration levels, 10, 25, and 50 μg L^{-1}, and analyzed using the proposed procedure. Nonfortified samples were also checked to exclude the presence of naturally contaminating antibiotics.

For validation purposes, the samples were also analyzed by HPLC with diode array detection (DAD). For sample preconcentration, SPE cartridges (Oasis MAX from Waters, Barcelona, Spain) were preconditioned as described elsewhere (*37*). Chromatographic analysis was carried out with a HP-1100 series high performance liquid chromatograph from Agilent Technologies (Palo Alto, CA) equipped with a quaternary pump, autosampler, automatic injector, and DAD detector. The analytical column was a LUNA C_{18} (2) (150 mm × 4.6 mm, 5 μm) HPLC column protected by a RP18 guard column (4.0 mm × 3.0 mm, 5 μm), both from Phenomenex (Torrance, CA). A gradient program was used for the mobile phase, combining solvent A (Milli-Q water 0.01% TFA) and solvent B (ACN with 0.01% TFA) as follows: 0% B (3 min), 0−37% B (5 min), 37% B (11 min), 37−67% B (5 min), 67% solvent B (5 min). Analyses were performed at a flow rate of 1.5 mL min^{-1}, and the column temperature was kept at 35 °C. The injection volume was 200 μL, and the UV detector wavelength was set at 220 nm. For the recovery studies, matrix-matched calibration standards were prepared for each wastewater sample by diluting one-fold the adequate amounts of the BLAs stock solutions with the corresponding SPE extracts. All of the analyses were carried out in triplicate.

RESULTS AND DISCUSSION

Nature of the Support, Size of the Reactor, and Reusability. The retention of immunocomplex in the reactor is highly dependent on the nature of the immunosorbent and will have a large impact on the performance of the assay. Three different supports have been tested for the development of the immunosensor: protein A covalently bound to controlled pore glass (CPGA) (Prosep A), protein A covalently bound to an azlactone-activated polymeric material (PA) (Ultralink Immobilized Protein A Plus), and protein A/G covalently bound to the same polymeric material (PAG) (Ultralink Immobilized Protein A/G Plus). The sorbents were packed into a reactor (10 mm × 4 mm, inner length × inner i.d.), and the long-term response of the supports to a negative control (B_0: PAAP 25 nmol L^{-1}) was measured using a constant Ab concentration (1:550). A higher reproducibility and signal response were obtained when PAG was used as the immunosorbent (PAG, RSD = 7%; PA and CPGA, RSD = 22%; n = 15), so this material was selected for the development of the immunosensor.

To evaluate the optimum amount of sorbent for the assay, several reactor sizes were tested. The response and the reproducibility of the immunosensors to a blank sample (PAAP 25 nmol L^{-1}) and to 100 ng mL^{-1} PENG were better when using a 125.7 μL (10 mm inner length × 4 mm inner inner diameter) volume reactor packed with PAG (**Table 1**). The use of larger reactors did not increase the sensitivity of the assay while requiring larger volumes of immunosorbent with the corresponding increment of hydrodynamic drawbacks as well as in the assay price. The reactor could be reused for more than 200 assay cycles without detectable activity losses (**Figure 3**).

To evaluate the sample volume and flow rate on sensor performance, the response of the device to a blank sample (B_0) and to a standard solution of PENG (100 ng mL^{-1}) was measured using a constant Ab concentration (1:550). The B_0

Table 1. Effect of the Reactor Size on the Response Obtained with the Immunosensor to a Blank Sample (B_0) and to 100 ng mL^{-1} PENG Standard Solution ($n = 3$)[a]

length (mm)	internal diameter (mm)	B_0 (s)	B/B_0 (s)
2	2	7.6 (5.0)	
4	4	11.9 (2.3)	0.66 (0.03)
4	6	14.0 (1.9)	0.54 (0.01)
10	4	33.0 (1.0)	0.35 (0.02)
15	4	30.1 (1.0)	0.40 (0.02)

[a] (s), Standard deviation ($n = 3$).

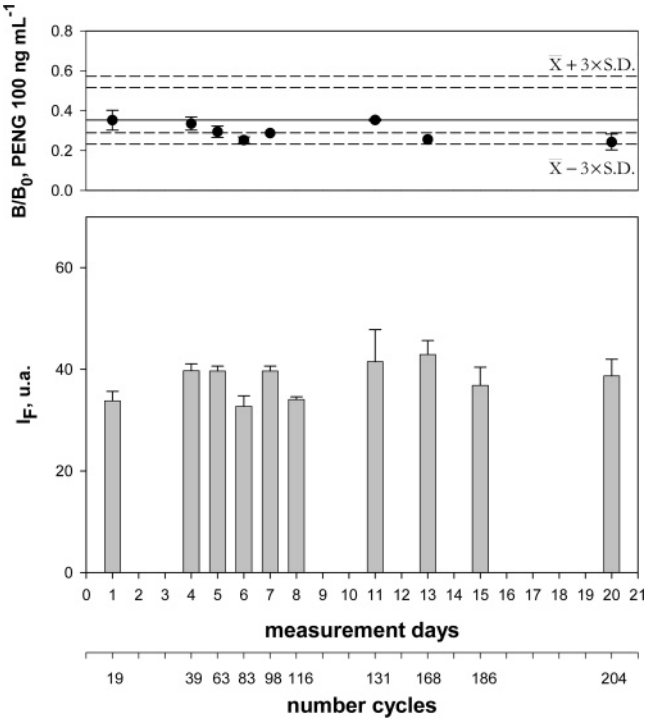

Figure 3. Long-term response obtained with the immunosensor to (■) PAAP (25 nmol L^{-1}) (B_0) and to a (●) 100 ng mL^{-1} PENG solution standard (Ab, 1:550) ($n = 3$).

signal decreased with increasing sample volumes and faster flow rates. However, sensitivity to PENG increased with higher sample volumes. Thus, the assay was optimized for a suitable assay speed and an acceptable signal by injecting 1 mL of sample/PAAP/Ab mixture at a flow rate of 0.25 mL min^{-1}.

Immunosensor Optimization and Analytical Characterization. To evaluate the effect of the buffer composition on the response, the assay was performed using phosphate buffer (PB), TRIS, or Gly/NaOH (pH 8.0) as carrier solutions. The first one provided the best results in terms of reproducibility and sensitivity. Variations of the buffer concentration in the 20−100 mM range (**Figure 4a**) showed no effect on the B_0 value and the relative response of the device to 100 ng mL^{-1} PENG. When the assay was carried out in nonbuffered solutions, the response of the device dramatically decreased both in the absence and in the presence of the antibiotic. In addition to decreased conformational stability of proteins (including antibodies) in low salt media, some authors have suggested that the lack of salts in the media could also prevent the formation of salt bridges between the antigen and the antibody that contribute positively to the noncovalent interactions between the antigen and the antibody (*38*). Finally, the concentration of the buffer was set at 20 mM.

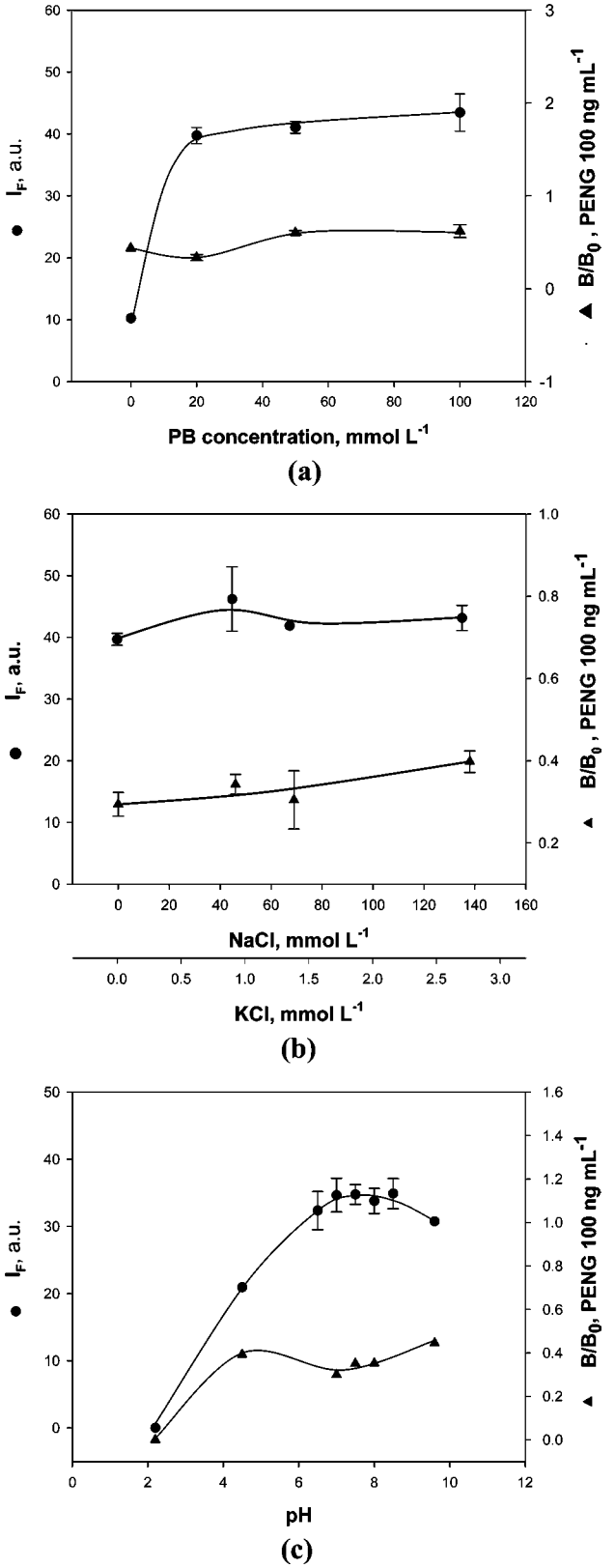

Figure 4. Effect of (**a**) buffer concentration (PB), (**b**) ionic strength, and (**c**) pH on the immunoassay performace in the absence (I_F, a.u.) and the presence (B/B_0) of 100 ng mL^{-1} PENG; [PAAP] = 25 nmol L^{-1} ($n = 3$).

The effect of the ionic strength and the pH of the media are depicted in **Figure 4b**,**c**. Both B_0 and B/B_0 for 100 ng mL^{-1} PENG remained almost constant at different concentrations of NaCl (0−140 mmol L^{-1}) and KCl (0−2.8 mmol L^{-1}), suggesting that the response of the immunosensor can be stable in the presence of different kind of matrices. With respect to the

Table 2. Optimized Operating Conditions for the Analysis of PENG Using the Automated Immunosensor

parameter	optimized value
incubation time Ab + Ag/Ag* (s)	200
antibody dilution factor	1:550
fluorescent tracer concn (nmol L^{-1})	25
carrier	phosphate buffer, pH 8.0, 20 mmol L^{-1}
carrier rate (mL min^{-1})	0.25
ionic strength NaCl/KCl (mmol L^{-1})	0/0
regenerating solvent	Gly/HCl, pH 2.0, 0.1 mol L^{-1}
regeneration rate (mL min^{-1})	0.5
reactor size (length × i.d., mm^2)	10 × 4 (volume, 125.7 μL)
immunosuport	Ultralink Immobilized Protein AG Plus
T (°C)	15–20
total analysis time (min/determination)	23

Table 3. Cross-Reactivity of Some BLAs and Other Related Compounds in the PENG Immunosensor

compound	50% inhibition (IC_{50}) (ng mL^{-1})	cross-reactivity (RC) (%)
PENG	30	100
6-APA	174	17
AMOX	58	50
AMPI	62	47
PENV	20	145
OXA	241	12
CLOX	741	3.9
DICLOX	1988	1.5
NAFCI	13408	<0.002
CEFAP	NC[a]	NC
CLAV	NC	NC
ENRO	NC	NC
CIPRO	NC	NC
CLOR	NC	NC

[a] NC, no competition.

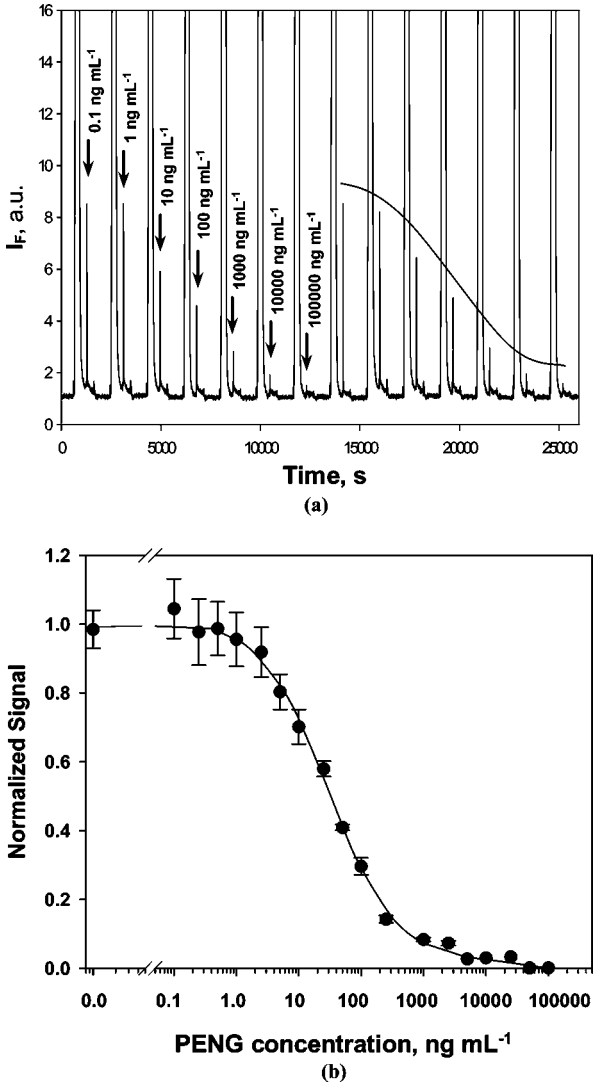

Figure 5. (**a**) Dose–response curve obtained with the immunosensor as a function of the PENG concentration, in buffered standard solutions, using the R543 antiserum (dilution of 1:550) and the labeled antigen PAAP (25 nmol L^{-1}) for 7 h of continuous work. (**b**) Calibration curve obtained for PENG using the optimized immunosensor. The experimental points have been fitted to eq 1 ($n = 5$).

pH, almost no changes were observed on the immunoassay features between pH 6.0 and 8.5 and 8.0 was chosen as the optimum pH to keep an acceptable signal and maximum sensitivity.

It has been reported (*39*, *40*) that the contact time between the reagents may have a direct effect on the sensitivity of competitive immunoassays. We observed that the sensitivity of the sensor increased by preincubating the antibody, the analyte, and the tracer in the syringe during 200 s, although longer incubation times did not improve the assay performance. In an alternative approach, the antibody was retained in the reactor in a first step and afterward the antibiotic−tracer mixture was pumped through the immunosupport at a constant flow rate (0.125 mL min^{-1}). Although the IC_{50} value calculated in this case (24 ng mL^{-1}) was similar to that obtained using the preincubation step (20 ng mL^{-1}), the measuring times were higher and the first approach was selected for the assays.

The amount of antibody (Ab) and tracer (Ag*) applied in the assay should be kept low enough to achieve a good sensitivity but high enough to provide an acceptable signal. The best results were obtained using 25 nmol L^{-1} of Ag* (PAAP) and Ab dilutions of 1:550. This combination allowed a good sensitivity as well as a wide dynamic range. **Table 2** summarizes the optimized conditions for the analysis of PENG using the immunosensor.

Figure 5a shows a typical dose−response curve measured with the immunosensor over long measuring times. The normalized competition curves obtained for PENG standards in phosphate buffer at concentrations ranging from 0 to 100 μg L^{-1} are depicted in **Figure 5b**. The IC_{50} value corresponds to 30 ng mL^{-1} PENG, and the limit of detection was 2.4 ng mL^{-1}. The immunosensor showed a dynamic range (normalized signal in the 20−80% range) from 6.0 to 191 ng mL^{-1} PENG. The LOD for the analysis of PENG with the generic antiserum on the immunosensor is only a bit higher than some specific immunosensor applications developed for the cephalosporanic β-lactam cephalexin (*32*, *34*, *35*) or for some aminoglycoside antibiotics (*30*, *31*). However, it compares favorably with other applications (*17*, *23*) or is even lower (*41*, *42*) than other enzyme immunoassays (EIAs) described in the literature, whereas the dynamic range of the fluoroimmunosensor is even wider, and the analysis time is much shorter.

The intraday relative standard deviation (RSD) was below 6% in the calibration range. Interday reproducibility was evaluated by measuring the calibration standards along different days ($n = 5$). The IC_{50} values ranged from 20 to 30 ng mL^{-1} (RSD 17%, $n = 5$). Typical RSD values, reported in the literature for immunoassays (*43*, *44*), were between 10 and 25%.

Table 4. Recovery Results for the Immunosensor and HPLC Waste Water Samples Analysis, Spiked at 10, 50, and 75 ng mL^{-1} PENG and AMOX ($n = 3$)[a]

STP wastewater sample	antibiotic	spiked level (ng mL^{-1})	immunoassay found (ng mL^{-1})	immunoassay recovery (%)	SPE/HPLC-DAD found (ng mL^{-1})	SPE/HPLC-DAD recovery (%)
influent	PENG	10	9 ± 2	90	<LOQ	
		50	52 ± 13	104	20 ± 7	40
		75	73 ± 7	97	35 ± 8	47
	AMOX	10	12 ± 3	120	<LOQ	
		50	51 ± 4	102	<LOQ	
		75	74 ± 9	99	<LOQ	
effluent	PENG	10	10 ± 1	100	<LOQ	
		50	52 ± 4	104	51 ± 3	102
		75	74 ± 7	99	70 ± 10	93
	AMOX	10	11 ± 5	110	<LOQ	
		50	50 ± 19	100	48 ± 10	96
		75	73 ± 28	97	74 ± 13	99

[a] 95% confidence interval ± $t_{\alpha,\nu} \times s_{n-1}/n^{1/2}$; ND, nondetected.

Cross-Reactivity. Cross-reactivity was calculated as the percentage between the IC_{50} value for PENG and the IC_{50} for the interfering compound, and the results are shown in **Table 3**. The polyclonal antibodies showed a remarkable group specificity, and the relative cross-reactivity was most pronounced for other penicillins, such as amoxicillin (50%) and ampicillin (47%) meeting MRLs as set by the European Union. It appeared to be possible to raise an antiserum (R543) against the common structure of the penicillanic β-lactam antibiotics, i.e., 6-APA, that recognizes various members from the group of the penicillanic β-lactams as was reported for aminoglycoside antibiotics by using neamin as a common structural moiety for preparation of the immunogen (*45*). Lower cross-reactivities with this antiserum were obtained for the isoxazolyl penicillins such as OXA (12%), CLOXA (3.9%), and DICLOX (1.5%), and cross-reactivity was negligible for nafcillin, cephapirin (a cephalosporin type BLA), or clavulanic acid (a β-lactamase inhibitor). Fluoroquinolones such as ENRO or CIPRO with a very different structure as compared to penicillin did not react either. Surprisingly, 6-APA showed a low CR (17%) relative to PENG, as has also been observed in enzyme-linked immunosorbent assays (*17*, *18*, *46*). According to Usleber et al. (*17*), the substitution of the free 6-amino group enhances cross-reactivity and the same behavior is observed when the fluorescent penicillin (PAAP) is used in the competitive assays instead of the enzymatic tracer.

Analysis of Wastewater Samples. The protein A/G flow-through immunosensor has been applied for the analysis of wastewater samples collected at the influent and effluent streams of a STP spiked with PENG and AMOX.

Calibration graphs were prepared by spiking blank matrix samples, previously found to contain no or undetectable levels of the antibiotics, with increasing concentrations of PENG and AMOX in the range of 0−100 μg L^{-1}. Because matrix effects were not observed for any of the samples, the immunosensor allowed the direct analysis of the antibiotics in wastewater. As shown in **Table 4**, excellent recoveries for PENG (mean value 99%) and AMOX (mean value 105%) in influent and effluent STP spiked wastewater samples were obtained at all concentration levels tested. Alternatively, the samples were also analyzed using a solid phase extraction method followed by HPLC with UV−visible (DAD) detection optimized previously (*37*). The immunosensor was especially suited for the analysis of influent STP samples waters where the chromatographic method does not show enough sensitivity. Moreover, the recovery of the amphoteric penicillin (AMOX) using the chromatographic method was strongly affected by the conductivity of the samples (influent, 849 μS cm^{-1}; effluent conductivity, 569 μS cm^{-1}). On the other hand, the fluoroimmunoassay provides excellent results regardless of sample complexity and without the need for further sample cleanup and preconcentration steps.

LITERATURE CITED

(1) Stan, H. J.; Heberer, T. H.; Linkerhägner, M. Occurrence of clofibric acid in theaquatic system—Is their therapeutic use responsible for the loads found in surface, ground and drinking water?. *Vom Wasser* **1994**, *83*, 57−68.

(2) Wilson, B. A.; Smith, V. H.; deNoyelles F., Jr.; Larive, C. K. Effects of three pharmaceutical and personal care products on natural freshwater algal assemblages. *Environ. Sci. Technol.* **2003**, *37*, 1713−1719.

(3) Sanderson, H.; Johnson, D. J.; Rietsma, T.; Brain, R. A.; Wilson, C. J.; Solomon, K. R. Ranking and prioritization of environmental risks of pharmaceuticals in surface waters. *Regul. Toxicol. Pharm.* **2004**, *39*, 158−183.

(4) Debska, J.; Kot-Wasik, A.; Namiesnik, J. Fate and analysis of pharmaceutical residues in the aquatic environment. *Crit. Rev. Anal. Chem.* **2004**, *67*, 34−51.

(5) Water Framework Directive (2000/60/EC), European Union, Brussels, 2000.

(6) http://europa.eu.int/com*m*/environment/water (March 30th, 2005).

(7) Díaz-Cruz, M. S.; López de Alda, M. J.; Barceló, D. Environmental behaviour and analysis of veterinary and human drugs in soils, sediments and sludge. *TrAC, Trends Anal. Chem.* **2003**, *22*, 340−351.

(8) Poole, C. F. New trends in solid-phase extraction. *TrAC, Trends Anal. Chem.* **2003**, *22*, 362−373.

(9) Bruno, F.; Curini, R.; Di Corcia, A.; Nazzari, M.; Samperi, R. Method development for measuring trace levels of penicillins in aqueous environmental samples. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 1391−1400.

(10) Ahrer, W.; Scherwenk, E.; Buchberger, W. Determination of drug residues in water by the combination of liquid chromatography or capillary electrophoresis with electrospray mass spectrometry. *J. Chromatogr. A* **2001**, *910*, 69−78.

(11) Kumar, K.; Thompson, A.; Singh, A. K.; Chander, Y.; Gupta, S. C. Enzyme-linked immunosorbent assay for ultratrace determination of antibiotics in aqueous samples. *J. Environ. Qual.* **2004**, *33*, 250−256.

(12) http://www.aoac.org/testkits/testedmethods.html (March 30th, 2005).

(13) Tschmelak, J.; Proll, G.; Gauglitz, G. Optical biosensor for pharmaceuticals, antibiotics, hormones, endocrine disrupting chemicals and pesticides in water: Assay optimization process for estrone as example. *Talanta* **2005**, *65*, 313−323.

(14) Aga, D. S.; Goldfish, R.; Kulshrestha, P. Application of ELISA in determining the fate of tetracyclines in land-applied livestock wastes. *Analyst* **2003**, *128*, 658−662.
(15) Yang, S.; Carlson, K. Routine monitoring of antibiotics in water and wastewater with a radioimmunoassay technique. *Water Res.* **2004**, *38*, 3155−3166.
(16) Van Emon, J. M.; Gerlach, C. L. Environmental monitoring and human exposure assessment using immunochemical techniques. *J. Microbiol. Methods* **1998**, *32*, 121−131.
(17) Usleber, E.; Litz, S.; Märtlbauer, E. Production and characterization of group-specific antibodies against penicillin antibiotics. *Food Agric. Immunol.* **1998**, *10*, 317−324.
(18) Strasser, A.; Usleber, E.; Schneider, E.; Dietrich, R.; Bürk, C.; Märtlbauer, E. Improved enzyme immunoassay for group-specific determination of penicillins in milk. *Food Agric. Immunol.* **2003**, *15*, 135−143.
(19) Nagakura, N.; Souma, S.; Shimizu, T.; Yanagihara, Y. Anti-ampicillin monoclonal antibodies and their cross-reactivities to various beta-lactams. *J. Antimicrob. Chemother.* **1991**, *28*, 357−368.
(20) Shirazi, M. F.; Hung, T. V.; Womersley, D. M. Polyclonal antibodies reactive to some beta-lactam antibodies. *Aust. J. Dairy Technol.* **1991**, *46*, 88−90.
(21) Usleber, E.; Lorber, M.; Straka, M.; Terplan, G.; Märtlbauer, E. Enzyme immunoassay for the detection of isoxazolyl penicillin antibiotics in milk. *Analyst* **1994**, *119*, 2765−2768.
(22) Jackman, R.; Mitchell, S. J.; Dyer, S. D.; Chesham, J. The use of a specific enzyme-linked immunosorbent assay to monitor the persistence of penicillin G residues in milk following intramamary antibody treatment. *Food Agric. Immunol.* **1991**, *3*, 3−12.
(23) Grubelnik, A.; Padeste, C.; Tiefenauer, L. Highly sensitive enzyme immunoassay for the detection of beta-lactam antibiotics. *Food Agric. Immunol.* **2001**, *13*, 161−169.
(24) Kitagawa, T.; Kanamuru, T.; Wakamatsu, H.; Kato, H.; Yano, S.; Asanuma, Y. A new method for preparation of an antiserum to penicillin and its application for novel enzyme immunoassay of penicillin. *J. Biochem.* **1978**, *84*, 491−494.
(25) http://probes.invitrogen.com (March 30th, 2005).
(26) Zhao, G. S.; Meier, T. I.; Kahl, S. D.; Gee, K. R.; Blaszczak, L. C. BOCILLIN FL, a sensitive and commercially available reagent for detection of penicillin-binding proteins. *Antimicrob. Agents Chemother.* **1999**, *43*, 1124−1128.
(27) Xing, B.; Khanamiryan, A.; Rao, J. Cell-permeable near-infrared fluorogenic substrates for imaging β-lactamase activity. *J. Am. Chem. Soc.* **2005**, *127*, 4158−4159.
(28) Urraca, J. L.; Benito-Peña, E.; Pérez-Conde, C.; Moreno-Bondi, M. C.; Pestka, J. J. Analysis of zearalenone in cereal and swine feed samples using an automated flow-through immunosensor. *J. Agric. Food Chem.* **2005**, *53*, 3338−3344.
(29) Gonzalez-Martinez, M. A.; Morais, S.; Puchades, R.; Maqueira, A.; Ferrer, I.; Marco, M. P.; Barceló, D. Reversible immunosensor for the automatic determination of atrazine. Selection and performance of three polyclonal antisera. *Anal. Chim. Acta* **1999**, *386*, 201−210.
(30) Haasnoot, W.; Loomans, E. E. M. G.; Cazemier, G.; Dietrich, R.; Verheijen, R.; Bergwerff, A. A.; Stephany, R. W. Direct versus competitive biosensor immunoassays for the detection of (dihydro)streptomycin residues in milk. *Food Agric. Immunol.* **2002**, *14*, 15−27.
(31) Haasnoot, W.; Cazemier, G.; Koets, M.; van Amerongen, A. Single biosensor immunoassay for the detection of five aminoglycosides in reconstituted skimmed milk. *Anal. Chim. Acta* **2003**, *488*, 53−60.
(32) Dillon, P.; Daly, S.; Browne, J.; Manning, B.; Loomans, E.; van Amerongen, A.; O'Kennedy, R. Application of an immunosensor for the detection of the β-lactam antibiotic, cephalexin. *Food Agric. Immunol.* **2003**, *15*, 225−234.
(33) González-Martínez, M. A.; Puchades, R.; Maquieira, A. On-line immunoanalysis for environmental pollutants: from batch assays to automated sensors. *TrAC, Trends Anal. Chem.* **1999**, *18*, 204−218.
(34) Meyer, U. J.; Zhi, Z.-L.; Loomans, E.; Spener, F.; Meusel, M. Automated stand-alone flow injection immunoanalysis system for the determination of cephalexin in milk. *Analyst* **1999**, *124*, 1605−1610.
(35) Zhi, Z.-L.; Meyer, U.; Van den Bedem, J. W.; Meusel, M. Evaluation of an automated and integrated flow-through immunoanalysis system for the rapid determination of cephalexin in raw milk. *Anal. Chim. Acta* **2001**, *442*, 207−219.
(36) Orellana, G.; Aparicio Lara, S.; Moreno-Bondi, M. C.; Benito-Peña, E. Síntesis de derivados fluorescentes de antiobióticos beta-lactámicos. Spanish Patent Application, 200200794.
(37) Benito-Peña, E.; Partal-Rodera, A. I.; de León-González, M. E.; Moreno-Bondi, M. C. Multiresidue determination of beta-lactam antibiotics in wastewater using solid-phase extraction and liquid chromatography with ultraviolet detection. Manuscript submitted for publication.
(38) Davies, D. R.; Chacko, S. Antibody structure. *Acc. Chem. Res.* **1993**, *26*, 421−427.
(39) Ballesteros, B.; Barceló, D.; Camps, F.; Marco, M. P. Preparation of antisera and development of a direct enzyme-linked immunosorbent assay for the determination of the antifouling agent Irgarol 1051. *Anal. Chim. Acta* **1997**, *347*, 139−147.
(40) Gascón, J.; Oubiña, A.; Onnerjord, P.; Ferrer, I.; Hammock, B. D.; Marco, M. P.; Barceló, D. Performance of two immunoassays for the determination of atrazine in seawater samples as compared with on-line solid-phase extraction-liquid chromatography-diode array detection. *Anal. Chim. Acta* **1996**, *330*, 41−51.
(41) Dietrich, R.; Usleber, E.; Märtlbauer, E. The potential of monoclonal antibodies against ampicillin for the preparation of a multi-immunoaffinity chromatography for penicillins. *Analyst* **1998**, *123*, 2749−2754.
(42) Delwiche, M.; Cox, E.; Goddeeris, B.; Van Dorpe, C.; De Baerdemaeker, J.; Decuypere, E.; Sansen, W. A biosensor to detect penicillin residues in food. *Trans. ASAE* **2000**, *43*, 153−159.
(43) Parker, G. A. Validation of methods used in the Florida Department of Agriculture and Consumer Services' Chemical Residue Laboratory. *J. Assoc. Off. Anal. Chem.* **1991**, *74*, 868−871.
(44) Horwitz, W.; Kamps, L. R.; Boyer, K. W. Quality assurance in the analysis of foods for trace constituents. *J. Assoc. Off. Anal. Chem.* **1980**, *63*, 1344−1354.
(45) de Leuw, P.; Kapa, G.; Petz, M. Generation and characterisation of multi-analyte antibodies against penicillins from egg yolk. *J. AOAC Int.* **1997**, *80*, 1220−1228.
(46) Loomans, E. E. M. G.; van Wiltenburg, J.; Koets, M.; van Amerongen, A. Neamin as an immunogen for the development of a generic ELISA detecting gentamicin, kanamycin, and neomycin in milk. *J. Agric. Food Chem.* **2003**, *51*, 587−593.

Received for review May 18, 2005. Revised manuscript received June 19, 2005. Accepted June 21, 2005. This work has been funded by the Spanish Ministry of Science and Technology (Project BQU 2002-04515-C0201) and the European Union (FAIR-CT96-1181 and QLK1-CT-1999-00902). E.B.P. thanks Complutense University for a predoctoral grant.

JF0511502