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Antibiotic immunosensing: Determination of sulfathiazole in water and honey

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ABSTRACT

The development of two sensitive and selective immunosensors for sulfathiazole, using immunoreagents – haptens, polyclonal antibodies, enzyme conjugates – previously obtained and characterized, is presented. One of them is based on the competitive immunocomplex capture format making use of an immobilized protein A/G sorbent, while the other employs a restricted access support in a novel homogeneous–heterogeneous (HH) assay mode. Maximum sensitivity, achieved with a total assay time of 18 min for the capture sensor, is traduced in a dynamic range from 0.4 to 24 $\mu g\,L^{-1}$, with a lower limit of detection of 0.11 $\mu g\,L^{-1}$, increasing to 1.2 $\mu g\,L^{-1}$ when employing an accelerated capture assay protocol that yields a sampling rate of 7 cycles per hour. The HH sensor shows the fastest response, performing each whole assay in only 2 min, with a limit of detection of 0.85 and a measurement interval of 3.9–181.0 $\mu g\,L^{-1}$, and with no need of support regeneration. Immunosensors are selective for sufathiazole, and only sulfamethoxypyridazine, sulfamethizole and sulfapyridine show non-negligible cross-reactivity, the same as in ELISA batch immunoassay. The application of the developed systems to the analysis of water, with no sample treatment, as well as honey samples after solid-phase extraction, demonstrate the reliability of the immunosensing for the monitoring of this type of pollutants.

1. Introduction

Immunochemistry has proven to be an interesting option for residue monitoring and control in clinical, food and environmental analysis. This methodology offers a range of possibilities for matching different analytical needs. Batch immunoassays show a huge working capacity because a high number of samples can be processed simultaneously, while immunosensing can provide a good solution for screening, when on-site analysis or low response time is required [1].

The development of immunosensors for environmental monitoring has been an intense research area for years [2], and many set-up devices show very good performances related to sensitivity [3,4], autonomy and portability [5,6], and multianalyte capacity [7,8]. Analysis rate is also a common goal to be achieved. In some published developments, the total assay time is around 15–20 min [9,10] for Total Internal Reflection Fluorescence (TIRF) and Surface Plasmon Resonance (SPR) immunosensors, both using immobilized conjugate format. In other approach [11,12], using a fluorescence-based immunosensor working with antibodies entrapped in sol-gel, this time is reduced to 5 min or less. The shortest response time found in the literature corresponds to the displacement immunosensor, that allows to run assays in only 2 min [13].

In general, flow immunosensors are based on heterogeneous competition formats performed in several steps (competition, tracing reaction, regeneration, etc.). Shortening assay time is easy in this kind of methodologies because there is no need to work under equilibrium conditions. It is only necessary to accelerate the basic operations, mainly by increasing flow rate. Also, for saving time, steps carried out on-line could be performed off-line in parallel. As counterpart, some analytical properties such as sensitivity can be partially lost, because the extent of the analytical reaction is lower. However, it must be told that rapidity is a property that should be set in function of the final application. In some cases, for instance alarm situations, response time must be as short as possible, while the sensitivity necessary for detecting the analyte at critical concentration could be lower than that provided by the original method. In other instances as continuous routine monitoring, sensitivity is more important than assay time, and a response time of 15 min or longer might be fine.

A different approach consists of the whole development of a simpler and faster immunosensing methodology. With this purpose, a novel homogeneous–heterogeneous (HH) immunoanalytical system has been recently studied [14]. The sensor carries out the competition between the antibody, the analyte and a fluorescent tracer in solution, and the immunocomplexes are further separated from the unbound species by means of a

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restricted access material (RAM) support that retains the small molecules but excludes the macromolecular dyes. No support regeneration is needed, and the sampling rate achieved is as high as 30 h^{-1}. The idea is not brand new, and similar approaches were carried out in the past [15,16], although it has been little exploited.

Enzymes cannot be used as labels with this assay format, because tracer needs to be a small molecule in order to access the inner surface of the RAM support and be retained. This way, markers used are fluorescent dyes. Coupling the hapten to the marker is a synthesis task, with the troubleshooting associated to this kind of operation: reaction conditions, product purification, yields, etc. Furthermore, changing the hapten or the marker implies to develop a new synthesis procedure. It is therefore recommendable to use a labeling method as general as possible. Oligonucleotides have been employed as bridge molecules between the label and the hapten [14], and a universal methodology has been set up to attach carboxylic acid-ended haptens to commercial amino-derivatized oligonucleotides carrying out fluorescein.

On the other hand, the application of immunosensors for pollution monitoring has been devoted mainly to pesticides, especially herbicides, and other typical pollutants such as PCBs and PAHs [2]. However, other important compounds to be controlled, such as pharmaceutical residues or toxins, have been scarcely targeted. One of the most relevant works of pharmaceutical immunosensing, not related to environmental applications, is devoted to the determination of paclitaxel [17], an anticancer drug, with a displacement immunosensor reaching a LOD of 1 μg L^{-1}. Another drug such as ibuprofen, at the lowest level of 0.2 μM, is determined using a commercial system based on SPR principle [18]. It is worth mentioning that the employment of methodologies based on biomolecular recognition for the determination of this kind of agents results especially suitable for differentiating between the active molecule and similar non-active ones.

On the other hand, sulfonamides are a kind of pharmaceuticals widely used [19]. Those are synthetic compounds very employed in the treatment and prevention of bacterial infection in veterinary and human medicine [20], as well as for feed additives in animal husbandry [21]. As the result, residues of sulfonamides have been found in food of animal origin such as honey, milk, eggs, meat or fish [22]. These antibiotics can also be considered as emerging pollutants, and different reviews of their occurrence and behaviour, as well as chromatographic methods applied for their determination in the environment, have been recently published [23,24].

Immunochemical methods for sulfonamides have been developed for years, using polyclonal and monoclonal antibodies raised from different kinds of haptens [25,26], as well as recombinant antibodies [27], with both specific [28] and generic [29] targeting mode. However, immunosensing applied to this kind of compounds is little common in the literature. Commercial SPR technology has been used in inhibition assays for the multiple determination of different sulfonamides [30,31], achieving LOD at low μg L^{-1}. Piezoelectric detection of antibody binding is also employed for the determination of sulfamethoxazole at the ng mL^{-1} level [32]. The most sensitive immunosensor for sulfonamides (LODs lower than 10 ng L^{-1}) using an immobilized hapten format, is presented by Tschmelak et al. [33].

In this work, the development of two immunosensors for sulfathiazole residues is described and their performances compared. One of them is based on an immunocomplex capture format [1,34], and the other makes use of the HH immunoanalysis mode. The basic management of the capture immunosensor is modified in order to decrease the assay time even though sensitivity or other properties are partially lost. Finally, the developed systems are applied to the analysis of water and honey samples with minimal sample pretreatment.

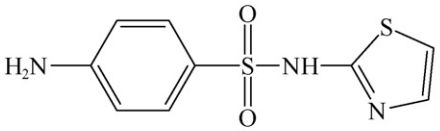
2. Experimental

2.1. Reagents

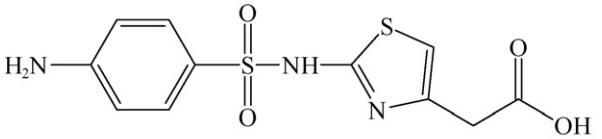
Sulfathiazole (STZ), N^4-Phthalylsulfathiazole (PSTZ), sulfacetamide (SAM), sulfadiazine (SDZ), sulfadimethoxine (SDM), sulfaguanidine (SG), sulfamerazine (SMR), sulfamethazine (SMZ), sulfamethizole (SMT), sulfamethoxazole (SMX), sulfamethoxypyridazine (SMP), sulfanilamide (SAN), sulfapyridine (SP), sulfasalazine (SSZ) and sulfisoxazole (SOX), as well as 3-(*p*-hydroxyphenyl)-propanoic acid (HPPA) and 3,3′,5,5′-tetramethylbenzidine (TMB), were purchased from Fluka-Sigma–Aldrich (Madrid, Spain). Horseradish peroxidase (HRP) was acquired from Boehringer Mannheim (Mannheim, Germany). Ultralink Immobilized Protein A/G was from Pierce (Rockford, IL), and Proclin 300 was provided by Supelco (Bellefonte, PA). HPLC-grade methanol and acetonitrile were obtained from Scharlab (Barcelona, Spain). Deionized water was generated by means of a Millipore Milli-Q system (Bedford, MA, USA). The oligonucleotide named SYM18, with sequence 5′ FL-TAG-C_7-NH_2 3′, was purchased from Molbiol (Berlin, Germany). All other reagents were analytical grade.

Buffers employed were phosphate buffer (PB, 0.02 M sodium phosphate, pH 8.0) and phosphate buffer saline (PBS, 10 mM phosphate, 137 mM NaCl, 2.7 mM KCl and HCl until pH 7.0). Twofold concentrated PBS and PBS with 0.8 M NaCl added were also used in HH sensing and oligonucleotide tracer purification by HPLC. Enzyme substrates HPPA and H_2O_2 were prepared in PB at concentrations 0.8 g L^{-1} and 0.012% (v/v), respectively. The dissociation agent employed in the capture sensor was 0.1 M glycine/HCl buffer, pH 2.0, containing 2 M NaCl.

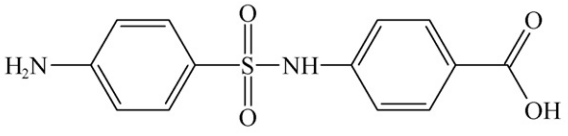
A set of immunoreagents – four haptens and eight sera – were previously obtained by our research group [28] and tested in batch immunoassay. The antibody employed in the immunosensors was S3-BSA, in combination with the hapten S4. The chemical structure of analyte and haptens S3 ([2-(4-amino-benzenesulfonylamino)-1,3-thiazol-4-yl]acetic acid) and S4 (6-(4-amino-benzenesulfonylamino)nicotinic acid) are shown in Fig. 1. Haptens were conjugated to HRP by means of the active ester method [35] employing gel permeation chromatography on



Sulfathiazole



S3 [2-(4-amino-benzenesulfonylamino)-1,3-thiazol-4-yl]acetic



S4 6-(4-amino-benzenesulfonylamino)nicotinic acid

Fig. 1. Chemical structures of the analyte and the two haptens employed.

Sephadex G-25 (Pharmacia, Uppsala, Sweden) for conjugate purification. The conjugation of haptens to SYM18 oligonucleotide was carried out via its amino derivatization employing the active ester reaction, but purification was accomplished by means of HPLC, as previously described [14].

For HH sensor, S4-BSA antibody and S4-SYM18 tracer were diluted in PBS 2× at 1/100 and 1/8 (v/v), respectively. In capture sensors, antibody solutions were prepared in PB containing 0.05% (v/v) Proclin 300, while 0.06 mM TMB was added to HRP-based tracer solutions.

2.2. Instrumentation

In capture immunosensing, a sequential injection device (Kloehn Ltd., Las Vegas, NV) was employed for flow management, in combination with a fluorescence detector (Turner model 450, Biomolecular Inc., Reno, NV, equipped with suitable filters at λ_{ex} 320 nm, λ_{em} 405 nm and a 15 μL Hellma flow cell). The manifold has been described in previous works [34,36].

HH immunoanalyses were performed using an 1100 Series HPLC from Agilent Technologies (Santa Clara, CA), equipped with a fluorescence detector (excitation and emission wavelengths set at 495 and 519, respectively), an RP 18 LiChrospher ADS 25 mm × 4 mm i.d. (Merck, Darmstadt, Germany) RAM column, and a Rheodyne 7725i valve with a 20-μL sample loop.

2.3. Immunoassay protocols

The immunocomplex capture format is based in the following basic operations: competition analyte-antibody-tracer in solution, capture of the formed immunocomplexes on the Protein A/G support, washing, signal display by measuring the activity of HRP bound to the support employing a fluorogenic substrate, and regeneration of the support by injection of the dissociation agent followed by washing with buffer. These operations were performed in the two types of immunosensors, the so-called "standard" and the "accelerated" one. Table 1 shows the details of the steps performed in both immunosensors, as well as their timing.

When working with standard immunosensors, each solution – antibody, tracer, analyte (standard or sample), HPPA, etc. – was in a separate vessel and the mixing processes were carried out on-line. In the accelerated immunosensing, a mixture of analyte, antibody and tracer was prepared off-line, which can be carried out manually or in automatic manner using an additional flow manager device. Also, a daily fresh mixture of HPPA and H_2O_2 was used instead of the on-line mixing of these reagents for each analytical cycle.

In HH immunosensing, analyte-antibody-tracer competition takes also place in solution, and the mixture is further injected through the RAM support, so that unbound small molecules are retained on the inner surface, while macromolecular immunocomplexes are excluded and eluted with the void volume. To carry out the analyses, 70 μL of analyte standard or sample in PBS were mixed with 5 μL of the antibody solution and 25 μL of the tracer solution. Then, 20 μL of the mixture were injected through the RAM column, using twofold concentrated PBS as carrier at 0.5 mL min^{-1}, and the fluorescence peak was registered. Each measurement was carried out in triplicate. At the end of a working day, the column was washed with 20 mL of methanol, thus eluting all the retained material, and kept in this solvent when not in use.

2.4. Standard and sample preparation

Standards for calibration of the target compound were prepared in buffer or in mixtures buffer/methanol and buffer/acetonitrile 90:10 (v/v). Water samples – a commercial bottled, a source water sample collected in Torre d'En Bessora village (Castellón, Spain) and tap water from Valencia (Spain) – were analyzed as native and spiked with STZ at levels from 0 to 50 μg L^{-1}. In order to adjust the pH and ionic strength of samples near that of PB or PBS, 5% (v/v) of 20× buffer was added as only sample treatment. When calcium phosphate precipitation was observed, filtration through a Whatman no. 40 filter paper was carried out prior to analysis.

Honey samples, collected from a local market, were fortified at 5, 10, 20, 50 and 100 mg kg^{-1} with STZ and extracted following basically the procedure described by Posyniak et al. [37]. Briefly, 1.5 g honey was diluted with 12.5 mL of 0.1 M sodium acetate buffer (pH 5.0) and immersed in ultrasonic water bath for 15 min. The solution was SPE extracted on Sep-Pak C_{18} disposable cartridges (Waters, Milford, MA) under depression of 0.5 bar. The column was preconditioned with 3 mL of methanol, 3 mL water and finally with 3 mL of acetate buffer (pH 5.0). After percolation of the whole solution, the bed of the column was washed with 3 mL of acetate buffer (pH 5.0), 3 mL of water and dried for 5 min. The analyte was eluted with 3 mL of methanol and the extract split in two 1.5 mL portions. One of them was diluted with buffer (1/10 and 1/5 (v/v) for capture and HH immunosensors, respectively) and directly analyzed by immunosensing. The other one was dried under nitrogen stream at 40 °C. The dry residue was dissolved in 0.5 mL of 0.5% acetic acid and 5% methanol in water (v/v/v) and injected onto the LC–MS system.

2.5. Chromatographic analysis

An Agilent Technologies 1100 liquid chromatographic system coupled to tandem mass spectrometry was used. The column was a ZORVAX C_{18} (50 mm × 2.1 mm i.d., with a particle size of 3.5 μm). Sample volume injected was 20 μL. A linear gradient from 100% solvent A (0.5% acetic acid and 5% methanol in water, v/v) at 0 min to 50% solvent A and 50% solvent B (methanol) at

Table 1
Protocols for the immunocomplex capture immunoassay format.

Step	Standard immunosensing		Accelerated immunosensing	
	Description	Time (min)	Description	Time (min)
1) Competition	Mixing analyte (800 μL), antibody (200 μL) and tracer (200 μL)	2.5	Mixing analyte (750 μL), antibody (250 μL) and tracer (250 μL)	Off-line
2) Capture	Injection of 1 mL of mixture at 0.25 mL min^{-1}	4	Injection of 1 mL of mixture at 1 mL min^{-1}	1
3) Washing	Injection of 4 × 1 mL of PB at 4 mL min^{-1}	1.25	Injection of 4 × 1 mL of PB at 4 mL min^{-1}	1.25
4) Display	Mixing HPPA and H_2O_2 solutions (200 μL each)	2	Mixing HPPA and H_2O_2 solutions (equal volumes)	Off-line
	Injection of 100 μL and incubation for 3 min	3	Injection of 100 μL and incubation for 1 min	1
	Injection of 1.5 mL PB at 2 mL min^{-1}. Signal display	1	Injection of 1,5 mL PB at 2 mL min^{-1}. Signal display	1
5) Dissociation	Injection of 1.5 mL of dissociation solution at 0.5 mL min^{-1}	3.25	Injection of 1.5 mL of dissociation solution at 0.5 mL min^{-1}	3.25
6) Regeneration	Injection of 3 × 1 mL of PB at 4 mL min^{-1}	1	Injection of 3 × 1 mL of PB at 4 mL min^{-1}	1

Time values are approximate, because previous to each step, the manifold runs a washing cycle of tubing and pump with the next solution to be used. The off-line steps can be automated, employing an additional device, and carried out the same as on-line, with no influence in analysis rate.

15 min was employed. The analyte was detected using electrospray in the positive ionization mode (ESI). Typical MS settings were: capillary voltage 110.2 V; lens 1 (6.8 V) and (−60 V) for lens 2; octopole amplitude 143.8 Vpp. The nebulizer gas flow (N_2) was set to 40 psi and the dried gas flow (N_2) to 10 L min^{-1}. The mass were monitored in the interval 90–400 *m*/*z*. Two different characteristic fragmentation reactions were monitored for STZ (256 > 156 and 256 > 108). The STZ precursor ion was 256 *m*/*z* and the chromatogram was obtained at a retention time of 5.8–6.8 min.

3. Results and discussion

3.1. Basic development of the immunosensors

3.1.1. Capture immunosensor

The immunocomplex capture format is not as popular as other assay modes employed in immunosensing [2]. However, our experience has demonstrated that this format is very useful for the reliable development of new immunosensors, provided that antibodies and competition haptens are available. This is due to the universal features of the protein A/G binding support, able to recognize many kinds of immunoglobulins, that allows the employment of any antibody, with independence of its affinity or other properties [1].

For STZ immunosensor development, antibodies obtained by means of the conjugates S3-BSA and S4-BSA, and tracers S3-HRP and S4-HRP, previously selected by ELISA from a pool of four haptens and eight antisera obtained by our research group [28], were tested. The four possible combinations did produce competition with I_{50} lower than 20 μg L^{-1}, and the best sensor results (I_{50} around 3 μg L^{-1}) were obtained with S3-BSA antibody and S4-HRP tracer, the same combination as the optimized antibody-coated ELISA format [28]. Using the standard competition protocol (Table 1, left), the application of immunoreagents was optimized. Table 2 shows the final conditions of application of the immunoreagents, as well as the sensitivity parameters achieved. The calibration curve obtained with the optimal conditions is shown in Fig. 2 (full symbols). These data indicate that the immunosensor is highly sensitive, similar to that obtained with the antibody-coated ELISA employing the same immunoreagents [28], and allows the measurement of sulfathiazole at concentrations under 1 μg L^{-1}.

Another important performance of the capture immunosensor is the working life and autonomy of the system. The regeneration and reusability of the protein A/G support has been deeply studied in the past [1], and more than 600 assay cycles can be performed without loss of binding properties, so the same reactor can be used for more than 2 weeks. The long-term stability

Table 2
Analytical performances of standard capture immunosensor for sulfathiazole.

[antibody] (dilution factor)	1/4000
[tracer] (mg L^{-1})	0.1
Blank signal (arbitrary units)	88,000
Slope at the inflexion point	1.6
I_{50} (μg L^{-1})[a]	3.2
LOD (μg L^{-1})[b]	0.11
DR (μg L^{-1})[c]	0.4–24.0

[a] I_{50}: analyte concentration that inhibits the binding of the tracer to the antibody by 50%.
[b] LOD: limit of detection, analyte concentration that inhibits the binding of the tracer by 10%.
[c] DR: dynamic range, analyte concentration interval for which the inhibition of the binding of tracer is between 20 and 80%.

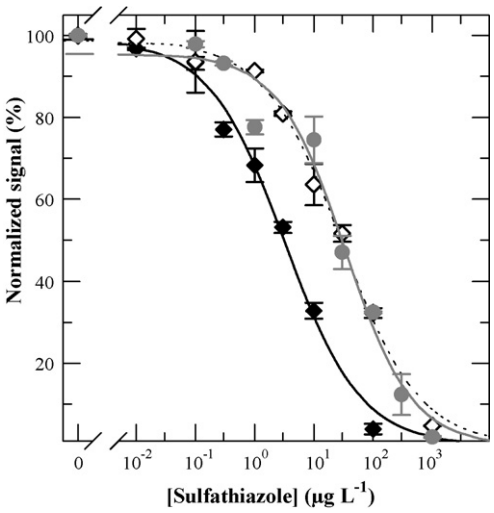


Fig. 2. Competition curve for sulfathiazole employing capture standard (full symbols), capture accelerated (hollow symbols) and HH (gray symbols) immunosensing.

of the solutions in the system was also studied, especially for the bioactive reagents. In the case of HRP tracer, two additives, which had been previously employed with success, were tested: TMB at concentration 0.06 M [38] and Proclin 300 at 0.05% (v/v) [39]; the addition of TMB produced best results, so it was used routinely. For antibody solutions, the addition of 0.05% (v/v) Proclin 300 as preservative produced better results than the employment of inert proteins such as ovalbumin or casein – no BSA could be applied in this case because the antibodies had been raised from hapten-BSA conjugates. By means of the use of these additives, immunoreagent solutions were stable for more than 3 consecutive days at room temperature, so this period was stated as the autonomy of the system.

The tolerance of the capture immunosensor to organic solvents was tested by carrying out whole calibrations in mixtures solvent/buffer at different concentrations. Methanol was selected for being the best performing solvent in previously developed immunosensors [36,38], and acetonitrile was also tested as modifier because it is a solvent widely employed in sample treatment protocols. When methanol was added at 10% (v/v), the calibration parameters were kept nearly the same as in the absence of modifier, and calibrations recorded showed I_{50} values between 3.1 and 3.6 μg L^{-1}, with limits of detection around 0.10–0.12 μg L^{-1}. When increasing the solvent concentration, interference was clearly manifested in a decrease of sensitivity, and I_{50} shifted to 23 and 91 μg L^{-1} for 25 and 50% (v/v), respectively. On the other hand, acetonitrile is more deleterious for immunoreagents than methanol, and 10% of this solvent did shift the sensitivity parameters to twice their value. So, employing acetonitrile, I_{50} and limit of detection were 7.5 and 0.25 μg L^{-1}, respectively, which allows the use of this solvent when no extreme sensitivity is required. The loss in sensitivity for high percentages of solvents had been previously observed in immunosensors for other analytes [36,38]. However, in the case of sulfathiazole, the possibility of employing 10% methanol with no loss of sensitivity is to be remarked.

3.1.2. Homogeneous–heterogeneous immunosensor

The HH developed assay format [14], also based on a competition protocol, but with a very simple and rapid operational mode. The competition takes place in solution (homogeneous) and the products are separated using a RAM support (heterogeneous) that binds the small species and excludes the macromolecular ones (immunocomplexes), so that the bound

tracer is detected downstream and the signal – fluorescence peak area – related to the analyte concentration, with a logistic calibration as in other competitive immunoassays. The homogeneous step is nearly instantaneous, and this process can be carried out while the previous sample is being separated, so each assay is as long as the separation process, typically 2 min [14].

The first assay was to check the elution profile of tracers (S3 and S4 coupled to SYM 18 oligonucleotide) and immunocomplexes, in the RP 18 ADS column, by injecting tracer alone and the complex (a mixture of tracer and antibody). The behaviour was that expected, since the tracer alone remained completely retained in the column, while the immunocomplex was eluted with the void volume.

The selection of the pair antibody-tracer, as well as their concentration, was carried out on the basis on maximal assay sensitivity in whole competition curves. The optimized calibration curve is shown in Fig. 2 (gray symbols), compared to those obtained for capture immunosensing. The lowest I_{50} was achieved with homologous S4-BSA antibody in combination with S4-SYM18 tracer, at 1/100 and 1/8 (v/v) dilution factors, respectively. The sensitivity parameters were I_{50} 29 $\mu g\,L^{-1}$, LOD 0.85 $\mu g\,L^{-1}$ and DR from 4 to 180 $\mu g\,L^{-1}$. For S3-BSA antibody and S4-SYM18 tracer, the same pair as in capture format, the values achieved are 40, 1.0, and 5 to 250 for I_{50}, LOD and DR, respectively. All these values are higher than those achieved with the capture immunosensor by one magnitude order, so the applicability of HH immunosensing without preconcentration is restricted to samples containing more than 1 $\mu g\,L^{-1}$ sulfathiazole. As counterpart, analysis rate for HH immunosensors, 30 samples per hour, is much better than capture sensors that need around 20 min for completing a whole cycle. On the other hand, it is worth mentioning that the optimal antibody-competition hapten pair is not the same as in capture immunosensing and batch immunoassay, which is indicative that in immunoanalytical techniques the conclusions drawn for an assay format are not always valid for a different format, even though the basic operations – in our case, the analyte-antibody-tracer competition in solution – are similar. It is supposed that the support and separation mechanism (bioaffinity in capture, RAM in HH) has strong influence in the whole assay performances.

The operational life of the HH system was better than that of other immunosensing approaches, because no support regeneration after each assay was necessary, since support capacity is very high [14]. Small molecules kept retained inside the support, but no saturation was observed after a whole working journey – more than 200 assay cycles in some cases, injecting 20 μL sample each. The complete regeneration of the support was carried out by washing with methanol at the end of a working journey, when the system was left in standby. A single column could be used for more than 10 months of continuous use, which is estimated to be equivalent to more than 8000 assays.

The tolerance of the HH immunosensor to the presence of organic solvents in sample was tested by running calibration curves with standards containing methanol and acetonitrile at different proportions. Very high organic percentages (more than 50%) led to unacceptable results, because the tracer was not completely retained in the column, and no proper competition was observed. For lower amounts of organic modifier (20%), when employing acetonitrile, results were also unacceptable, and this organic modifier was discarded. Employing methanol at 20%, calibration curves could be made, although sensitivity was partially lost, which was traduced in I_{50} value of 50 $\mu g\,L^{-1}$, as well as limit of detection of 1.4 $\mu g\,L^{-1}$. Higher concentrations of methanol, up to 40%, were also tolerated, and in this case, sensitivity drafted to an I_{50} value of 100 $\mu g\,L^{-1}$, with a limit of detection of 5 $\mu g\,L^{-1}$. It is clear that if high sensitivity is pursued, samples containing organic solvents should be avoided. However, the applicability of immunosensors to organic mixtures is the easiest way to join immunosensing with effective sample treatment in order to develop a whole analytical method able to solve a problem.

3.1.3. Reduction in assay time in capture immunosensing

One of the issues of this research was to achieve the maximal rapidity of the assay, even though other analytical properties were partially lost. HH immunosensing has shown to be nearly a real-time analytical method. Capture immunosensing is more sensitive, but much slower. However, this assay format can be modified so as to achieve a better sampling rate. For the basic standard immunosensing mode, the total assay time was 18 min (see Table 1). In order to reduce this period, some of the basic operations carried out by the immunosensor can be varied, shortening the analytical cycle.

Regarding the competition, this process is accomplished on-line by mixing the analyte solution (standard o sample) with the antibody and the tracer. If it is performed off-line, the time employed in mixing is saved, and the automation or autonomy is not necessarily jeopardized because a secondary flow manager can perform the mixing process, although the whole equipment becomes more complicated. However, competition off-line increases the sampling rate, because the total time devoted for each analytical cycle is reduced by more than 2 min, but it does not actually shorten the response time – that is, the period from sample intake to result display – because the mixing and competition are carried out the same manner on- and off-line.

The measurement of HRP activity, i.e. signal display, was also modified in order to accelerate the assay. A previously prepared mixture of HPPA and H_2O_2 reagents was employed, instead of carrying out the mixing on-line for each cycle. The mixture showed to be stable for no longer than a working day, although occasionally it went off in few hours, presumably due to contamination. The incubation time, typically 3 min, was also reduced to 1 min, so it was necessary to change antibody and tracer concentrations so as to obtain the same absolute fluorescence signals.

Only by modifying these two basic operations, the total assay time can be shortened from 18 to around 12 min, i.e. by more than 30%, although it is necessary to employ a more complex device for keeping automation.

Finally, the capture of immunocomplexes can be easily accelerated by increasing flow rate when the mixture is injected through the protein A/G support. This implies that capture is less effective, so immunoreagents (antibody and tracer) concentration must be higher, in order to keep the analytical signals (fluorescence peaks) at the same level. This increase in immunoreagent amounts does modify the competition process, so assay sensitivity should be affected. The influence of flow rate on assay performance is summarized in Table 3. It can be clearly seen that the higher the flow rate, the lower the total assay time, and the worse the sensitivity of the competition, as expected. The highest flow rate, 2 mL min^{-1}, leads to a sensitivity that can be unacceptable for many applications, due to the high limit of detection. However, the employment of 1 mL min^{-1} flow rate (see calibration curve in Fig. 1) could suppose a good compromise between high analysis speed (total assay time 8.5 min) and acceptable sensitivity (LOD 1.2 $\mu g\,L^{-1}$), so this flow rate is selected for further studies employing the accelerated protocol. It is worth mentioning that sensitivity of that capture immunoassay is similar to that of HH sensor, since I_{50} and LOD values are similar, but assay speed is much higher in HH sensing.

On the other hand, the effect of organic solvents on the performances of the accelerated immunosensor was similar to that of the standard one. For the above-described accelerated protocol, there were no significant differences between a calibration performed in

Table 3
Influence of flow rate in capture step on analytical performances of immunosensing for sulfathiazole.

Flow rate (mL min^{-1})	0.25	0.5	1	2
[antibody] (dilution factor)	1/4000	1/2000	1/1000	1/500
[tracer] (mg L^{-1})	0.1	0.2	0.4	0.8
Blank signal (arbitrary units)	39,000	49,000	58,000	80,000
I_{50} (μg L^{-1})[a]	5.3	12.6	30	48
LOD (μg L^{-1})[b]	0.15	1.0	1.2	3
Assay time (min)[c]	12	10	8.5	8

[a] I_{50}: analyte concentration that inhibits the binding of the tracer to the antibody by 50%.
[b] LOD: limit of detection, analyte concentration that inhibits the binding of the tracer by 10%.
[c] The assay time corresponds to the general protocol for accelerated immunosensor, i.e. employing competition off-line, a previously prepared mixture of HPPA/H_2O_2 substrates, and 1 min for substrate incubation time.

buffer and a calibration carried out in 10% methanol, and I_{50} value kept always around 30 μg L^{-1}. However, the presence of 10% acetonitrile did shift this parameter to 100 μg L^{-1} (LOD 10 μg L^{-1}), due to the denaturing capacity of this solvent.

In summary, the total assay time for the accelerated immunosensor can be reduced till 8 min, while each assay in the standard mode takes 18 min long. Time reduction is therefore higher than 50%, maintaining complete automation and autonomy. All these data allow to choose the final immunosensing conditions depending on the analytical needs: for routine continuous working, the standard protocol might be fine, depending on the analysis frequency needed, while if the sampling rate needs to be increased, the accelerated protocol could be a good issue, and the sensitivity can even be tuned for being good enough for the application.

3.2. Cross-reactivity

Specific selectivity (cross-reactivity) of all immunosensors was studied. Cross-reactivity was measured for a pool of the most employed sulfonamides. Table 4 shows the cross-reactivity values, compared to those obtained with antibody-coated format ELISA.

In general, selectivity of immunosensor is good because most interfering compounds show cross-reactivity lower than 10%. The only compounds that can potentially interfere in the capture immunoassays are sulfamethoxypyridazine, sulfamethizole and sulfapyridine, with cross-reactivities higher than 5%.

Table 4
Comparison of cross-reactivity values (%) of some analyte-related compounds employing different immunoassay protocols.[a]

Compound	ELISA[b]	Immunosensor		
		Capture		HH
		Standard	Accelerated	
Sulfamethoxypyridazine	19.2	11.5	11.7	1.9
Sulfamethizole	14.3	10.9	15.2	<0.3
Sulfapyridine	7.7	6.9	7.0	<0.3
Sulfamerazine	3.1	0.6	0.3	<0.3
Sulfadiazine	1.7	2.1	1.5	<0.3
Sulfamethoxazole	<0.03	0.6	1.1	<0.3
Phthalylsulfathiazole	<0.03	0.9	0.5	5.1
Sulfamethazine	<0.03	0.6	0.5	11.8
Sulfacetamide	<0.03	0.08	<0.15	<0.3
Sulfanilamide	<0.03	0.15	<0.14	<0.3
Sulfaguanidine	<0.03	0.3	0.1	<0.3
Sulfadimethoxine	Not determined	0.3	0.3	16.3
Sulfasalazine	<0.03	0.2	0.1	<0.3
Sulfisoxazole	<0.03	0.3	0.1	<0.3

[a] Cross-reactivity calculated as the percent ratio I_{50} for analyte/I_{50} for the interfering compound.
[b] Data from ELISA are published in Ref. [28], and correspond to the employment of S3-BSA antibody and S4-HRP tracer.

It is worth mentioning that cross-reactivity values for both kinds of immunosensors, although not exactly the same figures, are similar, and there is also an evident parallelism between cross-reactivity in immunosensing and in batch immunoassay. This fact is indicative that specific selectivity depends mainly on the recognition ability of the antibodies employed, and the influence of immunoassay mode is much less relevant. Also, this conclusion had been retrieved when comparing ELISA and immunosensing cross-reactivity for previously studied analytes [1,34].

In HH immunosensing, the cross-reactivity is quite different, because the antibody used is not the same, and in this case the interferers are sulfamethazine, sulfadimethoxine (CR > 10% in both cases) and phthalylsulfathiazole (CR 5.1%). The two first compounds are similar to the immunogenic S4 hapten, because in the three molecules the two rings are six-atoms large [29], and phtalylsulfathiazole also possess two bencenic rings. This similarity could explain that recognition ability of the antibody.

3.3. Application of immunosensors

The developed immunosensing systems were applied to the determination of sulfathiazole in water, as well as in methanolic honey extracts. Water samples, from different origin, were spiked with the analyte at levels ranging from 1 to 50 μg L^{-1}. The only treatment was to add sodium phosphate and sodium chloride in order to adjust the ionic strength to that of buffer employed in the standards, and to dilute the most concentrated samples when analyte concentration exceeds the dynamic range. As shown in Table 5(a), results are acceptable for most samples in all assay formats. No significant differences in behaviour are observed if comparing data from standard capture immunosensing to those of accelerated one, and HH immunosensor also generates suitable data. Most recovery results are in the range 80–120%, and the only values out of this range correspond to the application of the accelerated immunosensor to the analysis of tap water, when recovery values till 160% have been obtained. This is thought to be due to a chlorine matrix effect from this kind of water over immunoreagents, which is lower in standard immunosensing because the contact time of antibody and tracer with the matrix is lower and better controlled. A previous sample extraction could be applied in these cases, but it has not sense to employ the accelerated immunosensor when early-warning is required if a long sample pre-treatment is necessary for removing the matrix effect. As well, reproducibility found is also acceptable, and most results show a CV lower than 10%, the worst values also corresponding to tap water analysis.

The application of the standard capture immunosensor to honey extracts was also successful. Accelerated immunosensor was not applied in this case because its lower sensitivity would lead to no detection in many samples. Data correlate well with spiking values, as well as with HPLC–MS results. Only the most diluted sample (5 μg kg^{-1} in honey) shows a very high bias, which is expectable since the final concentration of sulfathiazole in the solution entering the immunosensor corresponds to the limit of detection for the buffer–methanol mixture. Repeatability for the measurement of honey extracts is also good, especially HPLC measurements, which also indicates that the extraction process is reproducible.

Methanolic honey extracts were also analyzed by the HH immunosensor, calibrating with 20% methanol standards. Results obtained are useful in this case only at semiquantitative scale, since biases are too high to be considered acceptable. Matrix effect is observed, even though a SPE procedure is applied. It is clear that

Table 5
(a) Analysis of water samples spiked with sulfathiazole by means of the immunosensor with capture and homogeneous–heterogeneous formats. (b) Analysis of honey samples spiked with sulfathiazole by means of the immunosensors with standard and HH format. Comparison with chromatography.

Sample[a]	Capture standard	Capture accelerated	HH
(a) Analysis of water samples spiked with sulfathiazole by means of the immunosensor with capture and homogeneous–heterogeneous formats			
B-0	<LOD[b]	<LOD	<LOD
B-1	1.1 ± 0.1	<LOD	<LOD
B-2	2.3 ± 0.2	1.9 ± 0.3	2.6 ± 0.6
B-5	5.2 ± 1.6	5.6 ± 0.2	4.2 ± 0.6
B-10	8.6 ± 1.0	9.6 ± 0.2	11.0 ± 1.0
B-20	17.8 ± 0.3	20.0 ± 1.0	17.0 ± 2.0
B-50	40.0 ± 2.0	46.0 ± 3.0	40.0 ± 4.0
S-0	<LOD	<LOD	<LOD
S-1	1.0 ± 0.2	<LOD	<LOD
S-2	2.4 ± 0.1	1.9 ± 0.6	2.8 ± 0.5
S-5	5.2 ± 0.6	4.8 ± 0.2	5.2 ± 0.6
S-10	12.0 ± 2.0	12.0 ± 0.4	10.0 ± 1.1
S-20	21.0 ± 2.0	17.0 ± 1.0	17.0 ± 2.0
S-50	41.0 ± 4.0	49.0 ± 3.0	48.0 ± 3.0
T-0	<LOD	<LOD	<LOD
T-1	0.8 ± 0.1	1.6 ± 0.4	<LOD
T-2	1.7 ± 0.2	2.3 ± 0.5	2.5 ± 0.7
T-5	4.6 ± 0.8	4.0 ± 0.7	4.0 ± 0.6
T-10	8.8 ± 1.0	13.9 ± 1.3	12.0 ± 1.2
T-20	22.0 ± 4.0	26.0 ± 3.0	27.0 ± 4.0
T-50	58.0 ± 5.0	42.0 ± 7.0	65.0 ± 7.0

Sample	Spiking level	Capture standard immunosensor	Homogeneous–heterogeneous immunosensor	HPLC
(b) Analysis of honey samples spiked with sulfathiazole by means of the immunosensors with standard and HH format. Comparison with chromatography				
1	0	<LOD	<LOD	<LOD
2	5	7.7 ± 0.7	<LOD	4.0 ± 0.1
3	10	8.2 ± 0.8	15 ± 3	8.5 ± 0.2
4	20	20.0 ± 1.2	16 ± 8	17.0 ± 1.0
5	50	47.0 ± 5.0	75 ± 14	41.1 ± 0.8
6	100	112.0 ± 6.0	140 ± 50	99.0 ± 12.0

All data expressed in μg STZ per kg of honey.
[a] B: commercial bottled water; S: source water taken in Torre d'en Bessora, Castellón, Spain; T: tap water. Numbers accompanying B, S and T correspond to the spiking level in $\mu g\,L^{-1}$. Each measurement represents the mean value ± S.D. of three replicates.
[b] LOD: limit of detection.

HH immunosensing is more prone to that effect than conventional immunosensing.

4. Conclusions

Immunosensing with capture and homogeneous–heterogeneous formats can be considered as reliable tools for the rapid and highly sensitive determination of sulfathiazole in water and honey with minimum sample preparation. The employment of protein A/G support in the immunocomplex capture format, as well as good-featured immunoreagents, leads to a system with high sensitivity and selectivity, able to measure automatically the presence of this antibiotic at low levels. The novel approach of separating the products of the homogeneous competition by means of a restricted access support provides an extremely rapid-response immunoanalysis tool. However, sensitivity for this assay mode should be improved, and the research should be focused on studying new tracers and employing labelling ratios higher than one. Analytical comparison on real samples between the immunosensors and other technologies such as chromatography shows good correlations at very low analyte levels.

Traditional immunosensing can be accelerated simply by adjusting the basic operations of the immunoassay format so as to minimize total assay time. Assay sensitivity is partially lost in the proposed system, although this loose can be controlled. As counterpart, sample throughput can be doubled, which is really interesting when screening a large number of samples or if response is to be retrieved immediately after sample collection. Performing a complete competition enzyme immunoassay in only 8.5 min, including regeneration of the sensing element, is a good achievement. Nevertheless, it cannot compete with 30 samples per hour that can be achieved with the HH immunosensor.

The rapidity of response and the full automation of the systems show the possibility of employing it in field analysis, alarm situations, survey planning, etc. in an advantageous manner when compared to chromatographic methods.

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References

[1] M.A. González-Martínez, R. Puchades, A. Maquieira, Trends Anal. Chem. 18 (1999) 204–218.
[2] M.A. González-Martínez, R. Puchades, A. Maquieira, Anal. Bioanal. Chem. 387 (2007) 205–218.
[3] J. Tschmelak, G. Proll, G. Gauglitz, Talanta 65 (2005) 313–323.
[4] J. Tschmelak, M. Kumpf, N. Käppel, G. Proll, G. Gauglitz, Talanta 69 (2006) 343–350.
[5] I.M. Ciumasu, P.M. Krämer, C.M. Weber, G. Kolb, D. Tiemann, S. Windisch, I. Frese, A.A. Kettrup, Biosens. Bioelectron. 21 (2005) 354–364.
[6] E. Mauriz, A. Calle, L.M. Lechuga, J. Quintana, A. Montoya, J.J. Manclús, Anal. Chim. Acta 561 (2006) 40–47.
[7] M.A. González-Martínez, R. Puchades, A. Maquieira, Anal. Chem. 73 (2001) 4326–4332.
[8] J. Tschmelak, G. Proy, J. Riedt, J. Kaiser, P. Kraemmer, L. Bárzaga, J.S. Wilkinson, P. Hua, J.P. Hole, R. Nudd, M. Jackson, R. Abuknesha, D. Barceló, S. Rodríguez-Mozaz, M.J. López de Alda, F. Sacher, J. Stien, J. Slobodník, P. Oswald, H. Kozmenko, E. Korenková, L. Tóthová, Z. Krascsentis, G. Gauglitz, Biosens. Bioelectron. 20 (2005) 1499–1508.
[9] S. Rodríguez-Mozaz, M.J. López de Alda, D. Barceló, Talanta 69 (2006) 377–384.

[10] E. Mauriz, A. Calle, A. Abad, A. Montoya, A. Hildebrant, D. Barceló, L.M. Lechuga, Biosens. Bioelectron. 21 (2006) 2129–2136.
[11] P. Pulido-Tofiño, J.M. Barrero-Moreno, M.C. Pérez-Conde, Anal. Chim. Acta 429 (2001) 337–345.
[12] P. Pulido-Tofiño, J.M. Barrero-Moreno, M.C. Pérez-Conde, Anal. Chim. Acta 562 (2006) 122–127.
[13] D.B. Holt, P.R. Gauger, A.W. Kusterbeck, F.S. Ligler, Biosens. Bioelectron. 17 (2002) 95–103.
[14] D. Jornet, M.A. González-Martínez, A. Maquieira, R. Puchades, Anal. Chem. 79 (2007) 9331–9339.
[15] P. Önnerjford, S.A. Eremin, J. Emenus, G. Marko-Varga, J. Chromatogr. A 800 (1998) 219–230.
[16] P. Önnerjford, G. Marko-Varga, Chromatographia 51 (2000) 199–204.
[17] S.H. Sheikh, A. Mulchandani, Biosens. Bioelectron. 16 (2001) 647–652.
[18] E.P. Meulenberg, G.O.H. Peelen, E. Lukkien, K. Koopal, Int. J. Environ. Anal. Chem. 85 (2005) 861–870.
[19] D.A. Franco, J. Webb, C.E. Taylor, J. Food Protect. 53 (1990) 178–185.
[20] G.L. Mandell, M.A. Sande, in: A. Goodman, T.W. Gilman, A.S. Rall, P. Nies, P. Taylor (Eds.), The Pharmacological Basis of Therapeutics, Pergamon Press, New York, 1990, pp. 1047–1064.
[21] S. Wang, H.Y. Zhang, L. Wang, Z.J. Duan, I. Kennedy, Food Addit. Contam. 23 (2006) 362–384.
[22] A. Adesiyun, N. Offiah, V. Lashey, N. Seepersadsingh, S. Rodrigo, K. Georges, J. Food Protect. 68 (2005) 1501–1505.
[23] M.S. Diaz-Cruz, D. Barceló, Anal. Bioanal. Chem. 386 (2006) 973–985.
[24] P. Sukul, M. Spiteller, Rev. Environ. Contam. Toxicol. 187 (2006) 67–101.
[25] W. Haasnoot, G. Cazemier, J. Du Pre, A. Kemmers-Voncken, M. Bienenmann-Ploum, R. Verheijen, Food Agric. Immunol. 12 (2000) 15–30.
[26] M. Franek, I. Diblikova, I. Cernoch, M. Vass, K. Hruska, Anal. Chem. 78 (2006) 1559–1567.
[27] T. Korpimäki, V. Hagren, E.C. Brockmann, M. Tuomola, Anal. Chem. 76 (2004) 3091–3098.
[28] N. Pastor-Navarro, C. García-Bover, A. Maquieira, R. Puchades, Anal. Bioanal. Chem. 379 (2004) 1088–1099.
[29] N. Pastor-Navarro, E. Gallego-Iglesias, A. Maquieira, R. Puchades, Talanta 71 (2007) 923–933.
[30] C. Situ, S.R.H. Crooks, A.G. Baxter, J. Ferguson, C.T. Elliott, Anal. Chim. Acta 473 (2002) 143–149.
[31] M. Bienenmann-Ploum, T. Korpimäki, W. Haasnoot, F. Kohen, Anal. Chim. Acta 529 (2005) 115–122.
[32] E.V. Melikhova, E.N. Kalmykova, S.A. Eremin, T.N. Ermolaeva, J. Anal. Chem. 61 (2006) 687–693.
[33] J. Tschmelak, M. Kumpf, G. Proll, G. Gauglitz, Anal. Lett. 37 (2004) 1701–1708.
[34] M.A. González-Martínez, S. Morais, R. Puchades, A. Maquieira, M.P. Marco, D. Barceló, Fresenius J. Anal. Chem. 361 (1998) 179–184.
[35] S. Bauminger, M. Wilcheck, Meth. Enzymol. 70 (1980) 151–159.
[36] J. Penalva, R. Puchades, A. Maquieira, Anal. Chem. 71 (1999) 3862–3872.
[37] A. Posyniak, T. Sniegocki, J. Zmudzki, Bull. Vet. Inst. Pullawy 46 (2002) 111–117.
[38] M.A. González-Martínez, R. Puchades, A. Maquieira, Int. J. Environ. Anal. Chem. 83 (2003) 633–642.
[39] M.A. González-Martínez, E.M. Brun, R. Puchades, A. Maquieira, K. Ramsey, F. Rubio, Anal. Chem. 77 (2005) 4219–4227.