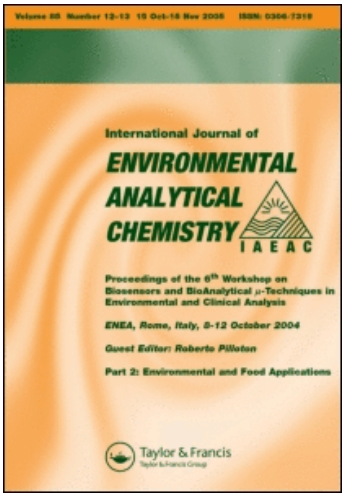

International Journal of Environmental Analytical Chemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713640455

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To cite this Article Meulenberg, Eline P. , Peelen, Gijsbert O. H. , Lukkien, Eddie and Koopal, Kees(2005) 'Immunochemical detection methods for bioactive pollutants', International Journal of Environmental Analytical Chemistry, 85: 12, 861 — 870

To link to this Article: DOI: 10.1080/03067310500158180

URL: http://dx.doi.org/10.1080/03067310500158180

Intern. J. Environ. Anal. Chem.
Vol. 85, No. 12–13, 15 October–15 November 2005, 861–870

Taylor & Francis
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Immunochemical detection methods for bioactive pollutants

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(*Received 8 October 2004; in final form 25 January 2005*)

Recently, environmental contaminants were found to include pharmaceuticals. Regarding the convenient applicability of the ELISA, especially in water samples, we have raised antibodies against two target compounds that have been found at rather high levels in effluents, groundwater, surface water and even drinking water, i.e. ibuprofen and naproxen. Several polyclonal antibody preparations were obtained from rabbits and used to design ELISAs. In addition, one of the antibodies against ibuprofen was also used in a preliminary fashion for the construction of an immunosensor. Ibuprofen has been found in the highest concentrations in waste water and influents of up to 16 nM, whereas in surface water it can reach levels of 7.8 nM. In our indirect competitive ELISA a detection limit of 0.1 nM was achieved, with a working range of 0.1–146 nM, which is well in the range required for monitoring purposes. The ELISA was validated in the usual way by determining several parameters, e.g. cross-reactivity (< 1% for all compounds tested (related and unrelated)), with the exception of naproxen (2.2%); matrix effects, linearity ($R^2 = 0.9580$), precision and recoveries (105–162% depending on the matrix and the concentration added). Naproxen has been detected in concentrations up to 0.126 μM in effluents and waste water. Our ELISA displayed a detection limit of 15 μM and a working range of 15–150 μM, which is not yet suitable to monitor this compound. One of the antibodies appeared to be rather specific for naproxen, showing less than 10% cross-reactivity with various related and unrelated substances. Similar to ibuprofen, linearity of the assay ($R^2 = 0.9850$), matrix effects, precision (intra-assay variation 11–24%, inter-assay variation 9–19% at levels of 62.5 to 250 μM) and recoveries were determined. From the results it appeared, for example, that reliable values were obtained by adding methanol to standards and samples before analysis. In addition, using the polyclonal antibodies against ibuprofen preliminary measurements have been performed on the Biacore™ 3000 in various spiked real water samples containing 200, 1000 and 8000 nM of ibuprofen. The results were compared with those determined in the ELISA; the correlation between both methods was highly significant showing an $R^2 = 0.9998$. Attempts are being made to transfer the Biacore immunoassay on a Spreeta™ format for field use. Herein, a standard curve with a detection limit of 3.5 μM was achieved. In conclusion, the pharmaceutical contaminant ibuprofen may be measured specifically both by ELISA and an immunosensor assay (Biacore). An ELISA for naproxen, a compound with comparable bioactivity, has been developed, but should either be optimized with respect to the required sensitivity or combined with a preconcentration step. This assay, however, is very robust and seems promising for further research.

Keywords: Environment; ELISA; Immunosensor; Pharmaceuticals

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International Journal of Environmental Analytical Chemistry
ISSN 0306-7319 print: ISSN 1029-0397 online © 2005 Taylor & Francis
DOI: 10.1080/03067310500158180

1. Introduction

With the increasing world population and the threatening shortage of clean (drinking) water in the near future there is a continuing concern about contaminants in water bodies. In the period 1960–1980, agriculture expanded to meet the need for food and this was accompanied by the application of huge amounts of pesticides to combat pests. As a result, pesticides were found all over in the environment and regulations and norms were established to limit the use, keep control and prevent excessive exposure of humans, animals and other non-target organisms. Then, at the end of the last century the world was alarmed by the finding that various pollutants could mimic endogenous hormonal effects and thereby affect reproduction and possibly lead to various cancers. Due to rapid advances in medicine and the availability of drugs, together with the development of sophisticated analytical methods, recent discovery of pharmaceuticals in water bodies and at low concentration even in drinking water [1–4] has shifted attention to this issue.

Pharmaceuticals and drugs are meant to be bioactive and to remain present within the body at a certain level to have their aimed effect. In the living body, pharmaceuticals are subject to oxidation, reduction, hydrolysis (phase I) and conjugation (phase II) [1]. As a result, both parent compounds and metabolites/conjugates are excreted and enter the environment. Other emission routes include deliberate and accidental disposal of excess drugs from industry and household use. The emission into the environment is approximately 8.3% of the total amount distributed and 3% of this is non-consumed.

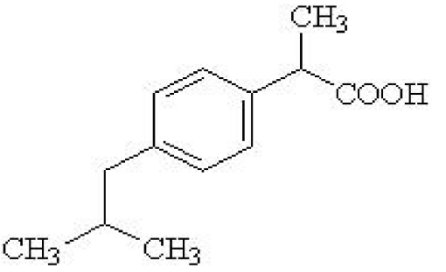
Once in the environment, degradation occurs through several pathways: physicochemical, microbial, photochemical degradation [1, 5]; further routes whereby these compounds may disappear from water is binding to particulate matter, sediment and finally by sewage treatment [6]. It should be noted that conjugated metabolites are partly converted back to their parent compounds by microorganisms in water, sediment and sewage treatment plants. The first reports of drugs in the environment were published in the 1970s. Methods used for detection are predominantly HPLC, GC/MS, LC/MS/MS, optionally after SPE pre-treatment, with a detection limit of about $0.05\,\mathrm{nmol\,L^{-1}}$ [4]. In 2001 a Dutch research paper was published about environmental effects of human pharmaceuticals, presence and risks [2]. It was found that the following compounds are at the top 10 of most prescribed pharmaceuticals: paracetamol (analgetic), oxazepam (sedative), diclofenac (analgetic, anti-rheumatic), salicylic acid acetate (analgetic, blood diluent), ethinylestradiol/levonorgestrel (contraceptives), temazepam (sedative), furosemide (diuretic), doxycycline (antibiotic), omeprazole (anti-pyrosis). The European guideline for pharmaceuticals in water is set at $0.05\,\mathrm{nmol\,L^{-1}}$ (EMEA guidelines, see [1, 7]). Accordingly, a monitoring study was performed in the Rhine catchment area including 78 pharmaceuticals covering 10 chemical groups. Twenty-four compounds were detected in a concentration range of $0.05\,\mathrm{nmol\,L^{-1}}$ to several tens of $\mathrm{nM\,L^{-1}}$. In our investigation we were interested in two analgetics that are commonly used in huge amounts, ibuprofen and naproxen. In particular ibuprofen is sometimes used by individuals in an amount of 1200–1600 mg per day and about 168–224 tons per year are introduced into the environment, of which about 29% are metabolized [6]. It should be noted that this

drug is also used for veterinary application. Ibuprofen has been found in effluents at levels up to 0.06 μmol L^{-1} and in surface water in a range of <0.02 to 0.20 nmol L^{-1} [1], which was confirmed in the Dutch monitoring study where it was detected in 10 of 31 samples at a level up to 0.26 nmol L^{-1}. Higher concentrations were reported in the Dutch survey where concentrations in effluent of >5 nmol L^{-1} and in surface water of >0.5 nmol L^{-1} were mentioned. Even in ground water ibuprofen appeared in concentrations of >0.5 nmol L^{-1}, whereas in drinking water it was below the detection limit. Naproxen has been determined in effluents and surface water at >0.4 nmol L^{-1} and at STPs at 2.3 nmol L^{-1}. The compounds have analgetic, anti-rheumatic, anti-fungal and anti-bacterial effects, although no effects on fish have been reported. In water they are predominantly degraded by aerobic and anaerobic microorganisms and photochemically, and partly adsorbed onto sediment; however, biodegradation of naproxen is much less than for ibuprofen.

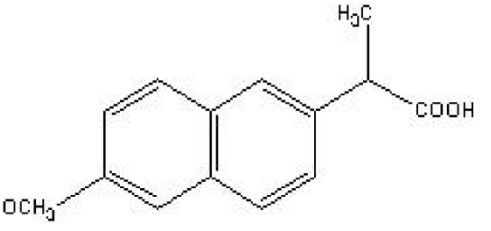
Generally, pharmaceuticals are measured by conventional analytical methods. These are inherently time-consuming and expensive and, consequently, not suitable for monitoring studies. Regarding the upper level according to the European guideline, the persistence of these compounds and their unknown overall effects and possible synergism, rapid cost-effective methods are needed. For these reasons we developed antibodies and ELISAs for both ibuprofen and naproxen. The results of our validation studies are described. In addition, for real-time measurements an attempt was made to transfer the immunochemical analysis to an SPR immunosensor.

2. Experimental

Ibuprofen and naproxen were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Their chemical structure is depicted in figure 1. Compounds used for the determination of cross-reactivity in the assays were from either Sigma-Aldrich (Zwijndrecht, The Netherlands) or Omnilabo (Breda, The Netherlands). Second antibody, goat anti-rabbit IgG conjugated to HRP was from ITK (Uithoorn, The Netherlands). For the ELISA the following buffers were used: coating buffer (0.05 M sodium carbonate, pH 9.6), 0.1 M PBS pH 7.4, ELISA buffer (PBS + 0.1% bovine serum albumine (BSA)), wash buffer (0.01 M PBS + 0.05% Tween-20). For the generation of antibodies, white New Zealand rabbits were immunized at the Central Animal Lab of the University of Nijmegen (The Netherlands) using initially 250 μg of immunogen (0.25 mL) mixed 1 : 1 with 0.25 mL of complete Freund's adjuvans, followed by booster injections in incomplete Freund's adjuvans with intervals of 3 weeks. The conjugates required for the immunization and the development of an indirect competitive ELISA, i.e. BSA-ibuprofen, OVA-ibuprofen, were synthesized using the well-known mixed anhydride method. Conjugates were purified with BioRad Econo-Pac 10DG columns (BioRad, Veenendaal, The Netherlands) following the manufacturer's instructions and using 0.1 M PBS as eluent with minimal dilution. Microtiter plates were flat-bottom middle-binding plates from Greiner (Alphen a/d Rijn, The Netherlands). The buffer used for the immunosensor assays consisted of 10 mM 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid, 150 mM sodium chloride, 3 mM EDTA, 0.005% (v/v) surfactant polysorbate, pH 7.4 (HBS-EP).



Ibuprofen: α-methyl-4-(2-methylpropyl)benzene acetic acid, MW 206.27



Naproxen: (S)-6-methoxy-α-methyl-2-naphthalene acetic acid, MW 230.26

Figure 1. Structure of ibuprofen and naproxen.

2.1 *Equipment*

ELISA readings were performed with a microtiter plate reader model EAR340 from SLT-Labinstruments (Abcoude, The Netherlands). For the design of immunosensor assays the Biacore™ 3000 (Biacore, Uppsala, Sweden) was used. A simplified version of an SPR sensor including the Spreeta™ (Texas Instruments, Dallas, USA) was also designed.

2.2 *Methods*

Immune serum of the rabbits was pooled after 55 weeks of immunization. During that period the titer of the antibodies was determined in an indirect ELISA format using OVA-ibuprofen (400 ng mL^{-1}) as coating conjugate and a dilution series of antiserum. At the end of the immunization period, aliquots of the pooled immune serum were extracted using the caprylic acid method. Herein, to 4 mL of serum 8 mL of acetate buffer, pH 4.0 was added and subsequently 0.3 mL of caprylic acid. After stirring for 30 min the mixture was centrifuged (10,000 rpm) and the supernatant collected. The pellet was resuspended in 4 mL of 0.015 M acetate buffer, pH 4.8. Following centrifugation for 20 min (10,000 rpm) both supernatants were combined, filtrated and dialysed against 0.15 M phosphate buffer, pH 8.0 for two days with several changes. The purified antibody was used to design indirect competitive ELISA. Appropriate controls were included in each assay. For the optimization of the ELISA, first a checker board was set up to establish optimal concentrations of coating-conjugate, purified antibody and HRP-labeled second antibody. Then the common parameters for

ELISA validation were determined, i.e. standard curve, detection limit and working range; precision (intra- and inter-assay variation), linearity, cross-reactivities, matrix effects and recovery in the usual way.

For the Biacore assay, onto a CM5 chip provided with a polymer layer a spacer (AVA, aminovaleric acid) was first coupled, followed by the coupling of OVA-ibuprofen. Antibody and ibuprofen in a concentration range of 1–10,000 nM were passed and the signal measured.

The same CM5 chip was directly coated with OVA-ibuprofen and used in the Spreeta embodiment that was coupled to an autosampler. Antibody and increasing concentrations of ibuprofen were passed over the sensor surface for 120 s for each sample and the chip was regenerated with 0.2 M NaOH for 40–60 s.

3. Results

3.1 *Ibuprofen*

Two rabbits, M50 and M51, were used for the generation of antibodies against ibuprofen. After approximately 13 weeks of immunizations, the serum titer remained rather stable showing values of ca. 50,000 for both rabbits. Preliminary displacement with excess ibuprofen showed that antibody M51 performed better for ELISA and this antibody was used for further validation. Standard curves were made to establish the working range and calculate the detection limit of this assay, as well as determine inter-plate variation. Using an antibody dilution of 1 : 15,000, a working range of 10–10,000 nM was determined and a detection limit of 5 nM could be achieved (4-parametric logiclog transformation for fitting the curve and 3 SD from B0 for calculation of the detection limit). The results are exemplified in figure 2. The linearity of the assay was determined by adding a known high amount of ibuprofen to PBS buffer and making serial dilutions before assay in quadruplicate. Regression analysis of the results showed a linear relation of $y = 0.7948x + 745$; $R^2 = 0.9628$, which is a highly significant correlation. The results are shown in figure 3. The cross-reactivity of the antibody M51 was determined for various substances and the antibody proved to be highly specific for ibuprofen as shown in table 1. Using spiked WWTP effluent and river water (Meuse) the matrix effects were determined in comparison to ibuprofen

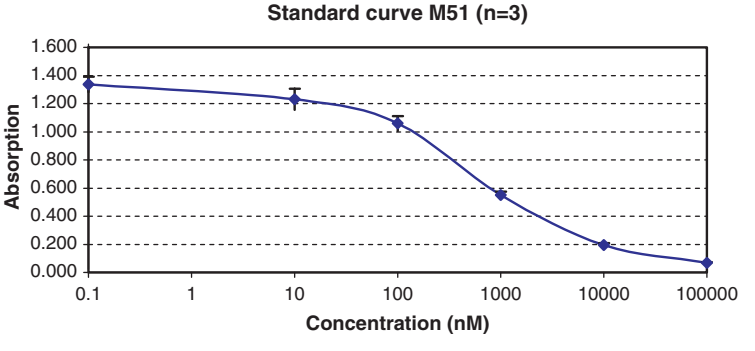


Figure 2. Ibuprofen standard curves were generated in three-fold. Given are the mean and SD of the standard points.

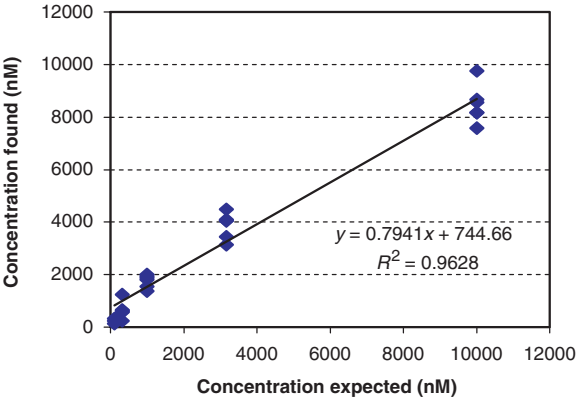


Figure 3. Ibuprofen was spiked in a relatively high concentration to sample and measured by ELISA. Shown is the relation between added and found levels.

Table 1. Cross-reactivity of anti-ibuprofen antibody M51.

Substance	% C.R.
Bezafibrate	0.4
Bisphenol A	0.6
Carazolol	0.04
Chlorpyrifos	0.3
Clofibrinic acid	0.3
Diclofenac	0.02
Erythromycine	0.1
Fenitrothion	0.5
Fenofibrate	0.5
Gemfibrozil	0.2
Ibuprofen	100
Metoprolol	0.1
Naproxen	2.2
Nonylphenol	1.3
Octylphenol	0.6
Propanolol	0.1
Vinclozolin	0.02
Zearalenon	0.2

in demi-water. Matrix effects in these types of water appeared negligible and the deduced recovery was approximately 100%. The precision of the ELISA expressed as intra- and inter-assay variation was determined by using a control sample containing about 3100 nM of ibuprofen and analysing in eight-fold on three different occasion. The intra-assay variation varied from 11 to 18% and the inter-assay variation was 18%.

3.2 *Naproxen*

Two rabbits, M79 and M80, were used for the production of antibodies against naproxen. The titer was found to be 500 and 1000, respectively, after 25 weeks of immunization. Antibody M80 was used for further investigation. Following checkerboard analysis with pooled and purified antibody to find optimal condition for the indirect competitive ELISA, the antibody was further validated as for ibuprofen.

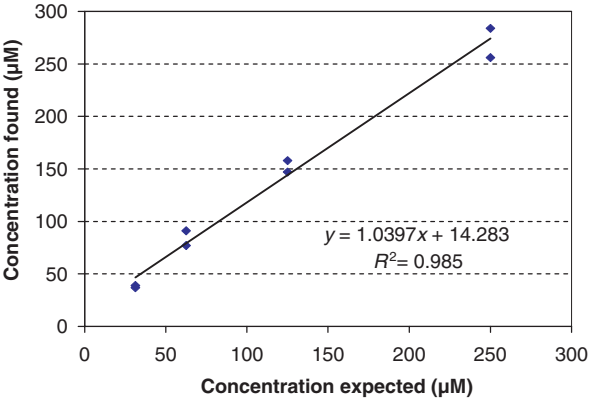


Figure 4. Naproxen was spiked in a relatively high concentration to sample and measured by ELISA. Shown is the relation between added and found levels.

Table 2. Cross-reactivity of anti-naproxen antibody M80.

Substance	% C.R.
Bezafibrate	5
Bisphenol A	5
Clofibrinic acid	5
Diclofenac	4
Erythromycine	3
Fenofibrate	6
Gemfibrozil	8
Ibuprofen	9
Metoprolol	6
Naproxen	100
Octylphenol	10
Propanolol	6

It appeared that the assay was not as sensitive as desired, showing a detection limit of about 15 μM and a working range of 15–150 μM. The linearity of a spiked surface water sample was highly significant, as shown in figure 4. The cross-reactivity of antibody M80 is shown in table 2, where it can be observed that this antibody is rather specific for naproxen. To assess matrix effects and recovery, various water types, demi-water, PBS, canal water and effluent, were spiked with several amounts of naproxen and measured. The results are shown in figure 5 and table 3, respectively. In view of the poor results, an attempt for optimization was made by adding 10% methanol to both standards and samples. As a result the mean recoveries in demi-water and effluent were lowered to more realistic values of $66 \pm 14\%$ (CV 21%; $n = 5$) for effluent samples.

3.3 *Immunosensor*

Attempts were made to transfer the ibuprofen ELISA to the Biacore 3000 in order to develop an immunosensor assay. An indirect assay was designed by coupling OVA-ibuprofen conjugate through an AVA (aminovaleric acid) spacer onto the

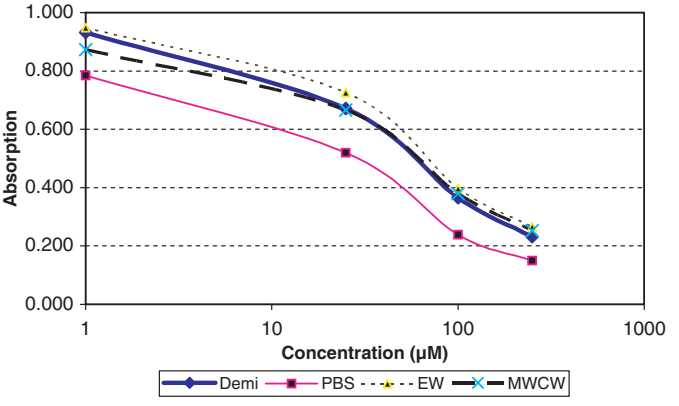


Figure 5. Naproxen was added in various concentrations to demineralized water (Demi), buffer (PBS), WTP effluent (EW) and river water (MWCW, Meuse Waal Canal Water) to determined effects of the matrix on the ELISA results.

Table 3. Recovery of naproxen from various water types.

Matrix	Concentration (μm)	Mean recovery (%)	SD (% CV)	No. of samples
Demi-water	25	177	59 (33)	9
	100	176	35 (20)	9
	250	184	70 (38)	8
PBS	25	340	55 (16)	9
	100	461	193 (42)	7
	250	238	238 (45)	5
Effluent	25	126	33 (26)	9
	100	137	21 (15)	9
	250	115	25 (22)	9
Canal water	25	119	31 (26)	9
	100	129	9 (7)	9
	250	118	30 (25)	9

polymer surface of a CM5 chip. A run was carried out by first mixing 75 μL antibody solution, 150 μL sample and 375 μL HBS-EP, followed by injection of 100 μL aliquots at a flow rate of 20 μL min^{-1}. Regeneration between runs was made using 5 μL of 20% acetonitrile/150 mM. An example of the standard curve made in various water types is shown in figure 6. A simpler embodiment of the immunosensor is the Spreeta™. This SPR device was provided with the same CM5 chip and treated as above. After establishing a standard curve, the same types of water samples as used for the Biacore were spiked with 200, 1000 and 8000 nM of ibuprofen, and measured with both with the Spreeta and with ELISA. Regression analysis revealed a high correlation between the results, i.e. $y = 0.9699x - 98.1$, $R^2 = 1$ for demi-water; $y = 0.8742x - 89.3$, $R^2 = 0.9998$, for canal water; and $y = 0.8416x - 10.8$, $R^2 = 0.9997$, for effluent.

4. Discussion

To the present knowledge of the authors, this is the first report of immunochemical methods for the detection of the analgetics ibuprofen and naproxen in the environment.

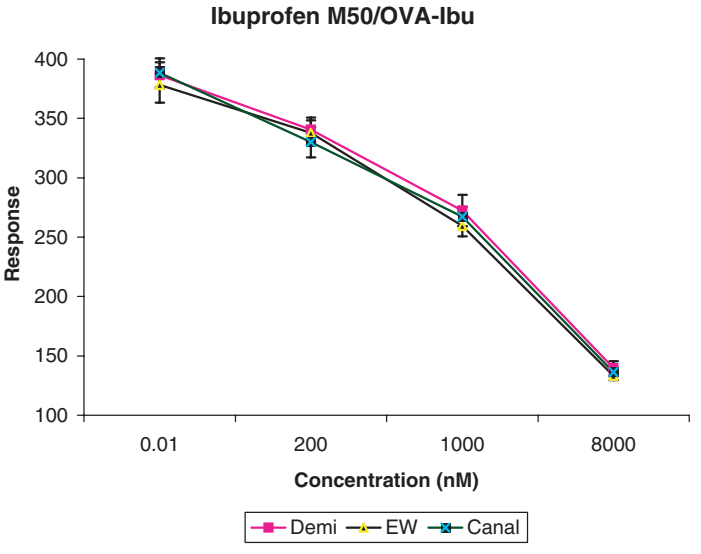


Figure 6. Ibuprofen was added in various concentrations to demineralised water (Demi), WTP effluent (EW) and river water (canal). These samples were analysed in the Biacore immunosensor assay. The results are expressed in response in resonance units.

In particular, our ELISA for ibuprofen displayed highly satisfying performance characteristics to be applied in monitoring studies. The detection limit achieved in the assay for ibuprofen is well below concentrations found in surface water, waste water and effluents [3, 7]. In addition, the antibody was found to be very specific for ibuprofen. Consequently, the assay may be used for monitoring studies in order to assess the load of this compound in the environment; potential effects on water organisms and possibly humans through drinking water may then be extrapolated. The latter issue may appear relevant in view of the reported influence of biologically active substances on biological water treatment facilities [8]. Naproxen, a comparable analgetic compound, has also been detected in effluents and waste water at levels of $>0.43\,\text{nmol}\,\text{L}^{-1}$. Although the immunochemical characteristics of the naproxen ELISA showed that the antibody used is rather specific and naproxen can be measured in various spiked water samples with high linearity, the recovery values indicated a profound influence of the matrix containing naproxen. This issue may be solved by adding an organic solvent such as methanol or ethanol to the samples, or the inclusion of a pre-incubation step in the assay. According to literature data, naproxen is found at levels of $>0.43\,\text{nmol}\,\text{L}^{-1}$ in surface water and about $2.2\,\text{nmol}\,\text{L}^{-1}$ in effluents of sewage treatment plants. Unfortunately, our ELISA could not achieve sufficient sensitivity for such concentrations. To solve this problem, attempts are being made to further optimize the assay. Another solution could be to produce IAC columns for purification and concentration of such samples.

Environmental studies may benefit from rapid, cost-effective, real-time measurements of target compounds. For this reason we tried to transfer the ELISA onto the Biacore™. Our preliminary results were such that the immunosensor assay was also performed on the Spreeta™, a much simpler and cheaper embodiment of the SPR based immunosensor. It may be concluded that using the appropriate antibody and suitable conditions in the immunosensor assay, ibuprofen may be measured in various

aqueous matrices showing a high correlation between the ELISA and immunosensor results. Further preliminary results indicate that the coupling of an autosampler to the Spreeta may enhance its efficiency with regard to handling time and costs. Because this type of assay seems very promising, further experiments are planned to optimize the immunosensor assays on the Biacore™ as well as on the Spreeta™.

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