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High sample throughput flow immunoassay utilising restricted access columns for the separation of bound and free label

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Abstract

A flow immunodetection system with high sample throughput capacity is described for the screening of various analytes. The immunochemical detection principle is based on the chromatographic separation of the formed immunocomplex (AbAg or AbAg*) and the free antigen (Ag) by a restricted access (RA) column, utilising size-exclusion and reversed-phase mechanism. A fluorescein labelled analyte (Ag*) was used in the competitive assay format with fluorescence detection. The speed and simplicity of the assay were the greatest advantages, allowing measurement of the analyte to be carried out in less than 1 min. The biocompatibility and capacity of the restricted access material allowed multiple injections of up to 5000, without any breakthrough of the fluorescent tracer molecule and thus need for regeneration. The flow immunoassay was developed using the well-known atrazine herbicide and some transformation products as model compounds, due to their human toxicity and widespread use. The sample throughput was 80 samples per hour and the detection limits were 1.4 n*M* (300 pg/ml) for atrazine (Ab I) and 2.3 n*M* (500 pg/ml) for the sum of triazines (Ab II–III). Different sample matrices, PBS buffer, creek water, and urine were successfully applied in the flow system without the need for any sample handling step. For plasma samples an additional clean-up step using solid-phase extraction had to be included. The resulting detection limits for atrazine in plasma and water samples using this clean-up and trace enrichment procedure were found to be 2 ng/ml and 20 pg/ml, respectively. The analysis could be performed at a sample throughput rate of 400 per 6-h working shift.
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1. Introduction

The problems involved in fast, sensitive and selective analysis of large number of samples require the development of new, highly efficient, automatable and more cost-effective screening techniques, which ultimately are aimed for unattended on-site screening, e.g. in order to alert on a sudden increase of environmental contaminants. Immunoassay techniques are potential candidates for fulfilling these requirements due to the high inherent selectivity of antibodies, the possible high sensitivity of such systems and the fact that antibodies can be raised against virtually any compound. Most commonly, immunoassays are performed in the batch wise mode, however, due to the cost of automation and the lack of discrimination between cross-reacting compounds, new immunodetection principles are

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under constant development. In recent years a number of continuous-flow immunodetection systems have been reported for either single or groups of analytes in flow-injection analysis [1–11], for multiresidue analysis in liquid chromatography with post-column immunodetection [12–16], and in capillary electrophoresis [17,18]. In the latter two systems, improved selectivity is obtained by combining both separation and biological recognition. The general advantages of continuous flow systems as compared to batch immunoassays are the comparatively low cost of automation, speed of analysis, improvement in sample through-put and the possibility of on-line coupling to sampling and sample clean-up systems.

The present paper deals with a further development of a heterogeneous competitive flow immunoassay [19], which can be used for the screening of smaller analytes in both environmental and biological samples. The method is based on off-line incubation of the analyte (Ag), a fluorescein labelled tracer (Ag*) and the corresponding polyclonal or monoclonal antibody (Ab). The separation of free (Ag*) and bound tracer (Ab–Ag*), is performed in a flow immunoassay system, which incorporates a small column containing an alkyl-diol-silica restricted access (RA) material [20], previously described for a non-competitive assay by Oosterkamp et al. [13]. Similar flowimmuno systems have recently been published [21–25].

The RA phase traps the free unbound tracer (Ag*) in its hydrophobic (C_{18}) inner cavity, but excludes the large Ab–Ag* complex, which is passed on and measured by a fluorescence detector. This type of system can, in principle, be applied to all small analyte molecules (≤3000 Da) if the corresponding antibodies are available, and is here exemplified for the screening of atrazine and its metabolites in a number of different environmental (surface water, waste water) and biological samples (human blood, urine).

2. Experimental

2.1. Method and instrumentation

The flow immunoassay system, shown in Fig. 1, consists of a Gilson (Villiers-le-Bel, France) ASPEC XL autosampler equipped with two Rheodyne (Berkeley, CA, USA) six-port injection valves (V1 and V2), a Gilson 305 pump. A manually packed restricted access (RA) precolumn (C_{18} alkyl-diol-silica, ADS, Merck, Darmstadt, Germany, I.D. 10×2.0 mm) was placed in V2, and injection loops of either 20 or 50 μl in V1. The RA column was first conditioned with 3 ml of methanol and then with 5 ml of a 1-mg/ml BSA solution to avoid unspecific binding of antibodies.

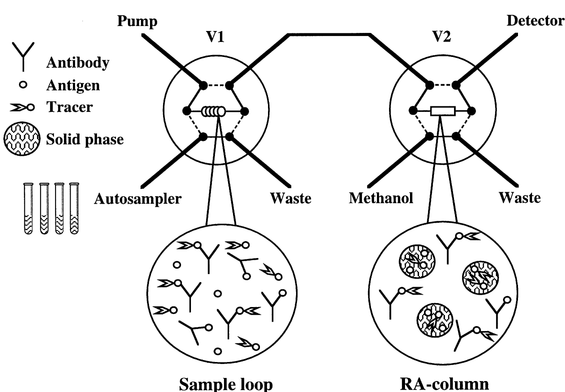


Fig. 1. Schematic overview of the flow immunoassay system. In the sample loop, all the reagents are present and at equilibrium. In the RA column only the immunocomplexes are passed through, while the unbound antigens are hydrophobically trapped on the reversed phase.

The analytical system was controlled using Gilson 719 software, all programmes being written in Turbo Pascal. Gilson 715 software was used for data acquisition. A fluorescence detector from Shimazdu RF-535 (Kyoto, Japan) was used and the excitation and emission wavelengths were set to 490 and 515 nm, respectively. Some environmental samples, from different locations in Russia, were analysed using the Envirogard Triazine plate kit (Millipore, Scarborough, ME, USA).

For sample enrichment with solid-phase extraction (SPE), a larger RA precolumn (I.D. 25×4 mm) was used. Conditioning (3 ml MeOH), washing (3 ml water), loading (20–22 ml sample), and desorption (400 μl 40% MeOH) of the precolumns were performed using a syringe pump (Dilutor 401C, Gilson) equipped with a 10-ml syringe. The plasma samples were spiked and diluted 25-fold (from 1 to

25 ml) before the pre-concentration of 20 ml were carried out on the ASPEC.

2.2. Chemicals and immunoreagents

The *s*-triazines, atrazine, IPO 005, and simazine, IPO 692, were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Stock solutions of the triazines were made in acetonitrile and kept in the dark at +4°C. The stock solutions were further diluted with 0.01 *M* phosphate-buffered saline (PBS) buffer (pH 7.4). All other chemicals were of analytical-reagent grade.

Fluorescein thiocarbamyl ethylene diamine (EDF) was synthesised as previously described [26] from fluorescein isothiocyanate (FITC) isomer I (Sigma Chem. Co., St. Louis, MO, USA). The derivative 4,6-dichloro-*N*-(isopropyl)-1,3,5-triazin-2-amine was synthesised from cyanuric chloride (Sigma Chem. Co., St. Louis, MO, USA) and isopropylamine (Merck, Darmstadt, Germany) as described by Goodrow et al. [27]. The tracer was synthesised by reacting 2 mg (10 μmol) of 4,6-dichloro-*N*-(isopropyl)-1,3,5-triazin-2-amine dissolved in 0.2 ml dimethyl formamide (DMF) (Fluka, Buchs, Switzerland) with 4 mg (8 μmol) of EDF. The reaction mixture was stirred for 1 h at room temperature and kept overnight at +4°C. Fifty-μl portions of the reaction mixture were then separated by thin-layer chromatography, TLC (Kieselgel 60, Merck, Darmstadt, Germany), using methylene chloride–methanol (5:1, v/v) as the eluent. The major yellow band at R_F=0.5, containing the tracer, was isolated and stored in methanol at +4°C. The concentration of tracer was estimated spectrophotometrically at 492 nm, assuming an absorptivity in sodium bicarbonate buffer (50 m*M*, pH 9.0) to be the same as for fluorescein ($\epsilon=8.78\times10^{4}$ M^{-1} cm^{-1}). The tracer solution was further diluted in 0.01 *M* PBS buffer (pH 7.4) before measurement.

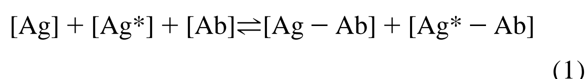
The different antibodies were obtained and designated according to the following: antibody I (polyclonal, from rabbit: 12/2d) was kindly provided by Dr. M.-P. Marco, CID-CSIC, Barcelona, Spain; antibody II (polyclonal, from rabbit: Sima); and antibody III (polyclonal, from rabbit: Arina), were kindly provided by Immunotech Inc., Moscow, Russia. All antibody preparations were purified using $(NH_4)_2SO_4$ precipitation (at 50% saturation) and centrifugation for 10 min (4000 *g*). The pellets were resuspended in 0.01 *M* PBS buffer and precipitated a second time before further clean-up was performed using dialysis for approximately 24 h in 0.01 *M* PBS buffer (three changes of buffer). Dialysis tubing was from Spectrum Med. Ind., Los Angeles, CA, USA, MW-cut-off 12–14 kDa. The protein concentrations were determined by calculating the difference in absorbance at 280 nm against a PBS blank. Assuming that the IgG concentration of 1 mg/ml corresponds to 1.35 absorbance units [28] the resulting [IgG] for Abs I–III were 1.9, 3.2 and 5.2 mg/ml, respectively.

3. Results and discussions

The specific characteristics of a given antibody preparation will ultimately be determined by the synthesised carrier protein conjugate (necessary for small antigens), the immunisation as such, and the preparations and purifications undertaken with the serum. Hence, variables such as the position of conjugation, as well as the structure and chain length of the anchoring group between target molecule and carrier protein, will have dramatic effects on the Ab properties and thus also on the sensitivity and selectivity of an immunoassay [29–31]. In a similar fashion, the chemical structure of the tracer molecule, i.e. the position of conjugation, structure and chain length of the linker between target molecule and the label, will influence the immunoassay. Theoretically, good competitive immunoassays can be developed when the antibody affinity constant (K_{aff}) for the tracer is in the same order of magnitude as for the analyte. However, for a small target analyte, the affinity for the tracer is normally higher than for the analyte due to the fact that the antibody can recognise part of the anchoring chain used for the antigen–carrier protein binding. To overcome this problem, antigens are labelled at a different position than was used for the immunogen conjugation, and/or linkers with different length and structure are used. The selection of optimal tracer structure and antibody preparation for flow immunoassay purposes was previously made [32] by kinetic in-

vestigations utilising fluorescence polarisation, a homogeneous assay technique based on the difference in rotational motion between bound and free fluorescent-labelled molecules [33].

The analysis in the flow immunoassay follows a competitive immunoassay format where the actual detection is based on the fluorescence intensity of the antibody-bound tracer fraction (Ag*–Ab) according to the following principle: The sample (Ag) is mixed and incubated off-line with optimised amounts of fluorescein-labelled tracer (Ag*) and antibody (Ab) until equilibrium conditions are attained:



$$[Ag] + [Ag^*] + [Ab] \rightleftharpoons [Ag - Ab] + [Ag^* - Ab] \quad (1)$$

The reaction mixture is then injected into the flow system depicted in Fig. 1, where the unbound Ag and Ag* are retained by reversed-phase partitioning in the pores of the RA column material. The modified hydrophilic outer surface prevents unspecific binding of proteins, and a molecular mass cut-off of approximately 15 kDa prevents proteins from entering the hydrophobic inner surface. The immunocomplexes Ag–Ab and Ag*–Ab are thus, due to their size, excluded from the pores of the RA material and pass unretained through to the fluorescence detector where the Ag*–Ab complex results in a signal peak that is stoichiometrically related to the Ag concentration. A low amount of target analyte in the sample gives a large fluorescence signal, resulting in a calibration plot with a negative slope. The time schedule for the analysis of four consecutive samples in Table 1 illustrates that the delay time from sample incubation, easily can be minimised by more efficient time programming.

In previous work, the fluorescein-labelled tracer iPr/Cl/EDF, containing a short two-carbon bridge between fluorescein and the antigen, resulted in the highest sensitivity using Ab II and Ab III [29,30], which is why this tracer was chosen in the following experiments. Capacity control experiments were made regularly by injecting iPr/Cl/EDF tracer without the presence of antibodies to ensure that the tracer was completely retained. Breakthrough was observed after approximately 5000 injections, whereby the RA column was regenerated.

3.1. Mobile phase dependence

The assay was optimised with a mobile phase of

Table 1
Time table over four consecutive samples

Time (min)	Event	Valve 1	Valve 2
Manual steps, off-line			
0.00	Mixing of sample 1 and immunoreagents	—	—
0.00–5.00	Incubation time (sample 1)	—	—
1.00	Mixing of sample 2 and immunoreagents	—	—
1.00–6.00	Incubation time (sample 2)	—	—
2.00	Mixing of sample 3 and immunoreagents	—	—
2.00–7.00	Incubation time (sample 3)	—	—
3.00	Mixing of sample 4 and immunoreagents	—	—
3.00–8.00	Incubation time (sample 4)	—	—
Steps in the automated ASPEC system			
5.00	Loop filling (sample 1)	Load	Load
5.10	Injection of sample 1 at equilibrium	Inject	Load
6.00	Loop filling (sample 2)	Load	Load
6.10	Injection of sample 2 at equilibrium	Inject	Load
7.00	Loop filling (sample 3)	Load	Load
7.10	Injection of sample 3 at equilibrium	Inject	Load
8.00	Loop filling (sample 4)	Load	Load
8.10	Injection of sample 4 at equilibrium	Inject	Load
5000 injections	Regeneration of column	Load	Inject

The events and valve positions for each individual time are listed.

0.01 *M* PBS buffer to allow as many injections as possible without regeneration and cleaning of the column. For some applications, using, e.g. solid-phase extraction and liquid chromatography, the analyte will be in an environment containing a polar organic modifier, such as methanol or acetonitrile. Accordingly, the detection step takes place under these conditions and the possibilities of using the flow immunoassay as an alternative detection unit was investigated. This is illustrated in Fig. 2, by running injections of the tracer alone (solid line) and the Ag*–Ab complex (dotted line) in mobile phases with various amounts of methanol. For 0 and 10% of methanol no peaks for free tracer injections were seen, however, as the methanol concentration increases the peaks appear, reaching the maximum signal at 40% of methanol. Comparison of the peaks from Ag* alone and the Ag*–Ab complex indicate that the immunocomplex is still formed at 30% of methanol, which is of major importance if the flow immunoassay is to be coupled as a post-column detection unit for column liquid chromatography (CLC), or if an SPE clean-up/enrichment step has to be introduced in order to obtain lower LOD values. Obviously, a mobile phase containing up to 10% of methanol can be used without any special considerations except that the tracer breakthrough will occur earlier.

The free label trapped on the RA column can be desorbed by using injections of 100% organic modifier. This phenomena can be used to obtain a second calibration curve. The first calibration curve with a negative slope (Fig. 3, solid line) can be obtained directly by determining the AbAg* fraction that passes the RA column unretained, due to the size-exclusion effect, while the second calibration curve (Fig. 3, dotted line) with a positive slope will be achieved after consecutive elution of the free tracer fraction using an organic modifier. The slope of the calibration curve obtained after tracer elution is steeper and can be explained by solution effects. The fluorescence intensity of the tracer, increases with amount of organic modifier up to approximately 80%, but close to 100% the signal is significantly reduced. However, since the methanol plug is dispersed during transportation, the methanol content decreases to approximately 80–90% and an intensity increase is observed instead, resulting in a steeper calibration curve.

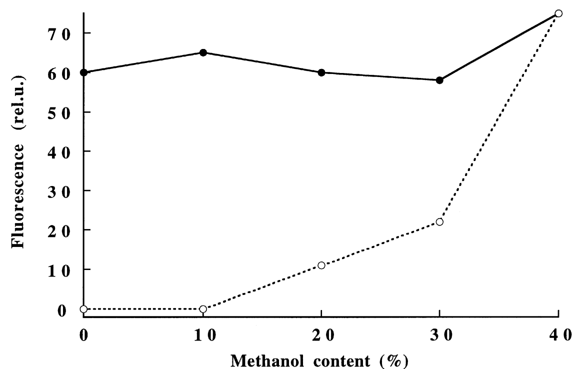


Fig. 2. Organic solvent influence on the flow immunoassay system. Various amounts of MeOH in the mobile phase were investigated making tracer injections (○), and tracer with antibody, Ag*–AbAg*, injections (●). Flow-rate, 0.8 ml/min; [Ab]= 100 μg/ml; [Ag*]=4.4 n*M*.

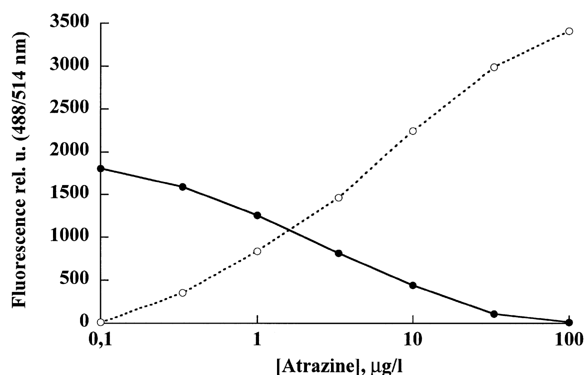


Fig. 3. Calibration curves obtained after direct measurements of the AbAg* fraction (●), or after desorption of the trapped Ag* fraction (○) with 100% methanol injections. Experimental conditions as in Fig. 2.

By using this method, where the tracer is first trapped on and then eluted off the solid phase, one sample will result in two calibration curves. Obviously, this 'sorption–desorption' technique would give more reliable and accurate results, since it can be based upon measurements of both free and bound tracer. Another advantage would be the possibility of studying the kinetics of slow immunoreactions by looking at the bound and free fractions. However, despite these advantages, the former method was preferable in order to obtain higher sample throughputs.

3.2. Selectivity

The selectivity, given by the immunoreagent specificitys, of an immunoassay can be chosen according to the particular purpose of an assay. In cases where the metabolites and transformation products are of special interest due to, e.g., toxicity, biological activity or other reasons, broad specificity of the antibody is desirable. If, on the other hand, the determination of a specific analyte is of interest, antibodies with narrow specificity are to be preferred.

The cross-reactivity of Ab I, II and III for seven different *s*-triazines were evaluated using fluorescence polarisation [32], where the sensitivity for atrazine was set to 100% (see Table 2). Table 2 shows that Ab I has 20 and 33% cross-reactivity for only hydroxyatrazine and propazine, respectively, and can thus be considered to be a rather atrazine-specific antibody. Ab II and III, however, show significant cross-reactivity for several of the other *s*-triazines, and in the case of propazine even higher sensitivity than for atrazine. Ab II and III can therefore be considered to be more group specific than Ab I. The flow immunoassay was therefore optimised with the more atrazine-specific antibody, Ab I, for the screening of atrazine.

3.3. Optimisation of the flow immunoassay using Ab I

In a competitive assay format the concentration of analyte, tracer and antibodies must be in the same order of magnitude. For very sensitive assays, the lowest possible tracer concentration should be used. This concentration was found to be 4.4 n*M* for the tracer injected into the flow immunoassay system. Antibody dilution curves were then recorded by incubating 4.4 n*M* of the tracer with various antibody concentrations. An incubation time of 5 min was found to be sufficient for obtaining >90% of the equilibrium, and was used in all further experiments [32]. The concentration of Ab I was chosen to be 100 μg/ml for further assay experiments, which corresponds to approximately 60% of the tracer molecules bound to antibodies. This concentration can easily be increased in order to expand the linear range of the assay, however, with an additional increase in antibody consumption.

3.4. Displacement and association assay principles

As already stated, the most sensitive competitive assay is obtained when the lowest possible amount of tracer is used. However, if the antibody affinity for the tracer and analyte differs considerably, the order of mixing the reagents can drastically change the sensitivity of the assay [32]. A significant difference in sensitivity has previously been observed using fluorescence polarisation immunoassay. Three mixing orders are possible giving different assay principles. (i) The association mode: pre-incubation of Ag with Ag* and the following addition of Ab will give the Ab equal possibilities to bind either Ag or Ag*. The assay sensitivity will be determined by the different affinities for the analyte and the tracer. (ii) The Ag displacement mode: pre-incubation of Ag with Ab to form the Ab–Ag complex and then the following addition of the tracer Ag*. This assay will be less sensitive if the affinity for the tracer is lower than the affinity for the Ag, since the tendency to shift the equilibrium from Ab–Ag to Ab–Ag* is

Table 2
The selectivity of Ab I, II and III are listed as cross-reactivity results, relative signal in comparison with atrazine (%)

s-Triazines	Ab I (%)	Ab II (%)	Ab III (%)
Atrazine	100	100	100
Simazine	1	69	6.4
Desisopropylatrazine	<0.1	6	2.3
Desethylatrazine	0.4	8	51
Propazine	33	450	250
Hydroxyatrazine	<0.1	0.1	3.9
Terbuthylazine	1	4.5	2.6

These values are obtained from the immunoreagent selection using fluorescence polarisation experiments in a preceding work.

low. (iii) The Ag* displacement mode: pre-incubation of Ag* with Ab to form the Ab–Ag* complex and then the following addition of the analyte Ag. For the same reasons as above this assay format will be less sensitive if the affinity for the Ag is lower than for the tracer Ag*. One advantage of the last method (iii) is that a single-reagent mixture, containing tracer and antibody (Ag*–Ab) can be used. The storage stability of such mixtures are usually much better than the two solutions separately [34]. In Fig. 4, the resulting calibration curves from the three competitive modes are compared for Ab I incubated with the tracer and different concentrations of atrazine. Since the curves are approximately the same, it can be concluded that the antibody affinities for Ag and Ag* are in the same order of magnitude. This means that in a mistake in the order of mixing reagents would not cause a large error in the analysis. However, the association mode would be preferred in order to reach equilibrium as fast as possible. It also shows that a single reagent mixture of AbAg* can be used, thereby simplifying sample handling and obtaining improved stability of the immunoreagents. The same experiment was carried out for Ab III with similar results.

3.5. Influence of flow-rate and injection volume

To obtain a high sample throughput as well as a low detection limit, it is of great importance to investigate the flow-rate and injection volume dependencies, which affect both peak height and peak width, and thereby also sample throughput. The theoretical maximum sample throughput was calculated by adding the time for all the necessary individual steps in the computer program. Using normal aspiration and dispension flow-rates, a 20-μl sample volume, and an average of three repetitive injections for each sample, a maximum sample through-put of 95 injections/hour was obtained, which corresponds to a maximum peak width of 0.63 min. The influence of the flow-rate on peak width and peak height is illustrated in Table 3, keeping the injection volume constant at 20 μl. This means that the maximum sample throughput was obtained at all flow-rates investigated, since all the peak widths are narrower than 0.63 min. However, at higher flow-rates there was a tendency of larger standard deviations, and at lower flow-rates the peaks were broader and less suitable for peak height measurements. As a compromise, 0.8 ml/min was selected as the optimum flow-rate.

Variation of the sample volume and the influence on peak width, sample throughput (observed), limit of detection (LOD) and linear range was investigated using the optimised flow-rate and is illustrated in Table 4. Also in this experiment, a maximum sample throughput was calculated keeping all parameters constant except the sample volume. The observed sample throughput was somewhat lower than the maximum, which was due to an additional waiting time in the program to obtain better baseline separation of the peaks. The LOD values for the flow immunoassay were calculated from the signal obtained with a 0-ppb atrazine sample (S_0) minus a 3× relative standard deviation (R.S.D.) of S_0, e.g., a R.S.D. of 2% would result in a LOD at the signal

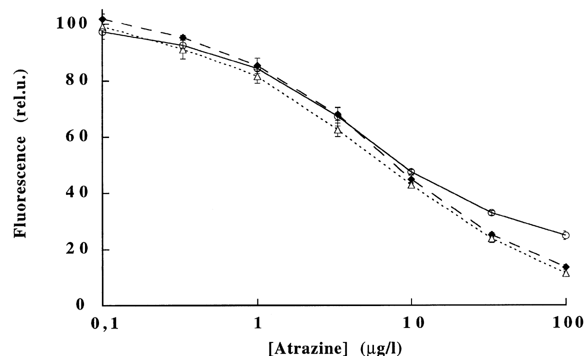


Fig. 4. Calibration curves for atrazine using different assay principles with the flow immunoassay using Ab I; (♦) mode i, (△) mode ii, (○) mode iii. A 20-μl loop, 0.8 ml/min flow-rate, a tracer concentration of 6.6 n*M* and [Ab] = 100 μg/ml were used.

Table 3
Flow-rate influence on peak parameters like peak height and peak width using a constant sample volume of 20 μl

Flow-rate (ml/min)	Peak width (min)	Peak height (mm)
0.40	0.42	48
0.60	0.37	51
0.80	0.31	54
1.00	0.26	57
1.20	0.23	63
1.40	0.20	69
1.60	0.18	69

Table 4
Sample volume influence on peak width, sample throughput, LOD and linear range using a mobile-phase flow-rate of 0.8 ml/min and constant aspiration/dispension flow-rates

Injection loop (μl)	Peak width (min)	Sample throughput (samples/h)	LOD (ng/ml)	Linear range (ng/ml)
2.5	0.25	100 (max. 115)	2	3–100
10	0.27	90 (max. 105)	1	2–100
20	0.31	75 (max. 95)	0.5	1–100
50	0.35	60 (max. 80)	0.3	0.5–100
100	0.39	50 (max. 52)	0.3	0.5–100

corresponding to 94% of S_0. As seen, the LOD values decrease with increasing sample volumes, however, a sample volume of 50 μl was selected as the optimum to minimise immunoreagent consumption.

3.6. Validation

The high sample throughput makes the assay very suitable for multiple injections of each sample resulting in better confidence limits and more reliable data for applications within both biomedical and environmental areas. For example, during one working day, typically 400 injections were made on 90 samples (four to six injections per sample). Within- and between-sample standard deviations were calculated using the (L.S.D.) one-way ANOVA for a 1-ng/ml atrazine sample. Six replicate measurements were run on eight separately prepared samples. The R.S.D. within and between samples were 1.9 (degrees of freedom, df=40) and 4.2% (df=7), respectively. The LOD for atrazine (Ab I) and triazines (Ab II) using the optimised system were 0.3 and 0.5 ng/ml, respectively. The long-term stability of the system is proven by one restricted access column that was used for approximately 5000 injections during a period of 6 months without regeneration. After regeneration with 3 ml of methanol, the column totally regained its separation capacity.

3.7. Applications

The extensive agricultural application of herbicides represented by triazine pesticides (atrazine, simazine, terbutylazine, and others) has created significant pollution problems around the world, as these pesticides are particularly persistent in the environment and are serious contaminants in food and drinking water. Concern about the pollution of drinking and ground water and its impact on human health have led the European Community (EU) to introduce regulations where the upper limit for a single pesticide at present is 0.1 ng/ml (0.5 n*M*) and for the total of all pesticides 0.5 ng/ml [35]. Toxicity aspects, where the metabolisation of the parent compound is of mandatory importance, are illustrated by the scheme shown in Fig. 5. The developed flow immunoassay system for screening of atrazine meets the necessary requirements, including high sample throughput, sensitivity, and selectivity, as presented above. The system was additionally applied to both real environmental (surface and waste waters) and spiked biological (blood and urine) samples, as described below.

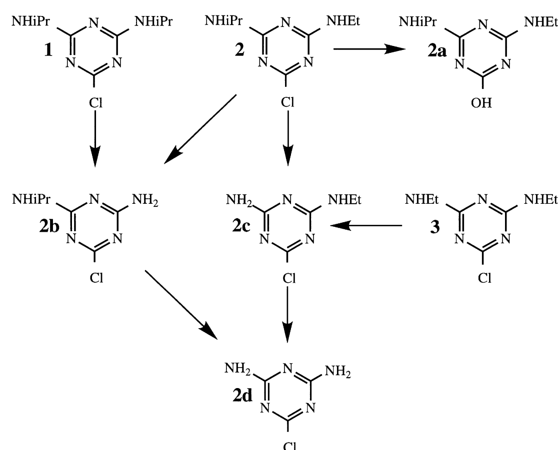


Fig. 5. Breakdown scheme including some of the metabolites obtained from biological and chemical degradation of parent triazine compound: 1, propazine; 2, atrazine; and 3, simazine). The metabolites correspond to: 2a, hydroxyatrazine; 2b, desethylatrazine; 2c, desisopropylatrazine; 2d, didealkylated atrazine.

Table 5
Results from biological and environmental applications using the flow immunoassay with Ab I (atrazine) or Ab II (atrazine+simazine) as well as an ELISA kit (atrazine)

Sample	Flow IA–Ab I	Flow IA–Ab II	ELISA env.
C10, spiked creek water at 10 ng/ml	++++	+++	not run
C3, spiked creek water at 3 ng/ml	++	++	++
C1, spiked creek water at 1 ng/ml	+	+	+
E1, Fishing lake	−	++	not run
E2, waste water, chem. plant A1	+	++++	+++
E3, river Oka-1	+	+++	+
E4, ground water	−	−	not run
E5, lake 'Boloto'	−	−	not run
E6, waste water, chem. plant B	−	−	not run
E7, waste water, chem. plant A2	not applicable	not run	not run
E8, river Oka-2	+++	++++	++
E9, tap water in Dzerinsk	+	−	not run
E10, river Seima	++	+++	+
U0, urine blank	+	not run	not run
U3, urine spiked at 3 ng/ml	+++	not run	not run
U10, urine spiked at 10 ng/ml	++++	not run	not run

Abbreviations for concentration intervals: −, [Ag] <0.3 ng/ml; +, [Ag] 0.3–1 ng/ml; ++, [Ag] 1–3 ng/ml; +++, [Ag] 3–10 ng/ml and finally; ++++, [Ag] >10 ng/ml.

3.7.1. Environmental samples

Water samples from Russia with unknown concentrations of atrazine (samples E1–E10 in Table 5) and creek samples from Sweden spiked at various concentrations of atrazine (samples C1, C3, and C10 in Table 5) were screened in the developed flow immunoassay, see Fig. 6a and Table 5. Some of these samples were, as seen in Table 5, also screened with a commercial ELISA kit for triazines (for description see Section 2). However, since simazine is more commonly used than atrazine in Russia, an additional flow immunoassay was developed and optimised for the detection of simazine. For this purpose, Ab II was used instead of Ab I, due to the high cross-reactivity of the former for simazine, as shown in Table 2. The LOD for the simazine assay was 0.3 ng/ml atrazine with a linear range of 1–30 ng/ml. The same samples as above were screened with the developed simazine flow immunoassay (see Fig. 6b and Table 5). The difference between the two flow immunoassays are thus that Ab I is more atrazine specific (1% cross-reactivity with simazine) and Ab II is both atrazine and simazine specific (100 and 69% cross-reactivity for atrazine and simazine, respectively). Since the developed flow immunoassays are screening methods, only positive (>LOD) or negative (<LOD) concentration intervals are given in Table 5. It can be seen that good agreements between the three methods were obtained for most of the samples. Some of the differences can be explained by the assay selectivity. The CR values for the ELISA kit are ATR 100%, SIM 17% and PROP 125%. All three assays show, as expected, strong positive atrazine signals for the spiked Swedish creek water (C1, C3, C10). The Russian samples, E1–E3, show no or slightly positive signals with the more atrazine-specific assay, but strong signals with the atrazine- and simazine-sensitive assay, indicating that these samples contain simazine and no or very little atrazine as expected. However, the Russian samples, E8 and E10, show strong positive signals with both assays, indicating that these samples definitely contain atrazine.

3.7.2. Biological samples

In order to be able to study toxicity aspects on occupational exposure of atrazine, spiked human blood and urine samples at 0, 3.3 and 10 ng/ml were processed in the same manner as the water samples above. As seen in Fig. 7a and Table 5, urine samples (peaks 1–3) were successfully applied, while plasma samples (peaks 4–6) were not. The high protein

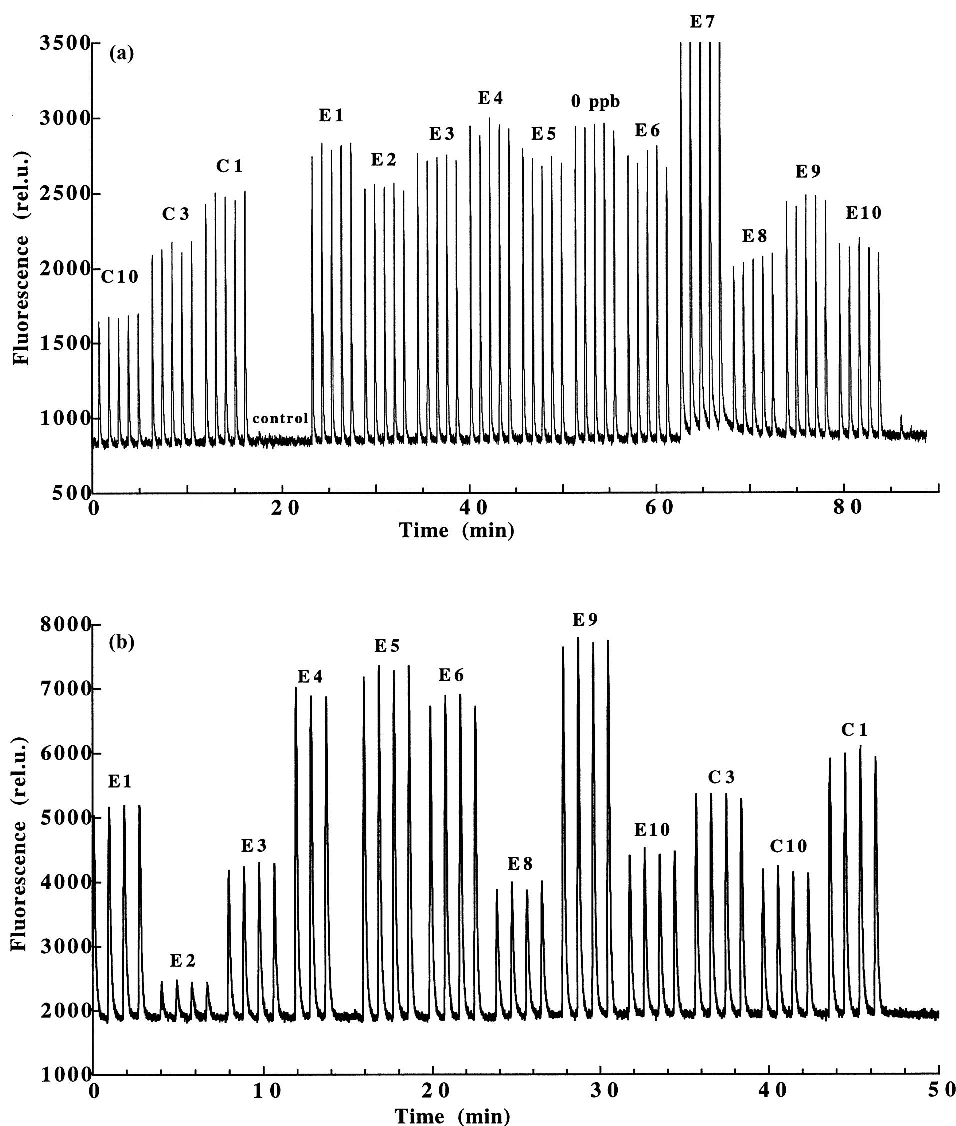


Fig. 6. Environmental applications of the flow immunoassay system for screening of triazines in different water samples. The sample 'control' corresponds to an injection of sample with tracer and no Ab, in order to check the system for tracer breakthrough. Sample E7 was not applicable without any clean-up, the signal is much higher than the 0-ppb on the calibration curve, and this is due to matrix effects from the black 'coffee-sample'. All other abbreviations are as described in Table 4. The flow immunoassay conditions were: 0.8 ml/min flow-rate and a 50-μl sample loop. The sample mixture was made by mixing 100 μl 8.8 n*M* tracer, 800 μl sample, and 100 μl antibody; (a) 100 μg/ml Ab I or (b) 200 μg/ml Ab II.

content in plasma with inherent native fluorescing properties resulted in peaks that were significantly higher than the normal AbAg* peaks for 0 ng/ml atrazine in 0.01 *M* PBS buffer. To remove these matrix effects and achieve lower detection limits, a trace enrichment step based on solid-phase extraction with the highly biocompatible RA phase was applied based upon work outlined in a previous paper [36]. Breakthrough volumes for simazine and atrazine were 30 and 50 ml, respectively, indicating that sample volumes below 30 ml should result in recoveries of ≈100%. Additional work on the

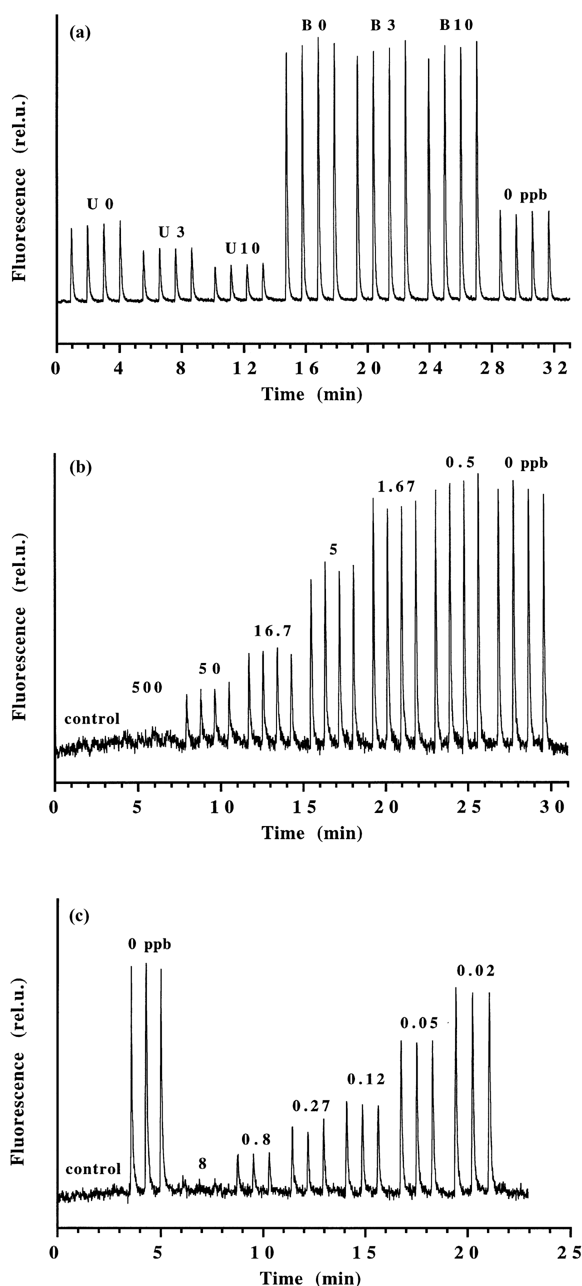


Fig. 7. (a) Biological applications of the flow immunoassay without any clean-up step, before analysis of urine (U0, U3, U10) and blood (B0, B3, B10), where 0, 3 and 10 corresponds to the spiked concentration (ng/ml atrazine). 0 ppb, corresponds to an unspiked 0.01 *M* PBS sample. All other conditions are as in Fig. 5. (b) Spiked blood samples diluted and then enriched using a solid-phase extraction (SPE) step before analysis with the flow immunoassay system as described above. (c) Spiked buffer samples with an SPE step as described above.

elution profile for atrazine, using various methanol contents in the eluent, revealed that 40% MeOH was sufficient to elute atrazine in a sharp peak. An SPE method with conditioning (3 ml MeOH), washing (3 ml H_2O), sample application ($\times$ml) and elution (400 μl of 40% MeOH) was used. The sample eluate was collected off-line and diluted four times in order to obtain a sample matrix completely compatible (10% MeOH) with the immunoreagents, before running the flow immunoassay. The SPE method was first applied on buffer samples, where 22 ml of sample was pre-concentrated. The resulting peaks for samples in buffer are depicted in Fig. 7c, showing that the LOD has been lowered from 0.3 to 0.02 ng/ml.

Plasma samples of 1 ml were diluted to 25 ml in order to simplify sample handling and reduce matrix effects, applied to the SPE column (20 ml) and eluted with 40% methanol. This procedure using SPE resulted in a detection limit of approximately 2 ng/ml (see also Fig. 7b). These results illustrate that lower detection limits can be obtained with simultaneous removal of difficult matrix effects, at the expense of analysis time. The SPE step takes approximately 10 min per sample using a volume of 20 ml.

4. Conclusions

General principles are outlined which should, due to the relatively low specificity of the RA phase, be applicable to any type of antigen with a molecular mass $\leq$3000 Da as long as the AbAg–AbAg* kinetic reaction conditions are favourable [32]. A flow immunoassay with a high sample throughput has been developed for screening of atrazine and simazine as model low-molecular-mass analytes. We performed 400 quantitative determinations during a 6-h cycle. It would be practically possible to increase the sample throughput to approximately 1000 samples per 24 h by introducing an automated sample handling robot with improved capacity. Fully automated robotic ELISAs for a 24-h assay have similar capacity as the system described. In comparison to conventional ELISAs advantages, such as simplicity, analysis time, instrument cost, repeatability and robustness, should be considered. Additionally, there is no need for error prone washing steps or any extra incubation steps. These factors makes the system

very suitable for use at on-site early-warning systems. The limitation of the system as such, is the requirement of larger amounts of immunoreagents and the molecular mass restrictions. However, with the progress within the field of cell and molecular biology, antibody production using designed cell expression systems can easily solve these limitations. Additional improvement of the detection limit or assay sensitivity, could be accomplished by including a solid-phase extraction step prior to the immunoassay. This sample handling step can easily be automated and coupled on-line to the system described. The immunoflow principle is additionally very suitable for the screening of possible antigens that can bind to a certain biological element (e.g., antibody or enzyme). This selection can easily be carried out within one working day, and the feedback from an experiment is fast.

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