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Problems in Immunoassay Development of Small and Volatile Molecules – Benzene, Toluene, and Xylenes (BTX)

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Summary

Indirect and direct enzyme immunoassay formats for the detection of the volatile organic compounds benzene, toluene, and xylenes have been developed based on polyclonal antibodies. In the indirect ELISA, the detection limit for the five analytes (equimolar amounts) in water was 210 μg/L and the center point value of the calibration curve was 1.7 mg/L. Because of the volatility of the analytes special precautions were necessary. Best conditions for assay procedure were found with an incubation at 4 °C and without covering the reaction vessel (microtiter plate or test tube) with a sealer (Parafilm®) during the competitive step. An incubation time of 10 min was sufficient and resulted in good sensitivity. With the addition of 10% dipolar aprotic organic solvent the analytes could be kept in water and the base value of the calibration curve could be decreased. With the use of a non-ionic surfactant the stability of the assay could not be improved. In contrast, the addition of Genapol C 080 or Triton X-100 led to interference with the ELISA. However, a slight increase in sensitivity was obtained (IC_{50}: 0.9 mg/L) by a transfer of the microtiter plate assay to test tubes, but with some loss of reproducibility. The tube ELISA can be used as a semiquantitative screening test.

Keywords: BTX, immunoassay, test format, organic solvents, surfactants, temperature

Introduction

Refined petroleum products are the most widely used products responsible for environmental contamination *(1)*. The contamination of soil and groundwater by petroleum products occurs frequently during their transport, processing, and storage. In the recent study made by the US Environmental Protection Agency (US-EPA) »Underground Storage Tank Program« it is estimated that in the United States there are approximately 1.4 million underground storage tanks, of which as many as 400,000 tanks may be leaking *(2)*. Key compounds for detecting contaminated sites include benzene, toluene, ethylbenzene, xylenes (BTEX), and petroleum hydrocarbons. These compounds are target analytes in the EPA methods and are found at relatively high concentrations in most gasolines. BTEX also have a relatively high aqueous solubility (Table 1) and at least in the case of benzene, are considered very toxic and carcinogenic *(3)*. The maximum contaminant level for benzene in drinking water is only 5 μg/L *(4)*. After accidental contamination of soil and water BTEX concentrations are generally several orders of magnitude higher. Typically, analytical methods require laboratory analysis by gas chromatography (GC) *(5–7)* and an extended period of time to obtain the results. Field analysis for total petroleum hydrocarbons by hydrocarbon vapor analyzer is almost instantaneous and therefore extremely useful. However, these instruments have shown poor correlation with laboratory-derived results *(8)*. Several factors are responsible for the poor correlation, including environmental conditions, sample aging, calibration procedures, and instrument response time *(9)*.

Usually immunochemical assays are accurate methods for field screening of contamination by pesticides or industrial products of environmental concern *(10–13)*. Their simplicity, reliability, and ability to provide information rapidly and on-site is enhancing the efficiency of many field and laboratory programs. The immunoassay methods rely upon antibody molecules to provide the

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sensitivity and specificity characteristics they exhibit. Normally, only substances with a molecular weight higher than $M_r = 1000$ and which are not found naturally in the immunized host will induce the formation of specific antibodies *(14)*. The industrial contaminants benzene, toluene, and xylenes (BTX) have a very low molecular weight (Table 1). These analytes are monocyclic aromatic organic compounds and the phenyl ring is an ubiquitous structure, for example also found in the amino acids phenylalanine and tyrosine. Toluene and xylenes have just a modified benzene molecular structure, in which the hydrogen atom(s) is (are) substituted by methyl groups. Therefore, the molecules are poorly immunogenic by themselves. However, it is possible to obtain specific antibodies also for small substances (haptens) as well as when these molecules are coupled covalently to a carrier of higher molecular weight, for example to a protein. Because of the absence of appropriate functional groups for coupling, BTX derivatives have to be synthesized to prepare immunogens, coating antigens and tracers.

Here we describe the development of an enzyme-linked immunosorbent assay (ELISA) for the detection of benzene, toluene, and xylenes (BTX) in water. The structural similarity and stability of BTX compounds results in similar and unique physical properties and geochemical behaviors in subsurface environments (Table 1).

Commercial immunological test kits

One of the advantages of immunological methods is the possibility of their application to complex matrices without or with only minor sample preparation. There is an exceptional interest in developing screening methods for environmental pollutants, especially with toxic and carcinogenic potential. For this reason and because of the common occurrence there are offered BT(E)X-immunological test methods from different companies. In these test kits different strategies are used, such as antibody source (polyclonal/monoclonal), no single analyte test but rather group specific assays, pre-extraction of sample with organic solvents and application of different test formats (tube, microtiter plate, immunofiltration). The detection is in the ppm- or in the high ppb-range for the mixture or for single analytes. There is no difference in sensitivity between the use of monoclonal or polyclonal antibodies.

The detailed evaluation of a BT(E)X-immunoassay for difficult environmental matrices, as was the ultimate objective of the research project, requires higher amounts of well-characterized antibodies. Therefore the development of an immunoassay based on an in-house antibody source was initiated. Table 2 summarizes the BT(E)X enzyme immunoassays which are commercially available or reported in the literature in comparison with the ELISA (TUM Assay) from the present investigation.

Table 1. Chemical and physical properties of BTX compounds *(15,16)*

Parameter	Compound		
	Benzene	Toluene	Xylene
Formula	C_6H_6	$C_6H_5CH_3$	$C_6H_4(CH_3)_2$
Molecular weight	78.12	92.15	106.18
Boiling point/°C	80.1	110.6	140.63*
Density/(g/mL)	0.8765	0.8669	0.8685*
Solubility/(mg/L)	1780	515	150*
K_{ow}**	135	490	*o*-X: 589 *m*-X: 1585 *p*-X: 1413
K_{oc}	97	242	570*
Vapour pressure at 20 °C/mm Hg	95.2	28.4	6.6*

*Average value, **(5)

Table 2. Comparison of different enzyme immunoassays for BT(E)X

Supplier's information and references	Benzene IC_{50}/(mg/L)	Toluene IC_{50}/(mg/L)	Xylenes IC_{50}/(mg/L)	Ethylbenzene IC_{50}/(mg/L)	BT(E)X IC_{50}/(mg/L)	Remarks
TUM Assay	38[a]	3.5[a]	*o*-X: 2.9[a] *m*-X: 0.3[a] *p*-X: 7.6[a]	2.3[a]	0.5[a]	in 10% DMSO, pAb
Agri Diagnostics Associates *(17)*	14[a] 250[b]	2.2[a] 25[b]	*o*-X: 8[a]; 160[b] *m*-X: 5.2[a]; 92.5[b] *p*-X: 0.6[a]; 6.3[b]	2.5[a] 25[b]	0.9[a] 18[b]	pAb
EnSys Inc. *(18)*	negative[a]	2.21[a]	*o*-X: 0.18[a] *m*-X: 0.48[a] *p*-X: 1.26[a]	0.17[a]	n.r.	mAb
EnSys Inc. *(1)*	0.005[a]	0.034[a]	0.056[a]	0.263[a]	n.r.	Extraction, mAb
EnSys Inc. *(19)*	0.5[a]	4[a]	15[a]	30[a]	n.r.	Extraction, nitration, mAb
Quantix Systems *(20)*	n.r.	n.r.	n.r.	n.r.	0.005–10[a,c]	Test tubes, pAb
Idetek Inc. *(21)*	n.r.	n.r.	n.r.	n.r.	0–0.6[a,c]	Capillary tubes
Mallinckrodt Baker *(22)*	51.0[a]	7.4[a]	*o*-X: 4.7[a] *m*-X: 1.8[a] *p*-X: 3.1[a]	7.8[a]	3.9[a]	Test tubes, Ab coupled on magnetic particles
Coring-System *(23)*	70[b,d]	7[b,d]	*o*-X: 3[b,d] *m*-X: 0.3[b,d] *p*-X: 0.5[b,d]	5[b,d]	2–50[b,c]	Test tubes, pAb
D-Tech *(24)*	12.5[a]	4.5[a]	4.3[a]	4.3[a]	0.6–10[a,c]	Immunofiltration, mAb

IC_{50}: Mass concentration of free analyte required to give 50% inhibition in the immunoassay, [a] In water, [b] In soil, [c] Working range. [d] LOD, n.r. – Not reported, pAb – Polyclonal antibody, mAb – Monoclonal antibody

Agri Diagnostics Associates *(17)* offers a direct competitive immunoassay system for the rapid on-site analysis of gasoline in soil and water. The system consists of a disposable assay device coated with polyclonal antibodies specific to gasoline components, and a small handheld reflectometer for quantitation of the test results. For the immunization a xylene derivative was used. The quantitative working range for the water assay system was from 250–3150 ppb (IC_{50}: 900 ppb) and 3–65 ppm total BTEX for the low and high ranges, after a simple dilution step during sample preparation. The quantitative range for the soil assay system was from 3.5–80 ppm (IC_{50}: 18 ppm) and 80–930 ppm total BTEX for the low and high ranges.

In 1992 EnSys Inc. reported on a monoclonal antibody immunoassay for the detection of gasoline and diesel fuel in the environment *(18)*. The assay principle was a direct competitive immunoassay on microtiter plates and the standards or samples were prepared in 10% methanol/water (volume fraction). The immunogen was prepared using a xylene derivative conjugated to bovine thyroglobulin (BTG). The center point of the calibration curve was 500 ppb for water and 10 ppm for soil samples for the mixture of gasoline, diesel, and kerosene. For detection of the target analytes in the ppb-range, a sample extraction with an organic solvent was applied *(1)*. Further investigations were described in 1995, where the samples were extracted with isooctane, then the analytes were converted to a nitroaromatic compound and detected with antibodies generated by an immunization with hydrazide derivatives of nitrobenzene conjugated to BTG *(19)*.

Quantix Systems published a direct competitive immunoassay in a test tube design *(20)*. For immunization they used 6-aminohexyl-p-tolylacetamide conjugated to BSA. The sensitivity level was in the range from 5 ppb to 10 ppm for volatile organic compounds. A solid-phase immunofluorescence assay system in a disposable cartridge was reported by Idetek Inc. *(21)*, with a determination range of 0-600 ppb BTEX. A borosilicate glass surface of capillary tubes was used as solid-phase and a cyanine dye was the fluorophore. Mallinckrodt Baker offer a test tube assay with antibodies coupled on magnetic particles *(22)*. The test range for BTEX in water is 0.12–18 ppm in water and 1.2–90 ppm in soil. Coring-System offer a BTEX tube test kit for soil analysis. The detection range is between 2–50 ppm depending on sample dilution *(23)*. Finally, an immunofiltration assay based on monoclonal antibodies is available from Merck. The working range in water is between 0.6–10 ppm *(24)*.

Materials and Methods

Synthesis of the immunogens, enzyme-tracers and coating antigens

For the first immunization a 6-phenylhexanoic acid (6-PHA, Sigma, Deisenhofen, Germany) was coupled covalently to KLH (keyhole limpet hemocyanine, Sigma) with the activated ester method *(25)*. The antisera obtained from this immunization (A) are noted as antiserum 1 and 2. Another two rabbits were immunized with a mixture of five different conjugates (6-PHA, *p*-tolyl acid and 2,5-, 3,4-, and 3,5-dimethylbenzoic acids), which were coupled first to 5-amino-n-pentanoic acid (Fluka, Deisenhofen, Germany), and then as before with the NHS/carbodiimide method to KLH (later designated as immunization (B) and antiserum 3 and 4). The enzyme conjugates were prepared by covalent attachment of 6-PHA, *p*-tolylbenzoylaminopentanoic acid and the three dimethyl benzoylaminopentanoic acids to horseradish peroxidase (HRP, Sigma) using the NHS/carbodiimid method. For the coating antigens the 6-PHA, *p*-tolylbenzoylaminopentanoic acid and three dimethyl benzoylaminopentanoic acids were coupled with the NHS/carbodiimide reaction to bovine serum albumin (BSA, Sigma). The hapten densities on the protein carriers were estimated with 2,4,6-trinitrobenzenesulfonic acid *(26)*. The comparison with the non-conjugated proteins gave the estimated percentage of coupling densities of 15% for KLH and 30–50% for the enzyme- and BSA-conjugates, based on the available amino functions of the carrier protein.

Preparation of polyclonal antibodies

The rabbits were immunized subcutaneously, intradermally and intramuscularly with 0.5 mg/mL KLH-conjugate mixed with complete Freund's adjuvant (Difco Labs, Detroit, USA). First booster immunization was given 8 weeks after priming with the incomplete Freund's adjuvant and then on a monthly basis, until the antibody response did not increase any more. For characterization the sera were tested every 4 weeks. Sera were stored at –20 °C or were used after storage at 4 °C with 0.1% NaN_3.

Direct and indirect competitive enzyme immunoassays

With the direct competitive assay format (Fig. 1A) microtiter plates (F-Form, Greiner, Frickenhausen, Germany) were coated overnight (16 h) at 4 °C with a dilution of 1:50,000 of polyclonal antiserum in coating buffer (35 mM $NaHCO_3$, 15 mM Na_2CO_3, 3 mM NaN_3, in 1 L pure water, pH = 9.6). 200-μL aliquots of the solution were added into each well of the plate. The plates were washed four times with buffer (43 mM KH_2PO_4, 375 mM K_2HPO_4, 873 mM NaCl, 30 mL Tween 20, in 1 L pure water, pH = 7.6) in an automated 8-channel-washer (washer, 812 SW1, SLT, Crailsheim, Germany). Free binding sites were blocked with a solution of 2% casein in PBS buffer (10 mM $NaH_2PO_4 \times 2\ H_2O$, 70 mM $Na_2HPO_4 \times 2\ H_2O$, 145 mM NaCl, in 1 L pure water, pH = 7.6) with 300-μL aliquots per well. The plates were shaken for 2 h at RT (microtiter plate shaker, EAS 2/4, SLT) or were stored for 24 h at 4 °C. After the final washing the plates were ready to use. A 100-μL aliquot of sample was added to each well. After 10 min pre-incubation, 100 μL of a 1:10,000 dilution of the enzyme conjugate was added. The incubation time was 30 min at RT and without shaking. The plates were washed and tetramethylbenzidine (TMB) substrate (1.5% TMB in methanol, 200 mM KH_2-citrate, 0.01% sorbic acid, 0.09% H_2O_2, in 1 L pure water, pH = 4.5) was added (150 μL per well) and incubated for 20 min. The reaction was stopped by the addition of 100 μL 5% sulfuric acid and absorbance was

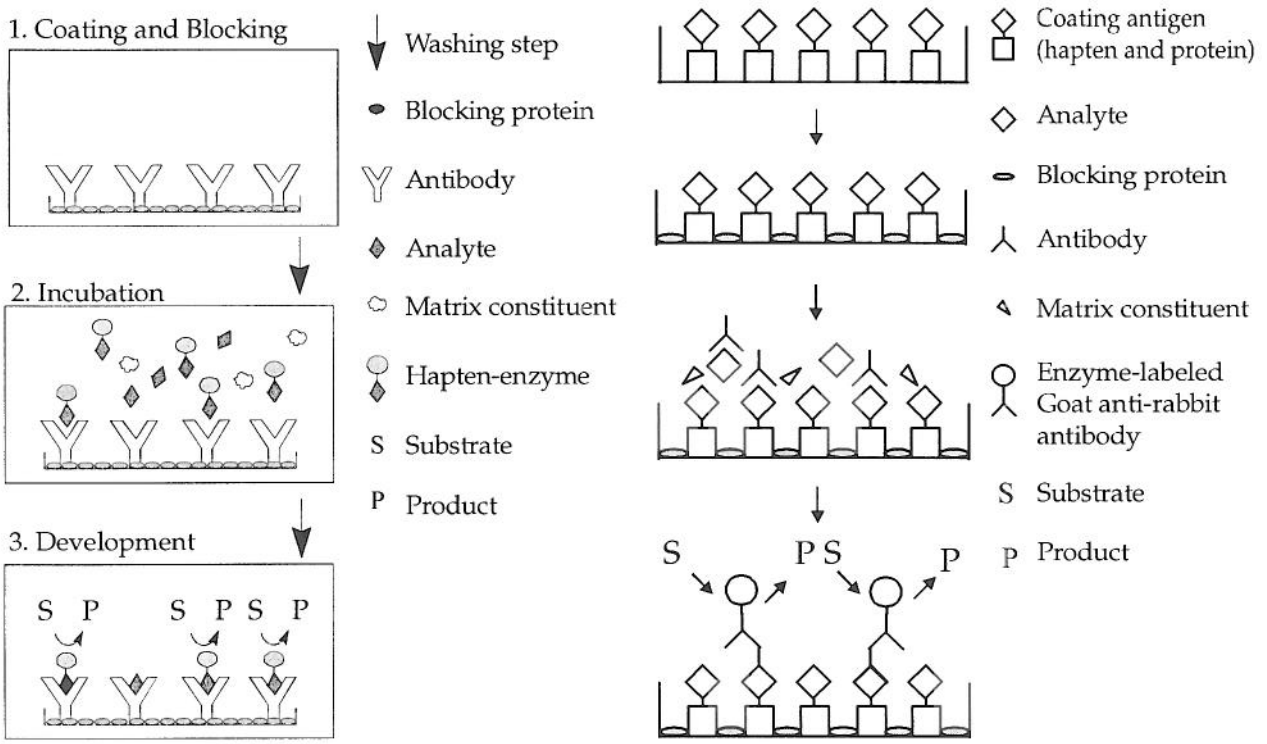

Fig. 1. Principle of the direct (A) and indirect (B) competitive enzyme immunoassay

read at 405 nm. All reagents were purchased from Merck (Darmstadt, Germany) if not specified otherwise.

The procedure of the indirect competitive enzyme immunoassay was described in detail elsewhere *(25)*. In short, the microtiter plates were coated with 0.01–0.025 μg/mL of 6-PHA-BSA or with other benzoylaminopentanoic acid-BSA conjugates (200-μL aliquots) in coating buffer overnight (16 h) at 4 °C. After washing, the plates were blocked with 2% casein in 300-μL aliquots. 100 μL of standard or sample and 100 μL of the antiserum (dilution 1:80,000) were transferred to each well of the microtiter plate. Incubation took place for 10 min at 4 °C. After washing, a goat anti-rabbit antibody labeled with HRP at a 1:20,000 dilution was added (150-μL aliquot) for 1 h. The plates were washed and the detection followed after the addition of substrate solution.

Development of a tube test design

The direct competitive microtiter plate test method on the microtiter plate was transferred to test tubes. The polystyrene tubes (Greiner) were incubated with 0.5 mL of antiserum dilution (1:20,000) in coating buffer overnight at 4 °C. After three-fold washing with distilled water and blocking with casein, the tubes were ready. In the competition step 2 mL of sample or standard were added and pre-incubated for 10 min. Then the enzyme conjugate was added into the tubes at a dilution of 1:10,000 and further incubated for 30 min. After washing with distilled water 2 mL of the substrate solution was added. The developing time was about 10 min, then the reaction was stopped with 5% H_2SO_4-solution, and finally aliquots were transferred to a microtiter plate for detection with a multichannel reader at 450 nm.

Results and Discussion

Comparison of different immunizations

Antisera obtained from two different immunization schedules were compared using the indirect ELISA format (Fig. 2). As can be realized from this figure, assay sensitivity was about one order of magnitude higher with antiserum 1 (immunization A) than with antiserum 3 (immunization B). Both sera were from bleedings performed after the same period of immunization.

It was noticed that the antiserum 3 was very sensitive to the original hapten dimethyl benzoic acid (IC_{50}: 8–10 μg/L). In contrast to that, the sensitivity (IC_{50}) of the antiserum 1 to 6-PHA was only 800 μg/L. For this reason we would consider that the heteroatoms in the direct neighbourhood of the aromatic moiety could be very important for antibody recognition. The influence of hydrogen bonds between heteroatoms for the antigen-antibody recognition was also discussed in the literature *(27–28)*.

Because of these results further experiments were carried out only with the antiserum 1 from immunization schedule (A).

Influence of organic solvents

Specially for hydrophobic analytes organic solvents are often used in immunoassays. Stöcklein and Scheller

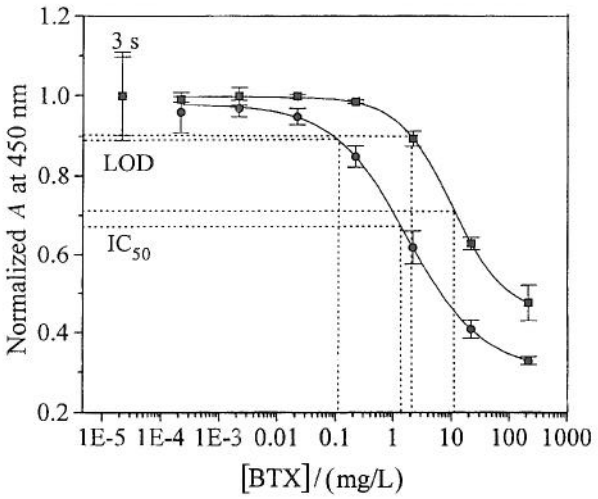

Fig. 2. Comparison of two different immunization schedules (n = 3, 1 s). ●Immunization (A): immunogen: 0.5 mg/mL 6-PHA-KLH; coating antigen: 0.01 μg/mL 6-PHA-BSA; blocking: 2% casein; antiserum 1:50,000; GAR (*g*oat *a*nti-*r*abbit antibodies)-HRP: 1:25,000; 14 min. IC_{50}: 1.3 mg/L BTX, LOD (3 s): 0.1 mg/L. ■ Immunization (B): immunogen: 0.5 mg/mL of a mixture of 6-PHA-KLH, *p*-tolylaminopentanoic acid-KLH, and *o*-, *m*-, *p*-dimethyl benzoylaminopentanoic acid-KLH; coating antigen: 0.0025 μg/mL *o*-dimethyl benzoylaminopentanoic acid-BSA; blocking: 2% casein; antiserum 3: 1:100,000; GAR-HRP: 1:25,000; 25 min. IC_{50}: 11 mg/L, LOD (3 s): 2.1 mg/L.

(*29*) reported on a change of the specificity (or cross-reactivity) of monoclonal anti-atrazine antibodies by the addition of 10 to 20% water-miscible organic solvent to the assay buffer. These observations could be explained by structural variation of antibody pleating. Further, each protein in the immunoassay could be influenced by an organic solvent. For comparison we performed several indirect competitive assays with zero-blanks (without BTX analytes) but with different concentrations of organic solvent. With this approach the influence of different solvents on the antibody and/or the hapten-protein conjugate was investigated.

As demonstrated in Fig. 3A, the organic solvents acetonitrile, acetone, dimethyl sulfoxide and dimethylformamide exhibit a similar behavior but to a different extent. First, the absorption in the test was only slightly affected until a solvent concentration in the samples of about 50% was reached. Higher concentrations such as 75% and 100% (final concentration in the test 37.5% and 50%) lead to an extreme increase in absorption. It is known that organic solvents can have a denaturing effect on antibodies, regardless of whether they are in solution or in the immobilized state, and can therefore only be used within a narrow range of concentrations *(30)*.

In contrast to that, another trend was observed with methanol and ethylene glycol (Fig. 3B). Even a slight increase of methanol had an effect on the absorption in the assay. This rise is different compared to acetonitrile, acetone, dimethyl sulfoxide and dimethylformamide. For this reason, methanol interferes much more than the other solvents tested so far. Interestingly, ethylene glycol shows a contrary influence: with increasing solvent content the optical density was decreased. The effect of organic solvents differs from antiserum to antiserum and has to be examined separately *(31–35)*. However, from the results outlined in Figure 3A and 3B an effect on different proteins (antibodies, coating antigen) cannot be predicted.

In addition, hydrophobic less water-miscible and volatile analytes will dissolve better in organic solvents and thus volatility could be reduced. To investigate the effect of organic solvents on the assay sensitivity, BTX--standards were prepared in different solutions containing 10% of organic solvent. It could be demonstrated that 10% of dipolar aprotic DMSO led to a minor but significant increase of the sensitivity of the ELISA *(25)*.

One problem, which was observed with the BTX immunoassay, is the relatively high base value of the sig-

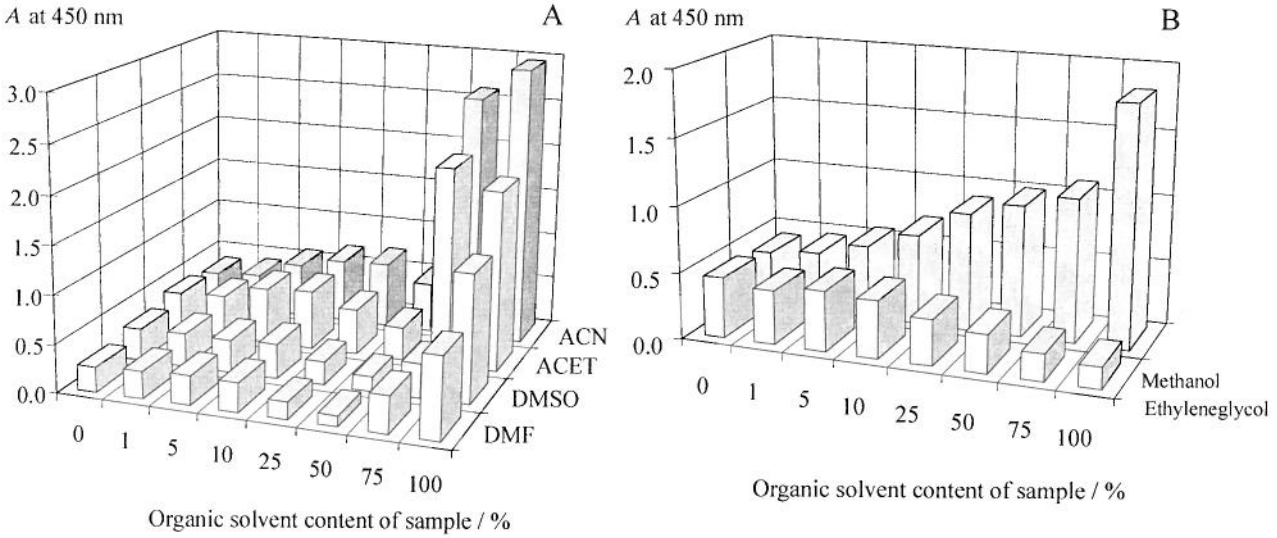

Fig. 3. Effect of different organic solvents on the indirect competitive ELISA (n = 3). Antiserum 1: 1:80,000; GAR-HRP: 1:20,000; 10 min: ACN = acetonitrile, ACET = acetone, DMSO = dimethyl sulfoxide and DMF = dimethylformamide.

moidal ELISA calibration curve. In general, this value should not be higher than 15% of maximum absorption *(36)*. In the past it was demonstrated that after the addition of polar or dipolar aprotic solvents the base value was reduced continuously *(25)*. Fig. 4 shows an ELISA calibration curve with different concentrations of BTX in pure water which exhibits a base value of 34%. In this figure a calibration curve prepared with BTX standards containing 10% of acetonitrile was also integrated. The center point of the calibration curve prepared in pure water was slightly better but the base value of the calibration curve in organic solvent was significantly lower. It is supposed that with the organic co-solvent the analytes could be retained much better in solution, especially with the highest concentrated solutions.

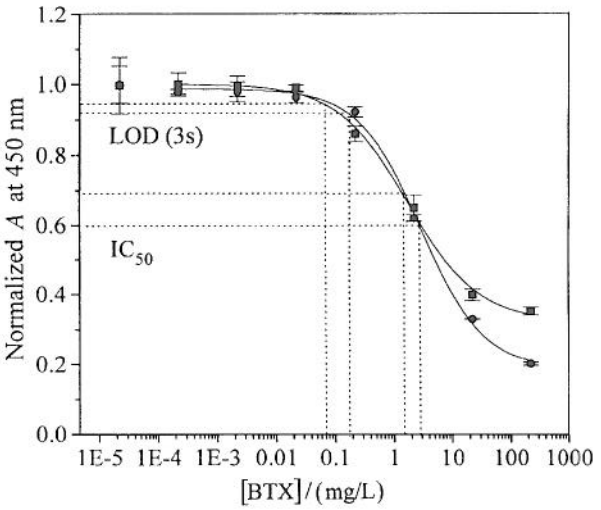

Fig. 4. The influence of organic solvents on the indirect competitive enzyme immunoassay sensitivity (n = 3, 1 s). Coating antigen: 0.025 μg/mL 6-PHA-BSA; blocking: 2% casein; antiserum 1: 1:80,000; GAR-HRP: 1:20,000; 15 min.
■ BTX standards in pure water (IC_{50} : 1.7 mg/L; LOD (3 s): 70 μg/L; base value: 34.4%). ● BTX standards in 10% ACN (IC_{50}: 2.9 mg/L; LOD (3 s): 0.2 mg/L; base value: 21.2%).

Variation of temperature and covering

It is very likely that the volatility of the analytes may be an important problem in the microtiter plate test design, because of the high surface/volume ratio. Especially the vapour pressure of benzene with 95.2 mm Hg at 20 °C is very high *(15)*. To minimize this interference the temperature during the competitive step of the assay procedure was reduced to 4 °C by incubation in the refrigerator. These plates were compared to those which were incubated at RT. Additionally, the influence of an adhesive plate sealer (Parafilm®), on the assay performance often used to avoid contamination or spill during the assay procedure, was investigated. So, plates which were covered during the competition step with the adhesive sealer were compared to those without a sealer. Experiments were carried out at 4 °C and RT (Fig. 5).

Each experiment was done at least is triplicate. It is shown that the best results are yielded at 4 °C. Similar results were reported by Piasiao *et al. (20)*. They developed a quantitative immunoassay for these analytes in a test tube format and the best competition took place at a temperature between 2 and 8 °C. As expected from theory, lower temperature will reduce the vapour pressure and thus loss of analytes from the incubation mixture. Further, the results show that there was no significant difference whether the competitive step of the assay was done with or without Parafilm®-covering of the plate. However, at both tested temperatures sensitivity of the ELISA was slightly higher without Parafilm®. For this reason in the present study the incubation step at 4 °C and without plate covering was also adopted in the indirect ELISA.

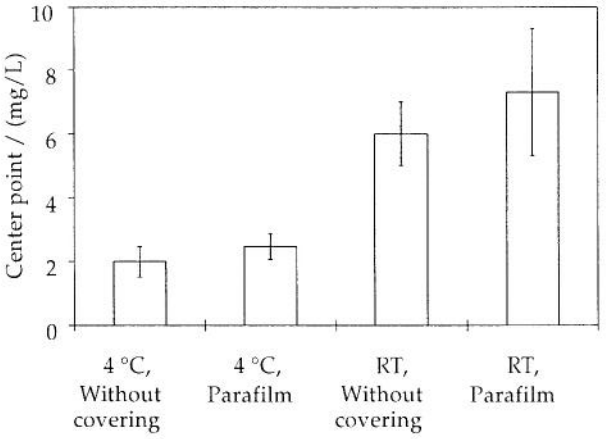

Fig. 5. Comparison of temperature and microtiter plate covering during the competition step in the direct ELISA (n = 3, 1 s). Antiserum 1: 1:50,000; blocking: 2% casein; 6-PHA-HRP: 1:10,000; 20 min.

Influence of pre-incubation time

In some cases pre-incubation of antibody and sample analyte results in better assay sensitivity *(37,38)*. Whether this is also true for the direct microtiter plate BTX-ELISA was studied as follows. BTX standards were filled into the wells of antibody coated plates and then, after different pre-incubation intervals (1 to 60 minutes) the enzyme tracer was added. Further steps were done as described above. As demonstrated in Fig. 6 pre-incubation had no favourable effect. On the contrary, longer pre-incubation times led to lower sensitivity, obviously caused by the loss of analyte through volatilization.

Influence of surfactants

The influence of surfactants was examined with 35 mg/L Triton X-100 (Merck) and 220 mg/L Genapol C 080 (Hoechst, Frankfurt, Germany). Applied concentration was about the critical micellar concentration (CMC) or ten-fold CMC-concentration *(39)*. The two non-ionic compounds were mixed with BTX standards at various concentrations to detect possible interference of these surfactants with the immunoassay or whether it can be used favourably to retain the volatile analytes in the solution (Fig. 7). Both surfactants led to a shift of the calibration curve to higher concentrations and to higher

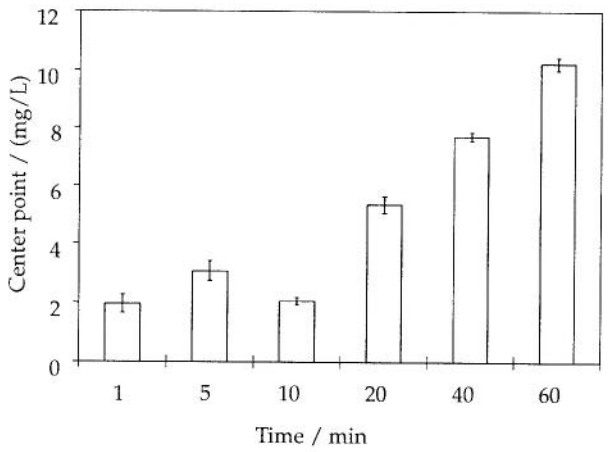

Fig. 6. Influence of pre-incubation time on the direct competitive assay (n = 3, 1 s). Antiserum 1: 1:50,000; blocking: 2% casein, 6-PHA-HRP: 1:10,000, 20 min.

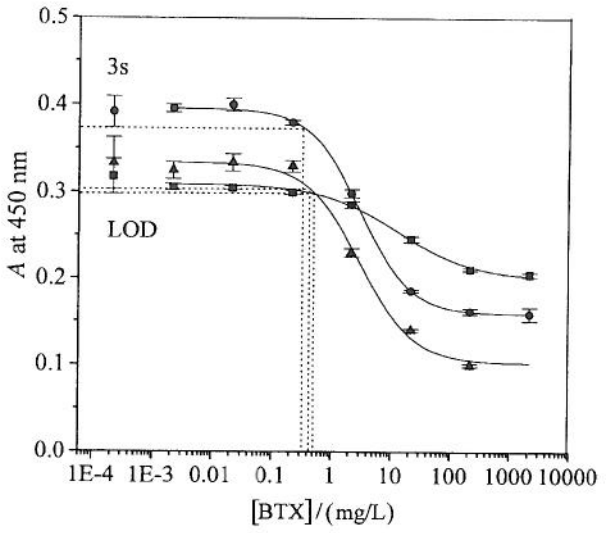

Fig. 7. Influence of surfactants on the indirect BTX-ELISA (n = 4, 1 s).
Coating antigen: 0.01 μg/mL 6-PHA-BSA; blocking: 2% casein; GAR-HRP: 1:20,000; 15 min.
● BTX standards in Triton X-100; IC_{50}: 3.1 mg/L; LOD (3 s): 0.3 mg/L; ▲ BTX standards in water; IC_{50}: 3 mg/L; LOD (3 s): 0.4 μg/L. ■ BTX standards in Genapol C 080; IC_{50}: 13 mg/L; LOD (3 s): 0.5 mg/L.

base values, indicating the encapsulation of the target analytes into micelles and thus its withdrawal from antibody binding reaction.

Yarmush *et al. (40)* had already discussed that surfactants could also have a detrimental effect on the protein structure, particularly when the solvating water which is necessary for bioactivity is lost. As a result, decreased antibody activity leads to lower assay sensitivity. In the present case, the non-ionic linear polyether showed a stronger effect on sensitivity and base value than Triton X-100. In contrast, Triton X-100 led to a higher maximum absorbance. Summarizing, the inclusion of surfactants in the sample had no beneficial effect on the BTX-ELISA but rather interferred with the assay.

Inter- and intra-assay variation

With the optimized incubation time, temperature and without plate covering the reproducibility of the indirect and direct competitive immunoassays was studied. It was based on a standard set containing 0.002 to 220 mg/L of BTX run 12-fold (indirect ELISA) or 4-fold (direct ELISA) on the same day (intra-assay) and on 5 plates on 5 different days (inter-assay of both indirect and direct ELISAs). The results are outlined in Table 3. The intra-assay coefficients of variation (CVs) ranged from 1.2 to 3.4% in the indirect ELISA. In the direct ELISA the CVs of the intra-assay were very similar: 1.5–4.1%. Inter-assay CVs were between 3.5 and 8.2% in the indirect immunoassay and between 2.5 and 8.4% in the direct ELISA. Variations were lowest at the center point of the calibration curve with (1.7 ± 0.5) mg/L in the indirect or with (4.2 ± 1) mg/L in the direct ELISA.

It is shown by these results that there is only a minor difference in reproducibility between the two assay formats. Because of some higher sensitivity of the indirect immunoassay this design is preferred when using microtiter plates.

Transfer of the microtiter plate assay to test tubes

One possibility of reducing the loss of highly volatile analytes during the sample incubation step in the immunoassays is to lower the surface/volume ratio. This can be done much better in tube assays compared to the microtiter plates. In this case, the direct ELISA format was used as it requires less incubation and washing steps. So 5-mL polystyrene tubes were coated with 0.5 mL of antiserum dilution. The sample and enzyme tracer were added in a volume of 2 mL each and incubated

Table 3. Intra- and inter-assay CVs for the indirect and direct competitive BTX-ELISAs

BTX-conc./(mg/L)	Indirect ELISA		Direct ELISA	
	CV/% Intra-assay (n = 12; 1 s)	CV/% Inter-assay (n = 3; 1 s)	CV/% Intra-assay (n = 4; 1 s)	CV/% Inter-assay (n = 3; 1 s)
Blank	3.4	3.5	3.8	7.9
0.0002	2.8	6.9	1.8	6.7
0.002	1.6	5.0	2.3	8.4
0.02	3.3	5.7	2.3	7.4
0.2	1.2	5.6	1.5	2.5
2.2	2.0	8.2	2.4	3.5
22	2.1	4.5	4.1	4.9
220	3.3	4.2	2.3	5.4

(open vial) for 30 min at RT. After washing, 0.5 mL of substrate were added and the enzymatic reaction was stopped after about 15 min. 200-μL aliquots (n = 3) were transferred to a microtiter plate and the absorbance was read with an automatic reader. As demonstrated in Fig. 8, a typical sigmoidal ELISA calibration curve can easily be obtained with the test tube assay.

The intra-assay CVs (measured on the same day) of the standards in the tube assay have been higher compared to the microtiter plate assay (2.2–8.4% in the test tube assay compared to 1.5–4.1% in the direct microtiter plate assay). The same effect occurs for the inter-assay CVs (measured at different days) at a considerably higher rate (15.3–49.2% in the test tube assay compared to 2.5–8.4% in the direct microtiter plate assay). This points to problems in handling higher numbers of tubes such as unreproducibility of washing (hand washing) and timing (Fig. 8 and Table 4).

Further, from Fig. 8 it can be seen that the base value was decreased in the test tube format (13% of minimum absorption in the test tube assay *vs.* 34% of minimum absorption in the microtiter plate assay). It can be speculated that more analyte will be retained by a smaller surface/volume ratio. Because of lower reproducibility the tube assay should be used only as a semi-quantitative test for rapid on-site screening to differentiate between positive and negative samples.

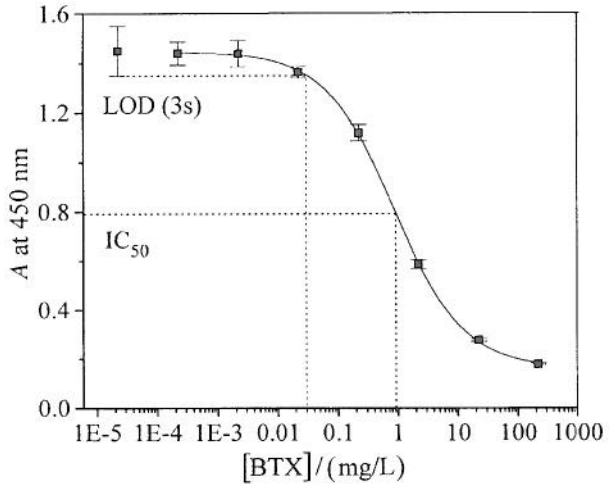

Fig. 8. Test tube ELISA in the direct format (n = 3, 1 s). Antiserum 1: 1:10,000; 6-PHA-HRP: 1:5,000; 20 min (IC_{50}: 0.9 mg/L; LOD (3 s): 30 μg/L).

Table 4. Intra- and inter-assay coefficients of variation (CVs)

BTX-conc./(mg/L)	CV/% Intra-assay (n = 3, 1 s)	CV/% Inter-assay (n = 4, 1 s)
Blank	2.8	20.8
0.0002	8.4	15.3
0.002	5.7	37.9
0.02	2.2	32.3
0.2	8.0	36.6
2.2	2.3	43.4
22	5.9	40.8
220	6.1	49.2

Conclusions

The development of an enzyme immunoassay for the analytes benzene, toluene, and xylenes (BTX) were examined in the indirect and direct microtiter plate method. Both methods showed comparable assay performance. The indirect ELISA gave slightly better sensitivity (indirect ELISA: IC_{50}: (1.7 ± 0.5) mg/L; direct ELISA: IC_{50}: (4 ± 1) mg/L). Several efforts were made to increase assay sensitivity and performance. The immunization with a 6-PHA-KLH resulted in an antiserum with higher affinity compared to the immunization with a mixture of 6-phenylhexanoic acid-KLH, *p*-tolylaminopentanoic acid-KLH and derivatives of dimethyl benzoylaminopentanoic acids-KLH. Because of the high volatility of the target analytes the parameter temperature and incubation time during the competitive step were changed. Best results were obtained using an incubation temperature of 4 °C (refrigerator) and no plate sealer. Further, pre-incubation of antibodies with sample analytes had no positive effect on assay sensitivity. The investigation of different organic solvents revealed that the addition of a 10% organic co-solvent obviously reduced loss of analyte through volatilization. In contrast, the addition of non-ionic surfactants had no benefitial effect, but rather interfered with the assay. Further, a direct competitive tube ELISA could be developed. With this design, the volatility of the analytes could be reduced and the format can be used for semiquantitative on-site screening. Present efforts are directed toward the development of immunological dip-sticks for BTX. Although of lower sensitivity, this design would be even faster and simpler compared to a tube test, and thus well suited for field screening applications.

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Problemi pri razradi imunokemijskog određivanja malih i hlapljivih molekula – benzen, toluen i ksilen (BTX)

Sažetak

Na osnovi poliklonskih antitijela razvijeni su izravni i neizravni postupci enzimskog imunokemijskog određivanja hlapljivih organskih spojeva, benzena, toluena i ksilena. Pri neizravnom postupku ELISA granica detekcije za pet ispitivanih spojeva (u ekvimolarnoj količini) u vodi iznosila je 210 μg/L, a vrijednost središnje točke na kalibracijskoj krivulji bila je 1,7 mg/L. Zbog hlapljivosti ispitivanih spojeva bile su potrebne specijalne mjere opreza. Najbolji uvjeti za provedbu određivanja nađeni su pri inkubaciji na 40 °C te u nepokrivenim reakcijskim posudama (mikrotitarske ploče ili epruvete) s Parafilmom® tijekom kompetitivne faze. Vrijeme inkubacije od 10 minuta bilo je prikladno i davalo je dobru osjetljivost. Dodatkom 10% dipolarnog aprotičkog organskog otapala ispitivani organski spojevi mogli su se čuvati u vodi, a snizila se osnovna vrijednost kalibracijske krivulje. Dodavanjem neionskih površinsko-aktivnih spojeva nije se poboljšala stabilnost imunokemijskog određivanja, a dodatak Genapola C 080 ili Tritona X-100 omeo je postupak ELISA. Manje povećanje osjetljivosti (IC_{50}: 0,9 mg/L) postignuto je promjenom mjesta određivanja s mikrotitarskih ploča u epruvete, ali uz određeni gubitak reproducibilnosti. Postupak ELISA u epruveti može se primjenjivati za semikvantitativni način ispitivanja.