Direct sub-ppt detection of the endocrine disruptor ethinylestradiol in water with a chemiluminescence enzyme-linked immunosorbent assay

Christian Schneider a, Heinz F. Schöler a, Rudolf J. Schneider b,∗

a Institute of Environmental Geochemistry, University of Heidelberg, Im Neuenheimer Feld 236, D-69120 Heidelberg, Germany
b Institute of Plant Nutrition, University of Bonn, Karlsruher Kreisstrasse 11, D-53113 Bonn, Germany

Received 6 May 2005; received in revised form 7 July 2005; accepted 13 July 2005

Available online 22 August 2005

Abstract

A chemiluminescence ELISA for the direct detection of ethinylestradiol (EE2) in water at sub-ppt levels was developed and validated. At a signal-to-noise ratio of three the detection limit is 0.2 ± 0.1 ng L⁻¹, at a ratio of 10 the LOQ is found to be 1.4 ± 0.8 ng L⁻¹. Based on a conservatively calculated precision profile the analytical working range is established from 0.8 to 100 ng L⁻¹. The ELISA was tested in four different matrices, including surface water and effluent of sewage treatment plants. All measurements were validated using an LC–MS/MS method. Typical results were consistent in both methods below 1 ng L⁻¹. Using this chemiluminescence ELISA facilitates for the first time the direct detection of EE2 at ecotoxicologically relevant concentrations.

© 2005 Elsevier B.V. All rights reserved.

Keywords: Chemiluminescence; ELISA; Ethinylestradiol; Sub-ppt concentrations; Direct measurement; Optimization

1. Introduction

Endocrine disrupting effects observed in the aquatic ecosystem have stimulated broad scientific and public interest. First studies already began in the 1970s when adverse effects of synthetic estrogens were discussed for the first time [1]. Research was intensified in the early 1990s with the advent of reproductive problems in some freshwater fish populations [2,3].

One of the most potent estrogenic hormones is the synthetic steroid ethinylestradiol (EE2). It has been shown that EE2 induces feminization in immature cyprinids at concentrations of 10 ng L⁻¹ in water and in rainbow trout at levels of 0.1 ng L⁻¹ [2]. In a thorough full life-cycle study using fathead minnow a NOEC value of 1 ng L⁻¹ was established for EE2 [4]. Therefore there is an urgent need for measurements in the lower nanogram per litre range.

EE2 and other estrogenic hormones are usually quantified in aqueous matrices using standard instrumental methods such as gas chromatography–mass spectrometry or high performance liquid chromatography–mass spectrometry. Current GC–MS/MS and LC–MS/MS methods achieve detection limits below 1 ng L⁻¹ after sample enrichment [5–7]. In addition to this enrichment that might cause problems with recovery, these chromatographic methods require an extensive sample clean-up due to ionization suppression. Enrichment and clean-up steps are by themselves error-prone and can lead to decreased precision and accuracy of results.

Alternatively immunoassays can be used for quantitation of EE2 in the lower nanogram per liter range. Several immunoassays based on RIA and ELISA formats have been developed for measuring EE2 in body fluids [8–14]. In recent years immunoassays for monitoring EE2 in environmental samples have been introduced, too [15–17]. Although most
of the latter assays exhibit improved sensitivity, none of them is capable of measuring in the ecotoxicologically relevant sub-ppt concentration range directly. In our work we present for the first time the development and optimization of an ELISA for the direct measurement of EE2 in the sub-ppt range.

2. Materials and methods

2.1. Reagents and materials

All reagents were of analytical or biochemical grade and were used as received. Horseradish peroxidase (E.A grade) was obtained from Roche (Mannheim, Germany), 1,3,5(10)-estratrien-17α-ethinyl-3,17-diol-6-one-6-carboxymethyl-oxime (EE2-6-CMO) was from Steraloids (London, UK), GuardianTM and SuperSignalTM (Fmno) was from Perbio (Bonn, Germany) 3,3',5,5'-Tetramethylbenzidine (TMB) and TweenTM 20 were purchased from Serva (Heidelberg, Germany), sodium azide was from VWR (Darmstadt, Germany), ultrapure water was obtained by running demineralized water through a Milli-Q water purification system from Millipore (Eschborn, Germany), N,N-Dimethylformamide, N-methylmorpholine, isobutylchloroformiate, ethenylstradiol, estradiol, tris(hydroxymethyl)aminoethane (Tris) and all buffer salts were supplied by Sigma–Aldrich (Taufkirchen, Germany). All metabolites of estradiol and ethinylestradiol were from Steraloids (London, UK). Sephadex columns were from Amersham Biosciences (Freiburg, Germany). Humic acid was from Carl Roth (Karlsruhe, Germany). The development of the polyclonal antibody and the preparation of the buffers have been described before [15], the TMB-based substrate (TMB-solution: 41 mM TMB, 8 mM tetrabuty-lammoniumborohydride in N,N-dimethylacetamide) was developed by Frey et al. [18]. The actual substrate solution consists of 540 μL TMB-solution in 21.5 ml substrate buffer (200 mM citric acid monopotassium salt buffer containing 3 mM hydrogen peroxide and 0.01% sorbic acid potassium salt, pH 3.8) and was freshly prepared for each run. Microtiter plates used were white and transparent 96 flat-bottom wells with high binding capacity (MaxiSorptTM and FluoroNuncTM from Nunc (Roskilde, Denmark). Washing steps were carried out using an automatic plate washer (Flexiwash, Asys Hitech, Eugendorf, Austria). Absorbance was measured at 450 nm and referenced to 650 nm with a plate reader (Vmax®, Molecular Devices, Munich, Germany). Luminescence was detected between 380 and 630 nm using a MicroLumat Plus LB 96V (Berthold Technolo-gies, Bad Wildbad, Germany). Filters were obtained from Macherey–Nagel (Düren, Germany) and Schleicher and Schuell (Dassel, Germany). Deuterated Estradiol (2,4,16,16-D4) and deuterated estrone (2,4,10,16-D4) were from CDN Isotopes (via Dr. Ehrenstorfer, Augsburg, Germany), respectively.

2.2. Preparation of enzyme conjugate

Horseradish peroxidase (POD) was coupled to the 6-CMO derivative of EE2 using modifications of a method described for coupling POD and derivatives of progesterone [19]. The oxime (0.958 mg EE2-6-CMO, 2.5 μmol) dissolved in 50 μL N,N-dimethylformamide in the presence of 0.4 μL N-methylmorpholine was activated with 0.4 μL isobutylchloroformiate under inert gas at –15°C. After stirring for three minutes this solution was slowly added to 2.5 mg POD (ca. 5.7 x 10−3 μmol with M = ca. 44,000 g mol−1) dissolved in a mixture of 25 μL water and 15 μL DME cooled to the same temperature. The reddish solution was stirred for 1 h and allowed to reach 0°C, using an ice-bath. Stirring continued for another 2 h at 0°C, after which the solution was directly poured on a Sephadex G-25 column previously conditioned with TBS (pH 7.5, 0.1 M Tris, 0.15 M NaCl). The same buffer was used for elution of the enzyme conjugate. Fractions were collected in a microtiter plate and their absorbance measured photometrically at 405 nm. Fractions of highest absorbance values were pooled, mixed with an equal amount of POD stabilizer (GuardianTM), aliquoted and stored at 4°C. Using –15°C as the initial temperature and 0°C for the termination of the reaction enables kinetic control in the activation of the steroid and coupling to lysine residues of POD. Of the total six lysine amino groups present in POD due to steric restrictions only three of them are accessible for coupling reactions. Conditions described above, using a 44-fold excess amount of oxime lead to a quantitative coupling to POD, resulting in a steroid to enzyme ratio of 3:1, as described in literature [20–22]. Thus the initial concentration of enzyme conjugate is estimated to be 6 x 10−7 mmol L−1.

2.3. Immunoassay procedures

Both immunoassays, the EE2 ELISA utilizing TMB and the EE2 CLEIA using SuperSignalTM Fmno as substrate, are based on the polyclonal antibody and the enzyme conjugate described above. The direct competitive ELISA format using sequential saturation was adapted for both assays [23]. The EE2 ELISA was performed as follows. Transparent microtiter plates were coated with the polyclonal antibody (dilution 1:200,000, 200 μL per well) in coating buffer (50 mM sodium carbonate buffer, pH 9.6). The plates were covered with Parafilm® to prevent evaporation. After overnight incubation at 20°C, the plates were washed three times with PBS 1 (10 mM PBS pH 7.6, containing 150 mM NaCl and 2.85% TweenTM 20) using the automatic plate washer. For construction of the calibration curve an EE2 stock solution was prepared in methanol and then further diluted with ultrapure water to obtain calibration solutions. After the three-cycle washing step the plates had EE2 standards added (100 μL per well) and were shaken at room temperature for 30 min. This was followed by the addition of the enzyme conjugate (dilution 1:50,000, 100 μL per well) in PBS 2 (8 mM PBS pH 7.6, containing 145 mM NaCl); the plates were shaken at room
temperature for 10 min, followed by a second three-cycle washing step. Finally, substrate solution was added (200 μL per well) and incubated for 30 min. The enzyme reaction was stopped by addition of sulfuric acid (1 M, 50 μL per well).

The EE2 CLEIA was performed as follows. White microtiter plates (FluoroNunc™) were coated according to the ELISA procedure using a serum dilution of 1:200,000. EE2 standards (100 μL per well) were incubated for 30 min, followed by a 10 min incubation together with the enzyme conjugate (dilution 1:50,000; 100 μL per well). After incubation plates were washed and placed in the luminometer. Two hundred microlitres of substrate were used per well, measurements were performed before addition of substrate (blank), immediately after addition and after a 3 min incubation time. Calibration curves were calculated using the difference of the third and the first measurement.

All determinations were at least made in triplicate on different plates. The mean values were fitted to a four-parametric logistic equation (4PL) [24], optical densities and relative light units were normalized according to Eq. (1) and plotted against the logarithmic concentration.

\[
N_N = \frac{Y - D}{A - D} \times 100 \quad (1)
\]

\[Y_N\] is the normalized optical density (OD) or the normalized relative light unit (RLU, respectively), \(Y\) is OD (RLU, respectively), \(D\) is a parameter of the 4PL ("upper asymptote" representing the OD (RLU, respectively) at infinitely low analyte concentration, \(A\) is the parameter of the 4PL (describing the OD (RLU, respectively) at infinitely high analyte concentration, the "lower asymptote", which is different from zero due to intrinsic optical density of substrate and microtiter plate and unspecific binding of the tracer).

2.4. Cross-reactivity determination

The relative sensitivity of the immunoassay towards other steroid hormones was determined for estradiol and estrone by assaying a dilution series of each substance in water. Cross-reactivity is calculated as the ratio of molar concentrations of the cross-reacting compound at its inflection point.

\[
CR = \frac{C_{\text{standard}}}{C_{\text{test}}} \times 100 \quad (2)
\]

where \(CR\) describes the cross-reactivity in percent, \(C_{\text{standard}}\) is a parameter of the 4PL giving the EE2 concentration at the inflection point and \(C_{\text{test}}\) refers to the concentration of the cross-reacting compound at its inflection point.

2.5. Matrix interference

Matrix constituents can be part of real samples and eventually affect antibody or enzyme performance. Effects were studied using serial dilutions of EE2 in solutions of commercial humic acid as interfering agent. Concentrations of humic acid covered ranged from 0.2 to 5 mg L\(^{-1}\).

2.6. Water samples

Water samples were collected in brown glass bottles, stored at 4 °C and analyzed within 24 h. Two spiked samples were prepared using ultrapure water and tap water (of origin 1/3 purified surface water and 2/3 groundwater). Eight surface water samples were taken at the river Rhine and two of its tributaries in Rhineland-Palatinate (Ahr) and North Rhine-Westphalia (Achter). Four effluent samples were taken from different municipal sewage treatment plants (STPs) in the surroundings of Bonn and Cologne.

2.7. Sample preparation

For CLEIA measurements surface water and effluent samples were passed subsequently through folded filter papers (Macherey-Nagel, ref. no. 619) and by vacuum through glass fibre filter GF 8 (Schleicher and Schuell, ref. no. 370120). For LC–MS/MS measurements all samples were enriched using solid phase extraction following a method described by Zühlke et al. [25].

2.8. LC–MS/MS procedure

LC–MS/MS determinations were run by the Institute for Hygiene and Public Health, University of Bonn. The HPLC system consisted of an Agilent 1100 system (Agilent, Böblingen, Germany) equipped with APCI source operated in the negative ionization mode. Gradient elution was applied using acetonitrile and water as solvents. Mass spectrometric measurements were performed using an API 2000™ (Applied Biosystems, Darmstadt, Germany) equipped with APCl source operated in the negative ionization mode. The method is an adaptation of a method described in literature [25]. The detection limits for EE2 after internal correction for the surrogate standards \(d_4\)-estrone and \(d_4\)-estradiol was established at a signal-to-noise ratio of three at 0.3 ng L\(^{-1}\).

3. Results and discussion

3.1. Assay optimization

Both immunoassays were optimized with regard to sensitivity. The initial serum dilution of 1:50,000 was increased to 1:200,000. Incubation time of the enzyme conjugate was varied from 10 to 180 min (see Table 1), the serial dilution of the conjugate in PBS 2 extended to 1:500,000 (see Table 2). Based on the optimized conditions, the EE2 calibration curve was constructed in the concentration range of 0.001–1000 ng L\(^{-1}\). A typical calibration curve and the
Table 1
Assay optimization: photometric ELISA

<table>
<thead>
<tr>
<th>Incubation time of enzyme conjugate (min)</th>
<th>10</th>
<th>30</th>
<th>60</th>
<th>180</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameter A&lt;sub&gt;a&lt;/sub&gt; (OD)</td>
<td>0.06 ± 0.001</td>
<td>0.32 ± 0.01</td>
<td>0.38 ± 0.01</td>
<td>0.51 ± 0.003</td>
</tr>
<tr>
<td>Parameter C&lt;sub&gt;a&lt;/sub&gt; (ng L&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.10 ± 0.01</td>
<td>0.51 ± 0.05</td>
<td>0.79 ± 0.11</td>
<td>149 ± 5.0</td>
</tr>
<tr>
<td>LOD (ng L&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.02 ± 0.02</td>
<td>0.06 ± 0.004</td>
<td>0.14 ± 0.01</td>
<td>2.3 ± 2.8</td>
</tr>
</tbody>
</table>

Influence of the tracer incubation time on signal intensity and test sensitivity using an antiserum dilution of 1:200,000 and a tracer dilution of 1:50,000.

* cf. Eqs. (1) and (2).

Table 2
Assay optimization: chemiluminescence enzyme immunoassay (CLEIA)

<table>
<thead>
<tr>
<th>Tracer dilution</th>
<th>1:500,000</th>
<th>1:250,000</th>
<th>1:50,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameter A&lt;sub&gt;a&lt;/sub&gt; (RLU 10&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>38.0 ± 9.3</td>
<td>114 ± 24</td>
<td>469 ± 136</td>
</tr>
<tr>
<td>Parameter C&lt;sub&gt;a&lt;/sub&gt; (ng L&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>7.39 ± 4.18</td>
<td>20.2 ± 4.5</td>
<td>102 ± 13</td>
</tr>
<tr>
<td>LOD (ng L&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.17 ± 0.10</td>
<td>0.77 ± 1.1</td>
<td>9.4 ± 4.6</td>
</tr>
</tbody>
</table>

Influence of the tracer dilution on signal intensity and test sensitivity using an antiserum dilution of 1:200,000 and a tracer incubation time of 10 min.

* cf. Eqs. (1) and (2).

corresponding precision profile [24] is compared to a calibration curve under suboptimal conditions in Fig. 1. Results of the optimization process can be clearly identified in the calibration curve shifted to lower analyte concentrations. The LOD of the optimized test at a signal-to-noise ratio (S/N) of 3 is found to be 0.2 ± 0.1 ng L<sup>-1</sup>, the LOQ (S/N of 10) is 1.4 ± 0.8 ng L<sup>-1</sup> calculated from eight individual calibration curves. The analytical working range of the optimized assay extends with a reasonable precision (CV < 20%) from 0.8 to 100 ng L<sup>-1</sup> making the assay usable over more than two orders of magnitude in analyte concentration.

3.2. Specificity of the antiserum

Specificity of the antiserum was tested in the EE2 CLEIA by determination of the molar cross-reactivities for estradiol and estrone, steroids frequently occurring in STP effluents. Cross-reactivity of an antiserum is characterized by intrinsic properties of the antibodies and binding kinetics applied during incubation of sample, enzyme conjugate and antiserum. Sequential saturation methods working under non-equilibrium conditions are to be distinguished from equilibrium type assays [26]. This assay and a Bio-LC EE2 ELISA described before [15] are both non-equilibrium type assays using the same antiserum. The values for estradiol were 0.2% in both the CLEIA and the Bio-LC-ELISA and for estrone were 0.1% (CLEIA) and <0.1% (Bio-LC-ELISA), respectively [15]. The antiserum proved highly specific for EE2, exhibiting only negligible reactivities for structurally similar compounds. Discrimination of these compounds can be explained through superior steric requirements of the IgG populations and the respective binding pockets involved in recognition and binding of the analyte.

3.3. Matrix interference

Humic acid was used as a model substance for the determination of matrix effects. The assay exhibited a rather robust behaviour in the presence of humic acids. In contrast to similar studies in literature the sigmoidal shape of the calibration curves could be maintained at elevated concentrations of humic acids [27]. However, curves were slightly shifted to higher concentrations of EE2 resulting in some loss of sensitivity. Effects are shown in detail in Fig. 2. The humic acid used in this study is produced from pulverized lignite, humic substances present in water samples might have a different and more complex composition than this commercially available one, though.

3.4. Water samples

The optimized CLEIA was tested with four different sample matrices: ultrapure water, tap water, surface water and effluent of sewage treatment plants. All samples were run at least in quadruplicate. Adsorption of EE2 to filters used during sample preparation was not observed, moreover use of these filters is a common procedure frequently applied as sample clean-up [6,28]. Relative standard deviations were
below 10%. EE2 was below the detection limit in three of the eight surface water samples. Two samples collected from the rivers Rhine and Agger showed values above the LOD. All effluent samples taken from local STPs showed values above the LOD from 0.4 to 0.7 ng L$^{-1}$ (Table 3). Results obtained in reference measurements using LC–MS/MS were generally in good agreement with the CLEIA results, the immunoassay showing false-positive results with some surface and waste-water samples.

![Figure 2](image.png)

**Fig. 2.** Influence of commercial humic acid on EE2 calibration curves. Curves are obtained using spiked calibrators: (squares) ultrapure water, (circles) 0.2 mg L$^{-1}$, (open circles) 0.5 mg L$^{-1}$, (triangles) 1 mg L$^{-1}$ and (open triangles) 5 mg L$^{-1}$ humic acid. Inset shows the influence on the corresponding midpoints (as a measure of sensitivity).

### Table 3

<table>
<thead>
<tr>
<th>Sample</th>
<th>CLEIA (ng L$^{-1}$)</th>
<th>LC–MS/MS (ng L$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ultrapure water</td>
<td>Tap water</td>
</tr>
<tr>
<td>Spiked samples$^a$</td>
<td>1.4</td>
<td>1.5</td>
</tr>
<tr>
<td>Surface water (river, location)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ahr, spring</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>Ahr, spring$^b$</td>
<td>&lt;0.2</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>Ahr, Dümpefied</td>
<td>&lt;0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>Ahr, Dümpefied$^b$</td>
<td>&lt;0.2</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>Ahr, Lohmar</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>Ahr, Lohmar$^b$</td>
<td>0.7</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>Rhine, Bonn</td>
<td>0.6</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>Rhine, Bonn$^b$</td>
<td>&lt;0.2</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>Wastewater</td>
<td></td>
<td></td>
</tr>
<tr>
<td>STP I</td>
<td>0.6</td>
<td>0.3</td>
</tr>
<tr>
<td>STP II</td>
<td>0.4</td>
<td>0.8</td>
</tr>
<tr>
<td>STP III</td>
<td>0.4</td>
<td>0.3</td>
</tr>
<tr>
<td>STP IV</td>
<td>0.7</td>
<td>0.6</td>
</tr>
</tbody>
</table>

$^a$ Samples were spiked with 2.5 ng L$^{-1}$ EE2.  
$^b$ Second sample collection, some weeks later.

### 4. Conclusion

Extensive discussions of factors affecting immunoassay sensitivity have been published [23,29,30]. The underlying concept of sensitivity in such reagent-limited assays is based on the antibody affinity. In contrast to immunometric assays the “detectability” of the label is of minor importance. However, if in reagent limited assays the prerequisite of optimum dilutions cannot be achieved due to sensitivity restrictions of the label, then the detectability becomes a significant factor impeding further improvements. Under identical conditions both labels, photometric and luminescent, yield identical calibration curves of the same sensitivity, yet the photometric assay is slow in developing a rather low absolute OD signal. Exemplary parameters of calibration curves obtained using both labels are as follows: \(A_{\text{luminescent}} = 100 \pm 6.2\), \(A_{\text{photometric}} = 100 \pm 7.6\); \(A_{\text{luminescent}} = 0.65 \pm 0.1\), \(A_{\text{photometric}} = 0.76 \pm 0.2\); \(C_{\text{luminescent}} = 42.7 \pm 14.0\) ng L$^{-1}$, \(C_{\text{photometric}} = 31.9 \pm 10.4\) ng L$^{-1}$, \(C_{\text{luminescent}} = 0 \pm 3.6\), \(C_{\text{photometric}} = 9 \pm 2.4\).

Although the photometric method based on the detection of peroxidase by the TMB/H$_2$O$_2$ substrate system with a detection limit of \(2 \times 10^{-15}\) mol L$^{-1}$ POD is already very sensitive [31], the switch to a luminol based substrate enabled us to achieve better detection limits due to its enhanced detectability. Our assessment of assay characteristics is based on conservative conventions: LOD and LOQ are calculated using signal-to-noise ratios of 3 and 10, respectively, the analytical working range is deduced using Ekins’ description of the “precision profile” with a comparatively low admissible coefficient of variation (CV) of 20% [32]. It should be mentioned that any use of empirical rules such as the Horwitz equation [33] would lead to arbitrary assumptions regarding the working range, simulating unsustainably low assay sensitivities. Furthermore application of the Horwitz equation to concentrations below 100 µg L$^{-1}$ yields unacceptably high values for reproducibility CVs [34].

EE2 values measured in surface water and STP effluent are in good accordance with the results obtained in LC–MS/MS measurements. Deviations observed in wastewater samples might be due to matrix effects regarding the antibody. The molecular mechanism of this potential interference is not understood, recognition of moieties from humic substances present in the sample might be an explanation for overestimated EE2 concentrations. Such false positive results due to matrix components interfering with the antibodies have been reported before [35]. As expected, with regard to human excretion, dilution factor and more recent observations by other researchers [5–7], all values are in the sub-ppt range. The antiserum employed in this study shows excellent specificity for EE2 with negligible cross-reactivities to metabolites and conjugates [36]. Additionally, according to Baronti [37] deconjugation of estrogens excreted as sulphates or glucuronides takes place already in the sewers. Therefore false-positive results due to cross-reacting compounds are not probable to occur.
As shown in this study antibody affinity and label detectability are of major importance for assay optimization. Under optimized conditions the reduction of experimental error and elimination of potential sources of imprecision becomes relevant. Although we were able to establish excellent detection limits in this manually performed assay, further advances are expected from implementing this assay on an automated system for water analysis [38].

Acknowledgement

C. Schneider thanks the German Federal Environmental Foundation (DBU) for a Ph.D. scholarship. We express our appreciation to Perbio and Nunc for excellent support and to D. Skutlarek and Dr. H. Färber of the Institute for Hygiene and Public Health, University of Bonn, for reference measurements.

References