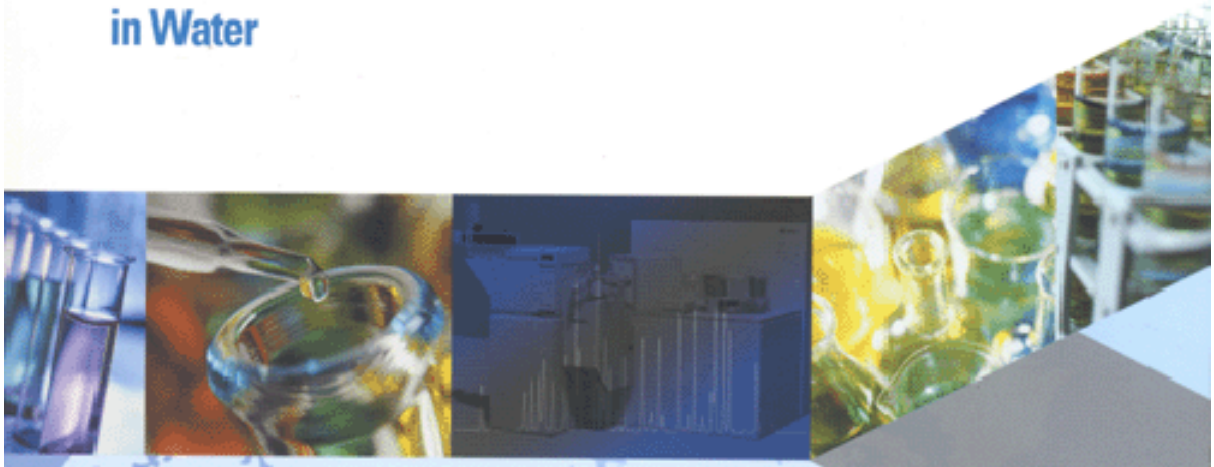
Reports on The International Collaborative Research

Development of Multi-residual Analytical Methods for Pharmaceuticals, Perfluorinated Compounds, Nitrosamines, Hormones, Persistent Organic Pollutants, in Water



Volume I

WARC · TZW · NIAES · OCWD

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Development of Multi-residual Analytical Methods for Pharmaceuticals, Perfluorinated Compounds, Nitrosamines, Hormones, Persistent Organic Pollutants, in Water

Volume I

WARC, TZW, NIAES, OCWD

Development of Multi-residual Analytical Methods for Pharmaceuticals, Perfluorinated Compounds, Nitrosamines, Hormones and POPs in Water

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PHARMACEUTICALS IN WATER BY HPLC/MS/MS

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1.0 SCOPE AND APPLICATION

1.1 Pharmaceuticals Method 1 is for determination of pharmaceuticals in aqueous samples by high performance liquid chromatography combined with tandem mass spectrometry (HPLC/MS/MS).

1.2 This method was developed for use in "International Collaboration for The Development of Multi-residual Analytical Methods for Pharmaceuticals, Nitrosamines, Hormones and POPs (persistent organic pollutants) in Water".

1.3 The target analytes and their corresponding Chemical Abstracts Service Registry Numbers are listed in Table 1-1.

1.4 The detection limits and quantitation levels in this method are usually dependent on the level of interferences rather than instrumental limitations. The method detection limits and practical quantitation limits (Standard Methods (1030C.)) are in Tables 1-3 and 1-5.

2.0 SUMMARY OF METHOD

The target analytes in this method are divided into two groups. Each group represents an LC/MS/MS run, as detailed in Table 1-2 to 1-5. Tables 1-2 and 1-3 are specific to Group 1. Tables 1-4 and 1-5 are specific to Group 2.

Group 1 is extracted under acidic (pH 2) condition and is run in the positive electrospray ionization (ESI+) mode. Group 2 is extracted under neutral (pH 6~8) condition and is run in the negative electrospray ionization (ESI-) mode.

2.1 Aqueous samples absent visible particles and filtrate from samples with visible particles. The pH of a 500 mL sample aliquot is adjusted to with acid. The pH of a second 500 mL aliquot of sample is adjusted with 6~8. Stable, isotopically labeled analogs of fraction is stabilized with tetrasodium ethylenediamine-tetraacetates dehydrate.

2.2 Determination by LC/MS/MS – The acid extract is analyzed in positive electro spray ionization (ESI+) LC/MS/MS run. The neutral extract is analyzed in negative electro spray ionization (ESI-) LC/MS/MS run. The analytes are separated by LC and detected by tandem

mass spectrometer. A productor m/z for each compound is monitored throughout a pre-determined retention time window.

2.3 An individual compound is identified by comparing the LC retention time and presence of the productor m/Z with the corresponding retention time and productor m/z of an authentic standard.

3.0 DEFINITIONS AND UNITS OF MEASURE

3.1 Internal Standard (IS)
A pure analyte(s) added to a sample, extract, or standard solution in known amount(s) and used to measure the relative response of other method analytes that are components of the same sample or solution. The internal standard must be an analyte that is not a sample component.

3.2 Laboratory Reagent Blank (LRB)
An aliquot of reagent water or other blank matrix that is treated exactly as a sample including exposure to all glassware, equipment, solvents, reagents, internal standards, and surrogates that are used with other samples. The LRB is used to determine if method analytes or other interferences are present in the laboratory environment, the reagents, or the apparatus.

3.3 Method Blank
An aliquot of reagent water that is treatment exactly as a sample including exposure to all glassware, equipment, solvents, reagent, internal standard that are used with samples. The method blank is used to determine if analytes or interferences are present in the laboratory environment, the reagent, or the apparatus.

3.4 Stock Standard Solution
A solution containing one or more method anlytes that are prepared in laboratory using assayed reference materials or purchased from reputable commercial sources.

3.5 Primary Dilution Standard Solution
A solution of several analytes prepared in the laboratory from stock standard solutions and diluted as needed to prepare calibration solutions and other needed analyte solutions.

3.6 Calibration Standard
A solution prepared for a secondary standard and/or stock solution and used to calibrate the

response of the HPLC/MS/MS instrument.

3.7 Quality Control Check Samples (QCS)
A solution of method analytes which is known concentrations used to fortify an aliquot of LRB or sample matrix. The QCS is obtained from a source external to the laboratory and different from the source of calibration standards. It is used to check laboratory performance with externally prepared test materials prepared external to the normal preparation process.

3.8 Native Compound
A molecule in which the atoms all have naturally occurring isotopic abundances.

3.9 Labeled Compound
A molecule in which one or more of the atoms is isotopically enriched, thereby increasing the mass of the molecule.

3.10 Reagent Water
Water demonstrated to be free from the analytes of interest and potentially interfering substances at the method detection limit for the analyte.

3.11 Internal Standard Quantitaion
A means of determining the concentration of a naturally occurring (native) compound by reference to a compound other than its labeled analog and a labeled compound by reference to another labeled compound.
A labeled compound used as a reference for quantitation of other labeled compounds and for quantitation of native compound other than the compound of which it is a labeled analog.

3.12 Method Detection Limit (MDL)
The constituent concentration that, when processed through the complete method, produces a signal with a 99% probability that it is different from the blank. For seven replicates of the sample, the mean must be 3.14s above the blank where s is the standard deviation of the seven replicates. Compute MDL from replicate measurements one to five times the actual MDL. The MDL will be larger than the LLD because of the few replications and the sample processing steps and may vary with constituent and matrix.

3.13 Practical Quantitaion Limit (PQL)
The lowest achievable level among laboratories within specified limits during routine

laboratory operations. The PQL is significant because different laboratories will produce different MDLs even though using the same analytical procedures, instruments, and sample matrices. The PQL is about five times the MDL and represents a practical and routinely achievable detection level with a relatively good certainty that any reported value is reliable.

4.0 INTERFERENCES

4.1 Solvents, reagents, glassware, and other sample processing hardware may yield artifacts, elevated baselines, matrix enhancement or matrix suppression causing misinterpretation of chromatograms. The specific selection of reagents and purification of solvents by distillation in all-glass systems may be required. Where possible, reagents are cleaned by extraction or solvent rinse.

4.2 Proper cleaning of glassware is extremely important, because glassware may not only contaminate the samples but may also remove the analytes of interest by adsorption on the glass surface.

4.2.1 Glassware should be rinsed with solvent and washed with a detergent solution as soon after use as is practical. Sonication of glassware containing a detergent solution for approximately 30 seconds may aid in cleaning. Glassware with removable parts, particularly separatory funnels with teflone stopcocks, must be disassembled prior to detergent washing.

4.2.2 After detergent washing, glassware should be rinsed immediately, first with methanol, then with hot DI water. The DI water rinse is followed by other solvents such as methanol rinse, then acetone, and then methylene chloride.

4.2.3 After drying and cooling, glassware should be sealed and stored in a clean environment to prevent any accumulation of dust or other contaminants. Store inverted or capped with solvent rinsed aluminum foil.

4.3 All materials used in the analysis must be demonstrated to be free from interferences by running reference matrix method blanks initially and with each sample batch (samples started through the extraction process on a given 12-hour shift, to a maximum of 20 samples).

4.4 Interferences co-extracted from samples will vary considerably from source to source,

depending on the diversity of the site being sampled. Interfering compounds may be present at concentrations several orders of magnitude higher than the analytes of interest. Because low levels of Pharmaceuticals are measured by this method, elimination of interferences is essential.

4.5 It may be useful to number reusable glassware is to associate that glassware with the processing of a particular sample. This will assist the laboratory in tracking possible sources of contamination for individual samples, identifying glassware associated with highly contaminated samples that may require extra cleaning, and determining when glassware should be discarded.

5.0 EQUIPMENT AND SUPPLIES

5.1 Sample bottles and caps

5.1.1 Liquid samples– Sample bottle, amber glass, 1 L minimum, with screw cap.

5.1.2 Bottle caps – Threaded to fit sample bottles. Caps are lined with Teflon.

5.1.3 Cleaning – Bottles are washed with detergent and water, then solvent rinsed before use.

5.2 Equipment for sample preparation

5.2.1 Laboratory fume hood of sufficient size to contain the sample preparation equipment listed below.

5.2.2 Vortex mixer

5.2.3 Desiccator

5.2.4 Balance, analytical – Capable of weighing 0.1 mg.

5.2.5 Balance, top loading – Capable of weighing 10 mg.

5.3 Apparatus for measuring pH

5.3.1 pH meter, with combination glass electrode

5.3.2 pH paper, wide range (Hydrion Papers, or equivalent)

5.4 Apparatus for solid-phase extraction

5.4.1 Visiprep 24tm DL(Disposal liner) Solid Phase Extraction Vacuum Manifold (Supelco, or equivalent).

5.4.2 Vacuum pump: Peristaltic pump – (Whatson marlow, or equivalent).

5.4.3 Vacuum source – Capable of maintaining 25 in. Hg, equipped with shutoff valve and vacuum gauge.

5.4.4 SPE cartridge – Hydrophilic-Lipophilic-Balance (HLB), Waters Oasis, 6CC, 200mg.

5.5 Filtration Apparatus

5.5.1 Vacuum filtration apparatus – 1-L , including glass funnel, frit support, clamp, adapter, stopper, filtration flask, and vacuum tubing. For wastewater samples, the apparatus should accept 90- or 144-mm disks.

5.5.2 Glass-fiber filter – Whatman GMF 150 (or equivalent), 1 micron pore size, to fitter vacuum filtration apparatus.

5.5.3 Whatman GF/F (0.45 μm), or equivalent, differing diameters, to fit the pressure filtration apparatus.

5.6 Centrifuge – Capable of rotating 500-mL centrifuge bottles or 50-mL centrifuge tubes at 5,000 rpm minimum, equipped with 500-mL centrifuge bottles (glass or polypropylene bottles) with screw-caps, and 50-mL centrifuge tubes with screw-caps, to fit centrifuge.

5.7 Pipet apparatus and pipets

5.7.1 Pipetter – variable volume

5.7.2 Pipet tips, disposable polypropylene, sizes from 1-10 L to 5 mL

5.7.3 Disposable, Pasteur, 150-mm long x 5-mm ID (Fisher Scientific 13-578-5A, or equivalent)

5.8 Concentrator – Turbo Vap Concentrator (0.5~1.0 mL) (Zymark Turbo Vap 500, or equivalent).

5.9 Amber glass vials, 2- to 5-mL PTFE (Polytetrafluoroethylene) lined screw-cap.

5.10 HPLC/MS/MS System.

5.10.1 HPLC system with high pressure inlet, multi-segment gradient capability. The system must be able to produce the LC separations for the analytical runs and the instrument conditions detailed in Tables 1-2, 1-4 and must meet other HPLC requirements in this method (API 4000).

5.10.2 LC columns

5.10.2.1 C18 – 15.0 cm, 2.0 mm i.d., 3.0 μm particle size (Phenomenex Gemmini C18, or equivalent).

5.10.2.2 C18 – 15.0 cm. 3.0 mm i.d., 3.0 μm particle size (Phenomenex Luna C18, or equivalent).

5.10.2.3 Alternative columns other than described above have not been tested and are not allowed for this method.

5.10.3 MS/MS system

5.10.3.1 Tandem MS with the necessary pumps, collision cell, makeup gases, high vacuum system, and capability for positive and negative ion electrospray ionization (ESI) of the effluent from the HPLC. (Applied Biosystems, API 4000 triple quadrupole MS, or equivalent). The system must be able to produce parent-daughter transitions for the groups of compounds in the acid and neutral fractions of the pharmaceuticals for the analytical runs detailed in Tables 1-3 and 1-4.

5.10.3.2 Instrument control and data system – Interfaced to the HPLC and MS/MS to control the LC gradient and other LC and MS/MS operating conditions, and to acquire, store, and reduce LC/MS/MS data. The data system must be able to identify a compound by retention time and parent-daughter m/z, and quantify the compound using linear or quadratic multi-point relative responses and response factors by internal standard techniques.

5.11 Miscellaneous labware – Beakers, 400- to 500-mL; Erlenmeyer flasks; volumetric flasks; etc.

6.0 REAGENTS AND STANDARDS

Note: All reagents are ACS Reagent Grade unless specified otherwise.

6.1 pH adjustment and solution stabilization

6.1.1 Ammonium hydroxide (NH_4OH) – Reagent grade, Sigma, or equivalent

6.1.2 Sulfuric acid – Reagent grade (specific gravity 1.84)

6.1.3 Hydrochloric acid – Reagent grade, 5N

6.2 Pre-purified nitrogen

6.3 Solvents, reagents, and solutions

6.3.1 Acetonitrile ammonium acetate, formic acid, methanol, HPLC water

6.3.2 Solvents and purchased solutions should be lot-certified to be free of interferences. If necessary, solvents should be analyzed by this method to demonstrate that they are interference free.

6.4 Elution solutions

6.4.1 Tetrasodium ethylenediamine tetraacetate hydrate ($Na_4EDTA•2H_2O$ ~+99.5%

titration), Sigma, used as received.

6.4.2 Formic acid solutions – Sigma Aldrich, >95 percent purity.

6.4.2.1 Acetonitrile:methanol (1:1) – mix 500 mL methanol and 500 mL of acetonitrile. Sonicate for 5 min.

6.4.2.2 0.1% formic water - dissolve 1 mL of formic acid in 1.0 L of HPLC water. Mix thoroughly and sonicate for 5 min.

6.4.2.3 20 mM Ammonium acetate/water - dissolve 1.54 g of ammonium acetate in 1.0 L of HPLC water. Mix thoroughly and sonicate for 5 min.

6.5 Reference matrices – Matrices in which the Pharmaceuticals and interfering compounds are not detected by this method.

6.5.1 Reagent water – Bottled water purchased locally, or prepared by passage through activated carbon.

6.6 Standard solution – Prepare from materials of known purity and composition or purchase as solutions or mixtures with certification to their purity, concentration, and authenticity. If the chemical purity is 98 % or greater, the weight may be used without correction to calculate the concentration of the standard.

6.6.1 Preparation and storage of solutions - For preparation of stock solutions from neat materials, dissolve an appropriate amount of assayed reference material in solvent.

6.6.2 Stock standard solution - For each of the target compounds a separate stock solution in methanol is prepared. Their concentrations depend on the solubility of the target compounds in methanol. For the preparation of each of the stock solutions, between 1 and 10 mg of the pharmaceutical compound are added on an analytical balance to a 10 mL volumetric flask. Then, the volumetric flask is filled up to volume with methanol. From the exact weight of the pharmaceutical and the volume of methanol, the exact concentration of the stock solution is calculated taking into account the purity of the target compounds. The stock solutions have to be stored in the dark at less than –10 in the screw-capped vials with PTFE caps.

6.6.3 Stock solutions should be prepared at a frequency necessary to preclude degradation from affecting the analysis.

6.6.4 Primary dilution standard solution - Few mL of methanol are added to a 50 mL volumetric flask. A defined volume of each stock solution of the method analytes is added with a syringe to achieve a concentration of 1 μg/L. The volumetric flask is filled up to volume with methanol.

6.6.5 Labeled injection internal standard spiking solutions – For the labeled injection internal standards for Groups 1 and 2, prepare labeled compound in methanol at the typical concentration.

6.6.6 Calibration standards – 500mL amber glass bottle are filled with reagent water. Different Volume of the Primary Dilution Standard solution and internal standard 50 μL are added with syringe.

6.7 QC check sample – A QC check sample should be obtained from a source independent of the calibration standards.

7.0 SAMPLE COLLECTION, PRESERVATION AND STORAGE

7.1 Collect samples in amber glass containers following conventional sampling practices.

7.2 Aqueous samples

7.2.1 Samples that flow freely are collected as grab samples or in refrigerated bottles using automatic sampling equipment. Collect 0.5-L each for the acid and neutral fractions (1 L total). If high concentrations of the analytes of interest are expected, collect two smaller volumes (e.g., 100 mL each) in addition to the 0.5-L samples.

7.2.2 If residual chlorine is present, add 80 mg sodium thiosulfate or Ascorbic acid per liter of water. Any method suitable for field use may be employed to test for residual chlorine.

7.2.3 Maintain aqueous samples in the dark at <4 from the time of collection until receipt at the laboratory.

8.0 QUALITY ASSURANCE/QUALITY CONTROL

The laboratory must make an initial demonstration of the ability to generate acceptable precision and recovery with this method.

8.1 Calibration

8.1.1 Instrument calibration: Perform instrument calibration, as well as maintenance, according to instrument manual instructions. Use instrument manufacturer's recommendations for calibration. Conform to instrument performance checks, such as those for LC/MS/MS analyses, according to method or SOP instructions.

8.1.2 Initial calibration: Perform initial calibration with a minimum of three concentrations of standards for linear curves. Choose a lowest concentration at the reporting limit, and highest concentration at the upper end of the calibration range. Ensure that the calibration range encompasses the analytical concentration values expected in the samples or required dilutions. Choose calibration standard concentration with no more than one order of magnitude between concentrations.

8.1.3 Calibration verification: Calibration verification is the periodic confirmation by analysis of a calibration standard that the instrument performance has not changed significantly from the initial calibration. Base this verification on time (e.g., every 12h) or on the number of samples analyzed (e.g., after every 10 samples). Verify calibration by analyzing a single standard at a concentration near or at the midpoint of the calibration range. The evaluation of the calibration verification analysis is based on allowable deviations (±20~30%) from the values obtained in the initial calibration. If the calibration verification is out of control, take corrective action, including reanalysis of any affected samples. Refer to the method of choice for the frequency of calibration verification and the acceptance criteria for calibration verification.

8.2 Determining Detection Levels

The MDL can be achieved by experienced analysts operating well-calibrated instruments on a non routine basis. For example, to determination the MDL, add a constituent to reagent water, or to the matrix of interest, to make a concentration near the estimated MDL. Prepare and analyze seven portions of this solution in the MDL determination. Calculate the standard deviation and compute the MDL. From a table of the one-sided t distribution selects the

Value of t for 7 – 1 = 6 degrees of freedom and at the 99% level; this value is 3.14. The product 3.14 times s is the desired MDL.

The practical quantitation limit (PQL) has been proposed as the lowest level achievable among laboratories within specified limits during routine laboratory operations. The PQL is significant because different laboratories will produce different MDLs even though using the same analytical procedures, instruments, and sample matrices. The PQL is about five times the MDL and represents a practical and routinely achievable detection level with a relatively good certainty that any reported value is reliable.

8.2.1 Accuracy (means) Control chart:

Control chart commonly used in laboratories is as follows: accuracy or means charts for QC samples, including reagent blanks, calibration check standards. This chart is essential tools for quality control. The accuracy chart for QC samples is constructed from the average and standard deviation of a specified number of measurements of the analyte of interest. The accuracy chart includes upper and lower warning levels (WL) and upper and lower control levels (CL). Common practice is to use ±2s and ±3s limits for the WL and CL, respectively, where s represents standard deviation. These values are derived from stated or measured values for reference materials. The number of measurements, n or n-1, used to determine the standard deviation, s, is specified relative to statistical confidence limits of 95% for WLs and 99% for CLs. Set up an accuracy chart by using either the calculated values for mean and standard deviation or the percent recovery. Percent recovery is necessary if the concentration varies. Construct a chart for each analytical method. Enter results on the chart each time the QC sample is analyzed. Examples of control charts for accuracy are given in Figure 1-1.

$$\bar{x} = \frac{\sum x_i}{n},\ \text{Standard deviation(s)} = \sqrt{\frac{\sum (\bar{x} - x_i)^2}{n-1}}$$

where, $\bar{x}$ = average of measured values

x_i = each measured value

n = the number of measurement

WL = ±2 × s (standard deviation)

CL = ±3 × s (standard deviation)

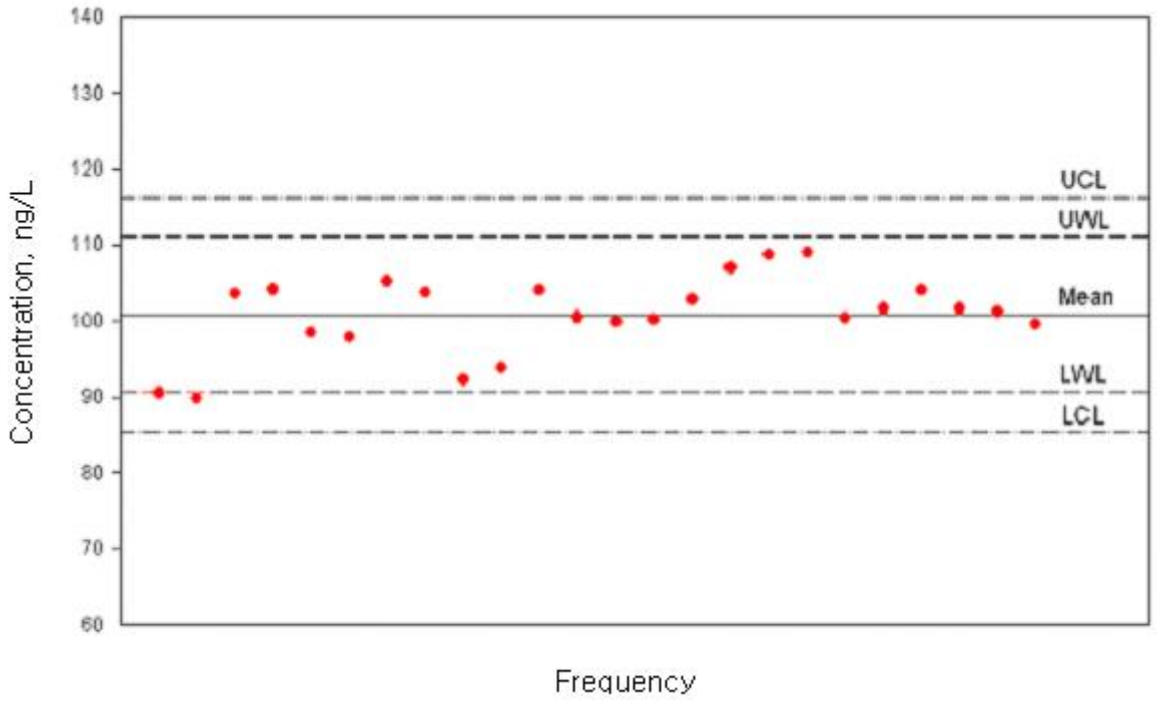

Fig. 1-1. Control charts for means

8.2.2 Chart analyses: If the warning limits (WL) are at the 95% confidence level, 1 out of 20 points, on the average, would exceed that limit, whereas only 1 out of 100 would exceed the control limits (CL).

Control limit: If one measurement exceeds a CL, repeat the analysis immediately. If the repeat measurement is within the CL, continue analysis: if it exceeds the CL, discontinue analyses and correct the problem. Another important function of the control chart is assessment of improvements in method precision. In the accuracy chart, if measurements never or rarely exceed the WL, recalculate the WL and CL using the 10 to 20 most recent data points.

9.0 SAMPLE PREPARATION

Aqueous samples – Because the analytes may be bound to suspended particles, the preparation of aqueous samples is depends on the presence of visible particles. Aqueous samples absent visible particles are prepared filtration.

Aqueous samples with visible particles – If visible particles can be seen in aqueous samples

they should be filtered and the solids and aqueous portions of these samples should be extracted and combined as follows. Filtration of particles – assemble a clean filtration apparatus. Apply vacuum to the apparatus, and pour the entire contents of the sample bottle through the filter, swirling the sample remaining in the bottle to suspend any particles. Rinse the sample bottle twice with approximately 5 mL portions of reagent water to transfer any remaining particles onto the filter. Rinse any particles off the sides of the filtration apparatus with small quantities of reagent water. Weigh the empty sample bottle to ±1g.

9.1 Preparation of aqueous samples absent I visible particles and corresponding QC samples - Two separate sample aliquots are required to analyze all of the target analytes in this procedure: one aliquot is adjusted to pH 2±0.5 and the other aliquot is adjusted to pH 6~8. Following this pH adjustment, both aliquots are filtered separately, and the two filtrates are extracted using the SPE HLB cartridge extraction and concentration.

9.1.1 Acid fraction – typically 500 mL

9.1.1.1 Acidify the filtrate for the acid fraction pH 2.0 ± 0.5 with H_2SO_4 while swirling or stirring the water. Re-adjust the pH as necessary to achieve pH 2.0 ± 0.5.

9.1.1.2 Spike the acid fraction native compounds and labeled compounds into the reagent water aliquot that will serve as the acid fraction standard. Acidify the standard aliquot and the blank aliquot in the same manner as the acid fraction of the field sample.

9.1.1.3 Add 4mL 5% EDTA to each of the acid fraction samples and QC aliquots. Cap the bottles and mix by shaking. Proceed to sample extraction.

9.1.2 Neutral fraction – typically 500 mL

9.1.2.1 Adjust the pH of the second of the two sample bottles to pH 6~8 with NH_4OH or H_2SO_4 while swirling or stirring the water.

9.1.2.2 Spike the neutral fraction native compounds and labeled compounds into the reagent water aliquot that will serve as the neutral fraction standard. Neutralize the standard aliquot and the blank aliquot in the same manner as the neutral fraction of the field sample.

9.1.2.3 Add 4mL 5% EDTA to each of the acid fraction samples and QC aliquots. Cap the bottles and mix by shaking. Proceed to sample extraction.

10.0 EXTRACTION AND CONCENTRATION

This method employs solid-phase extraction (SPE) procedures to extract the target analytes from aqueous samples.

10.1 Extraction of aqueous samples absent visible particles - Extraction of both the acid and neutral fractions of aqueous samples involve many of the same steps, beginning with the conditioning of the SPE cartridges.

10.1.1 Assemble the SPE extraction apparatus and attach the SPE HLB cartridges.

10.1.2 When extracting the acid fraction of a sample, condition an SPE HLB cartridge by eluting it with 5 mL of methanol, 5 mL reagent water and 5 mL of 0.5N HCl. Discard these eluants. Do not let the cartridge go dry ant any point during the conditioning process.

10.1.3 When extracting the neural fraction of a sample, condition an SPE HLB cartridge by eluting it with 5 mL of methanol and 5 mL of distilled water. Discard these eluants. Do not let the cartridge go dry ant any point during the conditioning process.

10.1.4 Using the SPE cartridge appropriate for the sample fraction (acid or neutral), load the sample onto the cartridge at a flow rate of 5 mL/min.

10.1.5 Once the entire sample has passed through the cartridge, wash the acid and neutral cartridge with 5 mL of distilled water.

10.1.6 Dry the cartridges for either fraction under vacuum for approximately 5 min.

10.2 Cartridge elution

10.2.1 Acid fraction: Elute the analytes with 10 mL 0.1% formic acid / methanol at a flow rate of 3 mL/min. Collect the eluant in a clean centrifuge tube. Proceed with

concentration of the extract.

10.2.2 Neutral fraction: Elute the analytes with 10 mL methanol at a flow rate of 3 mL/min. Collect the eluant in a clean centrifuge tube. Proceed with concentration of the extract.

10.3 Concentration of aqueous sample extracts
Extracts from the acid and neutral fractions of aqueous samples are concentrated separately to dryness and the solvent exchanged to methanol, as described below.

10.3.1 Concentrate the extract to near dryness under a gentle stream of nitrogen in a water bath held at 30 ± 5 .

10.3.2 Reconstitution with 0.5 mL of 20 mM Ammonium acetate: 0.1% formic acid / water:methanol (50:50) to the concentrated acid extracts and 0.5 mL 20mM Ammonium acetate / water:methanol (50:50) for neutral extracts, including the blank and standard aliquots.

10.3.3 Transfer 0.5 mL of each extract to an LC/MS/MS auto sampler vial for analysis.

11.0 LC/MS/MS ANALYSIS

Establish the same operating conditions established and optimized for the calibration appropriate to the fraction and Group to be analyzed. Analysis is performed using positive electrospray ionization (ESI+) for the acid fraction Group1 analytes. Analysis is performed by negative electrospray ionization (ESI-) for the neutral fraction Group2 analytes. Retention times, precursor-product transitions, quantitation references, method detection limits, and practical quantitative limit of quantitation for Groups1 and 2.

12.0 QUALITATIVE DETERMINATION

A native or labeled compound is identified in an standard, blank, or sample when the criteria.

12.1 The signal-to-noise ratio (S.N) at the LC peak maximum for each native compound at its

daughter m/z must be greater than or equal to 3 for each compound detected in a sample extract.

12.2 The retention time of the peak for a native compound must be within ±15 seconds of its RT.

13.0 QUANTITATIVE DETERMINATION (INTERNAL STANDARD QUANTITATION)

By adding a known amount of a labeled compound to every sample prior to extraction, correction for recovery of the native analog of that compound can be made because the native compound and its labeled analog exhibit similar effects upon extraction, concentration, and chromatography.

13.1 Concentrations in real samples are calculated by the software according to the following equation:

$$x = \frac{y - a_0}{a_1} \cdot C_{IS}$$

with x = concentration of the target analyte (ng/L)
y = peak area of the target analyte/peak area of the internal standard
a_1 = slope of the linear calibration function (= sensitivity)
a_0 = intercept of the linear calibration function
C_{IS} = Concentration of the internal standard (ng/L)

13.2 The recovery for the overall procedure is determined by a comparison of peak areas of pre-concentrated samples and direct injections according to the following equation:

$$R = \frac{Y_{pre-concentrated}}{Y_{direct}} \cdot 100\%$$

with $Y_{pre\text{-}concentrated}$ = peak area of a compound after pre-concentration
Y_{direct} = peak area of a compound after direct injection

The recovery is determined each time that the batch of SPE material or elution solvent is changed.

13.3 Reporting level - Report the result for each compound in each sample, blank, or standard at or above the minimum level of quantitation to 3 significant figures. Report the result below the

PQL in each samples as <PQL or as required by the regulatory authority or permit.

14.0 POLLUTION PREVENTION

14.1 Pollution prevention encompasses any technique that reduces or eliminates the quantity or toxicity of waste at the point of generation. Many opportunities for pollution prevention exist in laboratory operation.

14.2 The compounds in this method are used in extremely small amounts and pose little threat to the environment when managed properly. Standards should be prepared in volumes consistent with laboratory use to minimize the disposal of excess volumes of expired standards.

15.0 WASTE MANAGEMENT

15.1 SPE cartridges are only used once and are disposed with the domestic waste.

15.2 Solutions containing solvents are collected in glass bottles. Full glass bottles are shipped to a special unit for proper waste disposal.

16.0 REFERENCES

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ANNEX

Table 1-1. Names and CAS Registry numbers for pharmaceuticals determined by internal standard HPLC/MS/MS

Compound	CAS Registry	Labeled analog	CAS Registry
1,7-dimethylxanthine	511-59-5	$^{13}C_5$-Sulfamethoxazole	
Acetaminophen	103-902	$^{13}C_5$-Sulfamethazine	
Ampicillin	69-53-4	D_{10}-Carbamazepine	
Aztreonam	78110-38-0	$^{13}C_2$,^{15}N -Acetaminophene	
Bromohexin	3572-43-8	^{13}C-Naproxen	
Caffeine	58-08-2	$^{13}C_3$-Ibuprofen	
Carbadox	5804-07-5	D_4-Diclofenac	
Carbamazepine	298-45-4		
Ciprofloxacine	85721-33-1		
Diltiazem	42399-41-7		
Enrofloxacine	93105-50-5		
Furazolidone	67-45-8		
Lincomycin	154-21-2		
Midecamycin	35457-80-8		
Norfloxacine	70458-95-7		
Olaquindox	23696-28-8		
Oxolinicacid	14598-29-4		
Spiramycin	8025-81-8		
Streptomycin	57-92-1		
Sulfachloropyridazine	80-32-0		
Sulfadiazine	58-35-9		
Sulfadimethoxine	122-11-2		
Sulfamerazine	127-79-7		
Sulfameter	651-06-9		
Sulfamethazine	57-59-1		
Sulfamethoxazole	723-45-5		
Sulfamonomethoxine	1220-83-3		
Sulfaquinoxaline	59-40-5		
Sulfathiazole	72-14-0		
Sulfisoxazole	127-69-5		
Thiabendazole	148-79-8		

Compound	CAS Registry	Labeled analog	CAS Registry
Trimethoprime	738-70-5		
Virginiamycine	11005-75-1		
Cimetidine	51481-51-9		
Diclofenac	15307-86-5		
Florfenicol	73231-34-2		
Ibuprofen	15587-27-1		
Naproxen	22204-53-1		
Salicylic acid	69-72-7		

Table 1-2. Group 1 – Acidic extraction, positive electrospray ionization(ESI+) instrument conditions

Instrument	Agilent 1200SL HPLC, AB API 4000 MS/MS
LC Column	Phenomenex Gemini C18, 15.0 cm, 2.0 mm id, 3 um particle size
Ionization	Positive Ion Electrospray
Acqusition	MRM Mode, unit resolution
Injection Volume	5 uL

LC Gradient Program		LC Flow Rate (mL/min)	General LC Conditions	
Time(min)	Flow mixture			
10(Equilibrate)	100% Solvent B	0.25	Column Temp	40
10	30% Solvent A 70% Solvent B	0.25	Autosampler tray temperature	4
20	50% Solvent A 50% Solvent B	0.25	MS/MS Condition	
25	70% Solvent A 30% Solvent B	0.25	Source Temp	450
30	90% Solvent A 10% Solvent B	0.25	CUR	20
40	90% Solvent A 10% Solvent B	0.25	GS1/GS2	50/50

Solvent A : 1:1 Acetonitrile / Methanol

Solvent B : 0.1% Formic water

Table 1-3. Group 1 – Acidic extraction, positive electrospray ionization(ESI+) compound retention times(RTs), precursor-product transitions, quantitaion reference, method detection limit, quantitaion detection limit

Analyte	RT (min)	Precursor – product m/z	Internal Standard	Detection limits (ng/L)	
				MDL	PQL
Group 1	Analytes Extracted Under Acidic Conditions and analyzed Using ESI(+)				
Native Compounds					
1,7-dimethylxanthine	10.8	181.0/124.0	$^{13}C_5$-Sulfamethoxazole	3.2	15.1
Acetaminophen	10.3	152.0/110.0	$^{13}C_2$,^{15}N -Acetaminophene	1.3	5.5
Ampicillin	11	350.0/105.0	D_{10}-Carbamazepine	1.0	5.1
Aztreonam	13.1	435.0/355.0	$^{13}C_5$-Sulfamethoxazole	2.4	11.9
Bromohexin	15.5	377.0/114.0	D_{10}-Carbamazepine	0.8	3.9
Caffeine	12.1	195.1/138.0	$^{13}C_5$-Sulfamethoxazole	2.7	13.7
Carbadox	17.7	253.0/245.0	$^{13}C_5$-Sulfamethoxazole	1.5	7.9
Carbamazepine	20.2	237.0/194.0	D_{10}-Carbamazepine	1.8	8.9
Ciprofloxacine	12.1	332.2/314.1	$^{13}C_5$-Sulfamethoxazole	2.5	13.1
Diltiazem	15.3	415.0/178.0	$^{13}C_5$-Sulfamethoxazole	2.3	11.7
Enrofloxacine	12.5	350.0/315.0	$^{13}C_5$-Sulfamethoxazole	2.4	12.2
Furazolidone	14.9	225.1/122.1	D_{10}-Carbamazepine	2.0	9.8
Lincomycin	9.91	407.0/125.0	$^{13}C_5$-Sulfamethazine	3.2	15.9
Midecamycin	18.1	814.5/174.0	D_{10}-Carbamazepine	1.7	8.5
Norfloxacine	11.9	320.0/302.0	$^{13}C_5$-Sulfamethoxazole	1.2	5.1
Oxolinicacid	17.7	262.0/244.0	$^{13}C_5$-Sulfamethoxazole	1.1	5.7
Spiramycin	13.1	422.0/174.0	$^{13}C_5$-Sulfamethoxazole	1.9	9.5
Streptomycin	11	292.0/231.0	$^{13}C_2$,^{15}N -Acetaminophene	0.9	4.5
Sulfachloropyridazine	15.7	285.0/155.0	$^{13}C_5$-Sulfamethoxazole	1.5	8.2
Sulfadiazine	11.3	251.0/155.0	$^{13}C_5$-Sulfamethazine	0.5	2.7
Sulfadimethoxine	18.5	311.0/155.0	$^{13}C_5$-Sulfamethoxazole	0.9	4.7
Sulfamerazine	12.7	255.0/155.0	$^{13}C_5$-Sulfamethazine	1.1	5.4

Analyte	RT (min)	Precursor – product m/z	Internal Standard	Detection limits (ng/L)	
				MDL	PQL
Sulfameter	14.2	281.0/155.0	$^{13}C_5$-Sulfamethazine	0.9	4.5
Sulfamethazine	13.5	279.0/155.0	$^{13}C_5$-Sulfamethazine	1.1	5.5
Sulfamethoxazole	15.4	254.0/155.0	$^{13}C_5$-Sulfamethoxazole	1.2	5.0
Sulfamonomethoxine	15.2	281.0/155.0	$^{13}C_5$-Sulfamethoxazole	2.1	10.5
Sulfaquinoxaline	18.8	301.0/155.0	$^{13}C_5$-Sulfamethoxazole	1.0	4.9
Sulfathiazole	12.2	255.0/155.0	$^{13}C_5$-Sulfamethazine	1.1	5.5
Sulfisoxazole	17.2	258.0/155.0	$^{13}C_5$-Sulfamethoxazole	1.1	5.3
Thiabendazole	11.2	202.0/175.0	$^{13}C_2$,^{15}N -Acetaminophene	1.0	5.1
Trimethoprime	11.0	291.0/230.0	$^{13}C_2$,^{15}N -Acetaminophene	1.3	5.3
Virginiamycine	22.9	525.3/508.2	D_{10}-Carbamazepine	1.5	7.8
Labeled Compounds					
$^{13}C_5$-Sulfamethoxazole	15.4	250.2/98.2			
$^{13}C_5$-Sulfamethazine	13.5	285.2/98.1			
D_{10}-Carbamazepine	20	247.2/204.2			
$^{13}C_2$,^{15}N -Acetaminophene	10.2	155.1/111.1			

Table 1-4. Group 2 – Neutral extraction, negative electrospray ionization(ESI-) instrument conditions

Instrument	Agilent 1200SL HPLC, AB API 4000 MS/MS
LC Column	Phenomenex Luna C18, 15.0 cm, 3.0 mm id, 3 μ m particle size
Ionization	Positive Ion Electrospray
Acqusition	MRM Mode, unit resolution
Injection Volume	5 μ L

LC Gradient Program		LC Flow Rate (mL/min)	General LC Conditions	
Time(min)	Flow mixture			
10(Equilibrate)	100% Solvent B	0.25	Column Temp	40
10	25% Solvent A 75% Solvent B	0.25	Autosampler tray temperature	4
5	50% Solvent A 50% Solvent B	0.25	MS/MS Condition	
10	75% Solvent A 25% Solvent B	0.25	Source Temp	450
15	90% Solvent A 10% Solvent B	0.25	CUR	20
20	90% Solvent A 10% Solvent B	0.25	GS1/GS2	50/50

Solvent A : 1:1 Acetonitrile / Methanol

Solvent B : 20 mM Ammonium acetate/water

Table 1-4. Group 2 – Neutral extraction, negative electrospray ionization(ESI-) compound retention times (RTs), precursor-product transitions, quantitaion reference, method detection limit, practical quantitaion limit

Analyte	RT (min)	Precursor – product m/z	Internal Standard	Detection limits (ng/L)	
				MDL	PQL
Group 2	Analytes Extracted Under neutral Conditions and analyzed Using ESI(-)				
Native Compounds					
Cimetidine	15.9	251.1/205.1	^{13}C-Naproxen	1.1	5.5
Diclofenac	15.4	294.1/250.1	D_4-Diclofenac	0.5	2.5
Florfenicol	14.2	357.1/337.1	$^{13}C_3$-Ibuprofen	3.3	15.5
Ibuprofen	15.9	205.1/151.0	$^{13}C_3$-Ibuprofen	2.2	11.1
Naproxen	13.3	229.1/185.0	^{13}C-Naproxen	0.8	4.2
Salicylic acid	9.89	137.1/93.1	^{13}C-Naproxen	1.4	7.2
Labeled Compounds					
^{13}C-Naproxen	13.3	233.1/170.0			
$^{13}C_3$-Ibuprofen	15.9	208.2/153.1			
D_4-Diclofenac	15.4	298.1/254.2			

Table 1-5. Concentration of calibration solution (ng/L)

Calibration Standard No	Added Volume(μL) Primary Dilution Standard Solution(500 μg/L) /500mL	Concentration (ng/L)
STD-1	2.5	2.5
STD-2	5	5
STD-3	12.5	10
STD-4	25	25
STD-5	50	50
STD-5	125	100
STD-7	250	250
STD-8	500	500

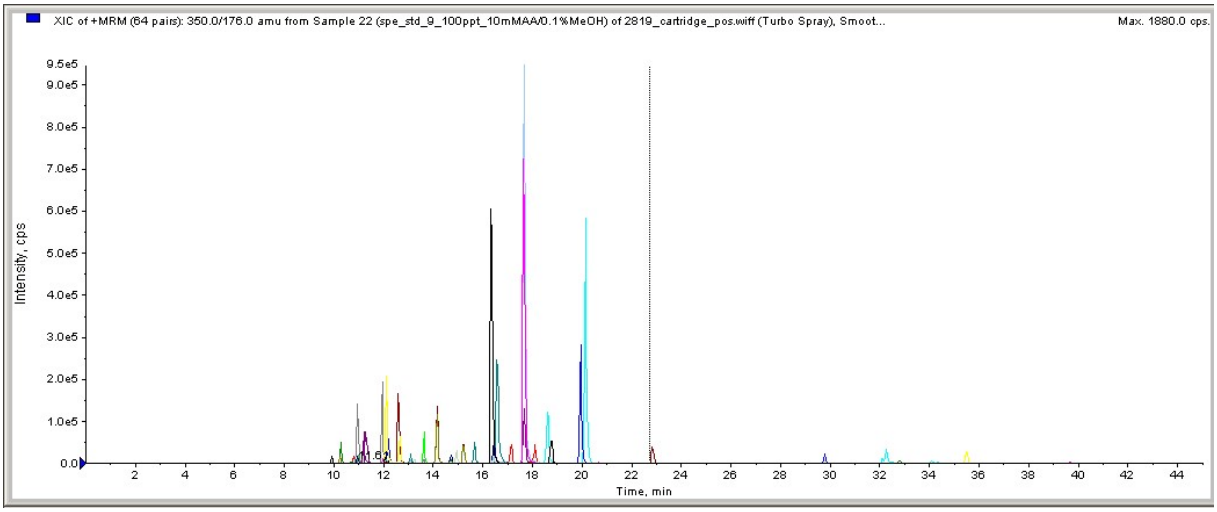

Fig. 1-2. Chromatogram of Pharmaceuticals (ESI Positive mode)

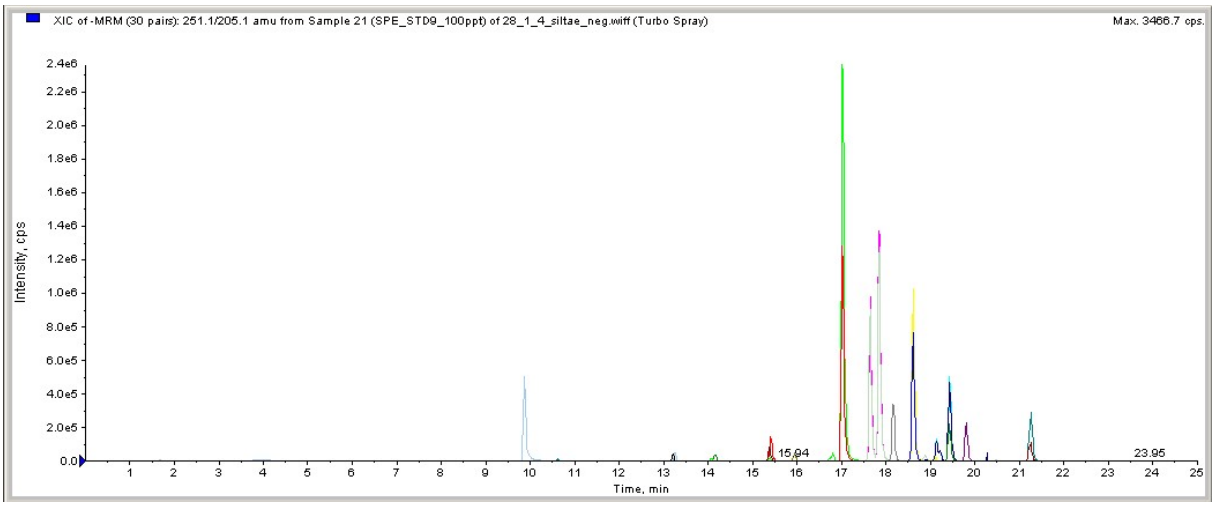

Fig. 1-3. Chromatogram of Pharmaceuticals (ESI Negative mode)

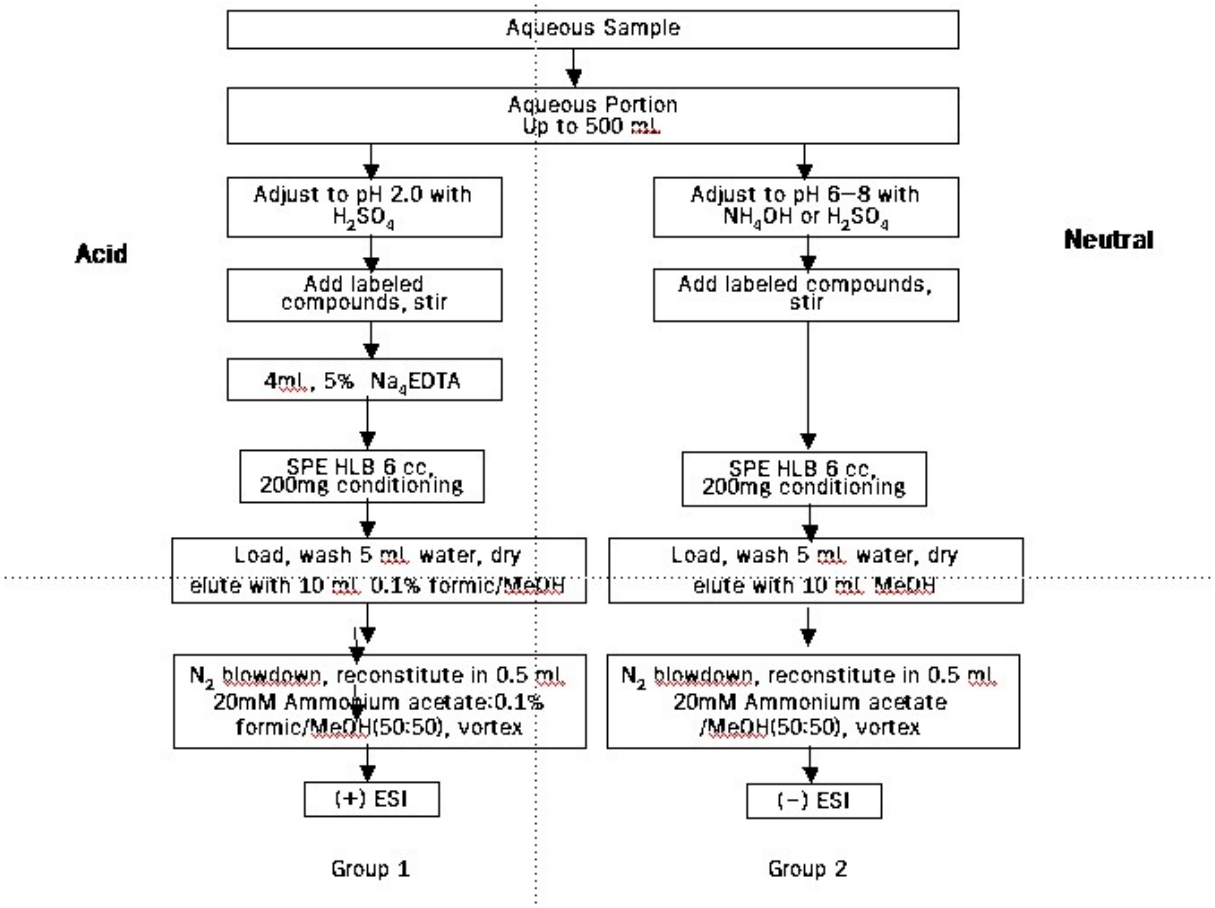

Fig. 1-4. Flow Chart for determination of pharmaceuticals by LC/MS/MS

DETERMINATION OF PERFLUORINATED COMPOUNDS (PFC) BY SOLID-PHASE EXTRACTION FOLLOWED BY LIQUID CHROMATOGRAPHY AND TANDEM MASS SPECTROMETRY

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1.0 SCOPE OF APPLICATION

The procedure can be used for analyzing selected perfluorinated compounds in surface, ground and drinking waters. The application to waste waters has to be checked (see also Section 3.0). The following compounds can be determined by this method.

Analyte	Abbreviation	CAS-No.
Perfluorohexanoic acid	PFHxA	307-24-4
Perfluoroheptanoic acid	PFHpA	375-85-9
Perfluorooctanoic acid	PFOA	335-67-1
Perfluorononanoic acid	PFNA	375-95-1
Perfluorodecanoic acid	PFDA	335-76-2
Perfluoroundecanoic acid	PFUnA	2058-94-8
Perfluorododecanoic acid	PFDoA	307-55-1
Perfluorobutansulfonic acid	PFBS	375-73-5
Perfluorohexansulfonic acid	PFHxS	355-46-4
Perfluorooctansulfonic acid	PFOS	2795-39-3

Analysis of additional perfluorinated compounds with this method may be possible, but has to be checked. The target compounds are pre-concentrated at the original pH of the water sample by solid-phase extraction onto a styrene-divinylbenzene polymer (SDB). Elution of the target compounds is done by methanol. After evaporation of the solvent and reconstitution of the dry residue, an aliquot is injected into the HPLC/MS-MS system.
Method detection limits (MDLs) depend on the compound and on the matrix. For surface, ground and drinking waters the MDLs for all method analytes are in the range of 1 ng/L. The application range is approximately 1 – 50 ng/L. Samples with higher concentrations have to be diluted before extraction.

2.0 DEFINITIONS

2.1 Internal Standard (IS)
A pure analyte(s) added to a sample, extract, or standard solution in known amount(s) and

used to measure the relative responses of other method analytes and surrogates that are components of the same sample or solution. The internal standard must be an analyte that is not a sample component.

2.2 Laboratory Reagent Blank (LRB)
An aliquot of reagent water or other blank matrix that is treated exactly as a sample including exposure to all glassware, equipment, solvents, reagents, internal standards, and surrogates that are used with other samples. The LRB is used to determine if method analytes or other interferences are present in the laboratory environment, the reagents, or the apparatus.

2.3 Laboratory Fortified Blank (LFB)
An aliquot of reagent water or other blank matrix to which known quantities of the method analytes are added in the laboratory. The LFB is analyzed exactly like a sample, and its purpose is to determine whether the methodology is in control, and whether the laboratory is capable of making accurate and precise measurements.

2.4 Stock Standard Solution
A concentrated solution containing one or more method analytes prepared in the laboratory using assayed reference materials or purchased from a reputable commercial source.

2.5 Primary Dilution Standard Solution (PDS)
A solution of several analytes prepared in the laboratory from stock standard solutions and diluted as needed to prepare calibration solutions and other needed analyte solutions.

2.6 Calibration Standard (CAL)
A solution prepared from the primary dilution standard solution or stock standard solutions and the internal standards and surrogate analytes. The CAL solutions are used to calibrate the instrument response with respect to analyte concentration.

2.7 Quality Control Sample (QCS)
A solution of method analytes of known concentrations which is used to fortify an aliquot of LRB or sample matrix. The QCS is obtained from a source external to the laboratory and different from the source of calibration standards. It is used to check laboratory performance with externally prepared test materials.

2.8 Procedural Standard Calibration

A calibration method where aqueous calibration standards are prepared and processed (e.g., purged, extracted, and/or derivatised) in exactly the same manner as a sample. All steps in the process from addition of sampling preservatives through instrumental analyses are included in the calibration. Using procedural standard calibration compensates for any inefficiencies in the processing procedure.

3.0 INTERFERENCES

3.1 Analyses of laboratory reagent blanks provide information about the presence of contaminants. When potential interfering peaks are noted in laboratory reagent blanks, the analyst should search for the source, including instrument, glassware, reagents, or solvents. Subtracting blank values from sample results is not permitted.

3.2 Interfering contamination may occur when a sample containing low concentrations of method analytes is analyzed immediately after a sample containing relatively high concentrations of method analytes. A preventive technique is analyzing blank samples between environmental samples.

3.3 Waste water samples have to be diluted prior to analysis in order to avoid any interference during pre-concentration and analysis.

4.0 INSTRUMENTATION

4.1 HPLC system: HP 1090 II from Agilent Technologies

4.2 Mass spectrometer: API 2000 from Applied Biosystems/MDS Sciex

4.3 Pre-concentration system from J.T. Baker

4.4 Equipment and supplies:

4.4.1 Separation column AquaPerfect 250 x 2.1 mm, 5 µm from MZ-Analysentechnik
4.4.2 Brown glass bottles, nominal volume 500 mL
4.4.3 Glass bottles with screw cap, volume: 500 mL

4.4.4 Vials: 1 mL and 10 mL from Supelco
4.4.5 Micro vial inserts from WGA, volume: 100 µL
4.4.6 Graduated cylinders, nominal volume: 50 mL, 100 mL, 250 mL, 500 mL
4.4.7 Volumetric flasks, nominal volume: 10 mL, 25 mL
4.4.8 Syringes (5 µL, 10 µL, 25 µL, 50 µL, 100 µL, 500 µL) from Hamilton or Agilent Technologies
4.4.9 Analytical balance from Sartorius
4.4.10 pH Meter from Schott instruments
4.4.11 High temperature drying oven (550 °C min.)

5.0 CHEMICALS

5.1 Solvents
All solvents have to be of p.a. purity and shall be stored at room temperature.

5.1.1 Methanol

5.2 Reagents
All reagents have to be of p.a. purity and shall be stored at room temperature.

5.2.1 Ammonium acetate
5.2.2 Ultrapure water

5.3 SPE Material

5.3.1 SDB1 from Bakerbond

5.4 Method analytes
All analytes must be purchased from a reputable commercial source and have to be of p.a. purity. The reference materials have to be stored according to the recommendations from the manufacturer.

5.4.1 Perfluorohexanoic acid
5.4.2 Perfluoroheptanoic acid
5.4.3 Perfluorooctanoic acid

5.4.4 Perfluorononanoic acid
5.4.5 Perfluorodecanoic acid
5.4.6 Perfluoroundecanoic acid
5.4.7 Perfluorododecanoic acid
5.4.8 Perfluorobutansulfonic acid
5.4.9 Perfluorohexansulfonic acid
5.4.10 Perfluorooctansulfonic acid

5.5 Internal Standards

As Internal Standards isotope-labeled chemicals are used. All Internal standards must be purchased from a reputable commercial source and have to be of p.a. purity. They must not contain any non-labeled compounds. The internal standards have to be stored according to the recommendations from the manufacturer.

5.5.1 $^{13}C_4$-Perfluorooctanoic acid
5.5.2 $^{13}C_4$-Perfluorooctansulfonic acid
5.5.3 $^{13}C_2$-Perfluorohexanoic acid

5.6 Stock standard solutions

For each of the target compounds a separate stock solution in methanol is prepared. Their concentrations depend on the solubility of the target compounds in methanol.
For the preparation of each of the stock solutions, between 1 and 10 mg of the pharmaceutical compound are added on an analytical balance to a 10 mL volumetric flask. Then, the volumetric flask is filled up to volume with methanol. From the exact weight of the pharmaceutical and the volume of methanol, the exact concentration of the stock solution is calculated taking into account the purity of the target compounds.
The stock solutions have to be stored in the freezer and are stable for 1 year.

5.7 Primary Dilution Standard (PDS) of the target compounds; $\beta_{\textbf{single compound}} = 0.05$ ng/µL

Few mL of methanol are added to a 25 mL volumetric flask. A defined volume of each stock solution of the method analytes is added with a syringe to achieve a concentration of 0.05 ng/µL. The volumetric flask is filled up to volume with methanol.
The standard mixture of the target compounds has to be stored in the freezer and is stable for 6 months.

5.8 Mixture of the internal standards (IS); β = 0.05 ng/µL

First, stock solutions of the internal standards $^{13}C_4$-perfluorooctanoic acid, $^{13}C_4$-perfluorooctansulfonic acid and $^{13}C_2$-perfluorohexanoic acid are prepared as described in Section 5.6. These stock solutions are diluted with methanol in a 25 mL volumetric flask to a final concentration of 0.05 ng/µL as described in Section 5.7.
The mixture of the internal standards has to be stored in the freezer and is stable for 6 months. Allocation of internal standards to target analytes for quantification is given in Table 2-4 in the Annex.

5.9 Calibration samples of target compounds

Twelve glass bottles (V = 500 mL) are filled with 500 mL tap water with a graduated cylinder. Different volumes of the standard mixture of the target compounds (see Section 5.7) and 100 µL of the standard mixture of the internal standards (see Section 5.8) are added with a syringe. The added volumes as well as the resulting concentration levels are given in Table 2-1 in the Annex.

5.10 Eluent A: Aqueous 10 mM ammonium acetate solution

An aqueous 10 mM ammonium acetate solution is prepared in a 500 mL glass bottle with screw cap. This solution can not be stored and has to be prepared freshly for each day of analysis.

5.11 Eluent B: 10 mM ammonium acetate solution in methanol

An aqueous 10 mM ammonium acetate solution in methanol is prepared in a 500 mL glass bottle with screw cap. This solution can be stored at room temperature for a maximum of 1 week.

6.0 SAMPLE COLLECTION, PRESERVATION, AND STORAGE

6.1 Sample Collection and Dechlorination

Sampling is done in 1000 mL brown glass bottles. If samples, such as finished drinking waters, are suspected to contain residual chlorine, 100 mg of sodium thiosulfate should be added for each 1000 mL of water sample.

6.2 Sample Storage

Storage of samples is done in the cold storage room at 4 °C and must not exceed a time period of 2 weeks. Extracts must be stored in the freezer and are stable for at least 2 weeks.

6.3 Sample preservation

For surface water, groundwater and drinking water samples, no sample preservation is required.

7.0 PROCEDURE

7.1 Extraction

100 µL of the mixture of the internal standards are added to 500 mL water sample. Conditioning of 200 mg SDB1 bakerbond material is done manually with 5 mL methanol and subsequently with 5 mL ultrapure water. For pre-concentration of the target analytes, the water sample is sucked through the conditioned SPE material. Flow rate is approx. 10 mL/min. After pre-concentration, the SPE material is dried for 30 min. with nitrogen gas. Elution of the target analytes is done manually with 10 mL methanol. After passing through the SPE material the elution solvent is collected in a 10 mL vial. Then the solvent is completely evaporated in a stream of nitrogen. The dry residue is reconstituted with 75 µL methanol and 75 µL ultrapure water. Then, the sample is ready for HPLC/MS-MS analysis.

7.2 HPLC/MS-MS analysis

Details on instrumentation and conditions of HPLC/MS-MS analysis are given in Tables 2-2 to 2-4 in the Annex.

7.2.1 Operation of the HPLC/MS-MS system

Operation of the HPLC/MS-MS system is done as described in the manufacturer's manual. Prior to analysis of environmental samples, a blank run is started to check the pressure conditions and to flush the column with solvent. For checking the MS detector, a standard solution is analysed. 50 µL of the standard mixture of the target compounds are evaporated to dryness in a stream of nitrogen and reconstituted with 150 µL of methanol and 150 µL of ultrapure water. Subsequently, 20 µL are injected into the HPLC/MS-MS system. Criteria for checking the performance of the system are signal height, retention time and peak performance.

7.2.2 Calibration

Calibration is done for the overall procedure. Calibration samples are prepared as described in Section 5.9 and analysed. A new calibration is prepared for each batch of samples or whenever the QA/QC sample gives striking results.

8.0 DATA ANALYSIS AND CALCULATIONS

8.1 Complete chromatographic resolution is not necessary for accurate and precise measurements of analyte concentrations if unique ions with adequate intensities are available for quantitation. If the response for any analyte exceeds the linear range of the calibration established in Section 7.2.2, obtain and dilute a duplicate sample. Do not extrapolate beyond the calibration range.

8.2 Evaluation of the chromatograms, i.e. correlation of retention times and mass spectra to target compounds and determination of the peak areas is done by the software tool Analyst 1.1. If necessary, manual integration is done.

8.3 Linear calibration functions are established by plotting the peak area ratios of the respective target analyte and the internal standard against the concentration of the target analyte in the respective calibration solution. Linear regression of the calibration functions provides the calibration coefficients a_1 und a_0 as well as the regression coefficient r.

8.4 Concentrations in real samples are calculated by the software according to the following equation:

$$x = \frac{y - a_0}{a_1} \cdot C_{IS}$$

with x = concentration of the target analyte (ng/L)
y = peak area of the target analyte/peak area of the internal standard
a_1 = slope of the linear calibration function (= sensitivity)
a_0 = intercept of the linear calibration function
C_{IS} = Concentration of the internal standard (ng/L)

8.5 The recovery for the overall procedure is determined by a comparison of peak areas of pre-concentrated samples and direct injections according to the following equation:

$$R = \frac{Y_{pre-concentrated}}{Y_{direct}} \cdot 100\%$$

with $Y_{pre\text{-}concentrated}$ = peak area of a compound after pre-concentration
Y_{direct} = peak area of a compound after direct injection

The recovery is determined each time that the batch of SPE material or elution solvent is changed.

9.0 REPORT

9.1 Specification of results
Results are reported in ng/L with two significant digits.

10.0 QUALITY CONTROL

10.1 QC requirements
Quality control (QC) requirements are the initial demonstration of laboratory capability followed by regular analyses of laboratory reagent blanks, field reagent blanks, and laboratory fortified blanks. A MDL for each analyte must also be determined. Each laboratory must maintain records to document the quality of the data generated. Additional quality control practices are recommended.

10.2 Figures of merit
Figures of merit such as LOD, LOQ, or repeatability are determined during method validation according to the German Standard Procedure DIN 32645.

10.3 Laboratory Blanks

With each batch of samples processed as a group within a work shift, analyze a LRB to determine the background system contamination.

10.4 Calibration Check

In order to check the calibration, a LFB is processed with each batch of samples. For each of the method analytes the result of the LFB must be within 30% of the spiked concentration. If the deviation between spiked and measured concentration exceeds this range, appropriate measures must be taken to improve the quality of analysis before analysis of environmental samples can be continued.

10.5 QC control chart

For QA/QC purposes control charts are used. A QA/QC sample with a concentration of 10 ng/L for each target analyte is analysed each day of analysis.

11.0 POLLUTION PREVENTION

11.1 No solvents are utilized in this method except the extremely small volumes of methanol needed to prepare calibration standards and elution solvents. The only other chemicals used in this method are the neat materials in preparing standards and sample preservatives. All are used in extremely small amounts and pose no threat to the environment.

12.0 WASTE MANAGEMENT

12.1 SPE cartridges are only used once and are disposed with the domestic waste.

12.2 Solutions containing solvents are collected in glass bottles. Full glass bottles are shipped to a special unit for proper waste disposal.

ANNEX

Table 2-1. Added volumes for the preparation of the calibration samples

Calibration sample	Added volume (µL)	Concentration (ng/L)
1	5	0.5
2	10	1
3	25	2.5
4	50	5
5	75	7.5
6	100	10
7	125	12.5
8	150	15
9	200	20
10	250	25
11	300	30
12	350	35

Table 2-2. LC conditions for analysis of perfluorinated compounds

Separation column: MZ-AquaPerfect, 250 mm*2.1 mm, 5 µm
Eluent A: Aqueous 10 mM ammonium acetate solution
Eluent B: 10 mM ammonium acetate solution in methanol
Flow rate: 0.2 mL/min (constant flow)
Injection volume: 20 µL
Gradient:

time in min	A %	B %
0.00	50	50
29.0	10	90
30.0	0	100
34.0	0	100
35.0	50	50

Table 2-3. MS-MS conditions for analysis of perfluorinated compounds

System: Applied Biosystems/MDS Sciex API 2000
Interface: Electrospray
Spray position: z = 1.0 y = 1.5
Heater temperature: 250 °C
Nebuliser Gas (GS1): 40 units
Heater Gas (GS2): 60 units
Ionisation mode: positive
Ionisation voltage: +5500 V
Curtain gas: 30 units
CAD: 3.00

All other parameters depend on the compounds and are documented in the laboratory.

Table 2-4. Detection conditions for analysis of perfluorinated compounds

All compounds are analysed in the Multiple Reaction Monitoring (MRM) mode. Details on the selection of precursor and product ions as well as on the allocation of target compounds to internal standards are summarised in the following table.

Compound	Precursor ion	Product ions	Internal standard
Perfluorohexanoic acid	313.0	269.0, 119.0	$^{13}C_2$-Perfluorohexanoic acid
Perfluoroheptanoic acid	363.0	318.8, 169.0	$^{13}C_4$-Perfluorooctanoic acid
Perfluorooctanoic acid	413.0	369.0, 168.9	$^{13}C_4$-Perfluorooctanoic acid
Perfluorononanoic acid	463.0	418.9, 169.0	$^{13}C_4$-Perfluorooctanoic acid
Perfluorodecanoic acid	513.0	469.0, 218.8	$^{13}C_4$-Perfluorooctanoic acid
Perfluoroundecanoic acid	562.9	519.1, 168.9	$^{13}C_4$-Perfluorooctanoic acid
Perfluorododecanoic acid	613.0	569.0, 169.0	$^{13}C_4$-Perfluorooctanoic acid
Perfluorobutansulfonic acid	298.9	98.9, 79.9	$^{13}C_4$-Perfluorooctansulfonic acid
Perfluorohexansulfonic acid	398.9	98.8, 79.9	$^{13}C_4$-Perfluorooctansulfonic acid
Perfluorooctansulfonic acid	499.0	99.0, 80.0	$^{13}C_4$-Perfluorooctansulfonic acid
$^{13}C_4$-Perfluorooctanoic acid	416.9	371.8, 168.8	-
$^{13}C_4$-Perfluorooctansulfonic acid	503.0	99.0, 80.0	-
$^{13}C_2$-Perfluorohexanoic acid	315.0	269.6	-

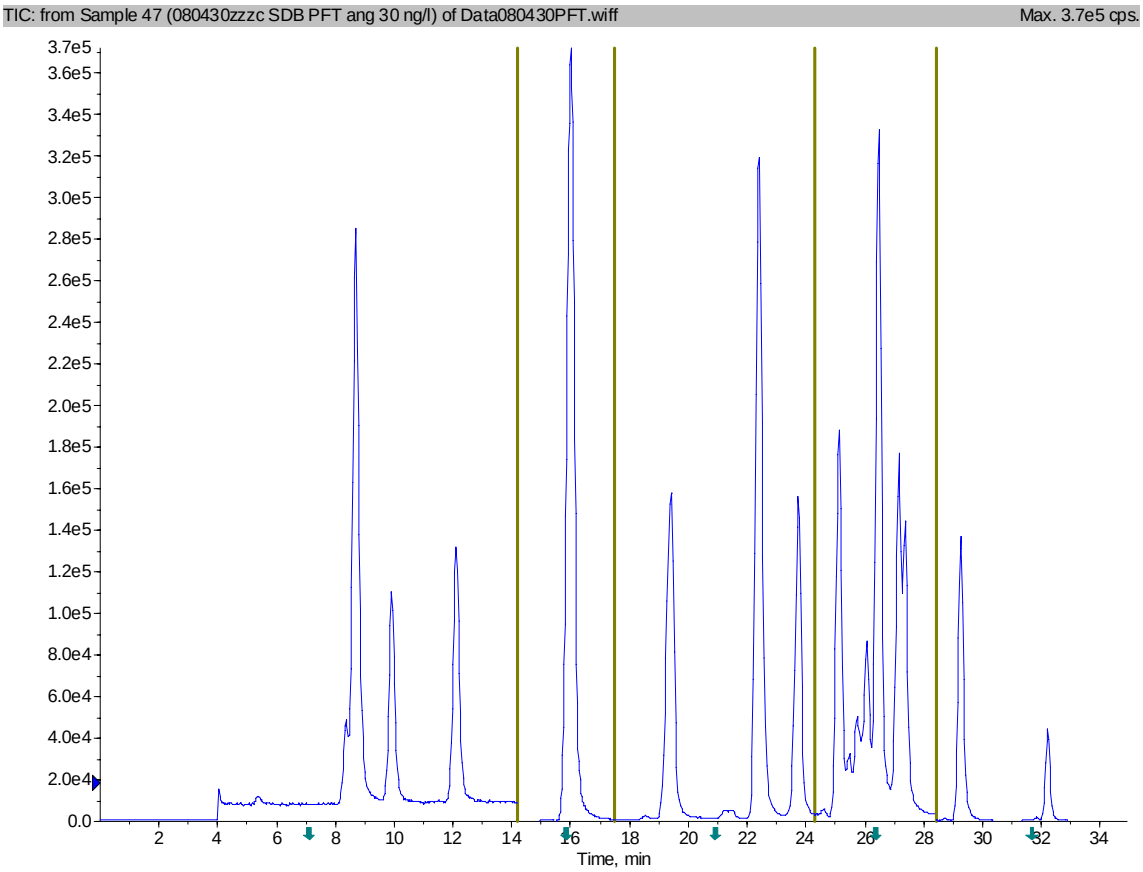

Fig. 2-1 Chromatogram of perfluorinated compounds.

DETERMINATION OF PHARMACEUTICAL RESIDUES BY SOLID-PHASE EXTRACTION FOLLOWED BY LIQUID CHROMATOGRAPHY AND TANDEM MASS SPECTROMETRY

Edited by Prof. H.-J. Brauch and Dr. F. Sacher

TECHNOLOGY CENTER FOR WATER (TZW), KARLSRUHE, GERMANY

1.0 SCOPE OF APPLICATION

The procedure can be used for analyzing selected pharmaceutical residues in surface, ground and drinking waters. The application to waste waters has to be checked (see also Section 3.0). The following compounds can be determined by this method.

Analyte	CAS-No.
Bezafibrate	41859-67-0
Carbamazepine	298-46-4
Clofibric acid	882-09-7
Diazepam	439-14-5
Diclofenac	15307-79-6
Etofibrate	31637-97-5
Fenofibrate	49562-28-9
Fenofibric acid	42017-89-0
Fenoprofen	31879-05-7
Gemfibrozil	25812-30-0
Ibuprofen	15687-27-1
Indometacin	53-86-1
Ketoprofen	22071-15-4
Naproxen	22204-53-1
Pentoxifylline	6493-05-6

Analysis of additional pharmaceuticals with this method may be possible, but has to be checked. After pH adjustment the target compounds are pre-concentrated by solid-phase extraction onto a styrene-divinylbenzene co-polymer (SDB). Elution of the target compounds is done by acetone. After evaporation of the solvent and reconstitution of the dry residue, an aliquot is injected into the HPLC/MS-MS system.
Method detection limits (MDLs) depend on the compound and on the matrix. For surface, ground and drinking waters the MDLs for all method analytes are in the range of 10 ng/L. The application range is approximately 10 – 1000 ng/L. Samples with higher concentrations have to be diluted before extraction.

2.0 DEFINITIONS

2.1 Internal Standard (IS)

A pure analyte(s) added to a sample, extract, or standard solution in known amount(s) and used to measure the relative responses of other method analytes and surrogates that are components of the same sample or solution. The internal standard must be an analyte that is not a sample component.

2.2 Laboratory Reagent Blank (LRB)

An aliquot of reagent water or other blank matrix that is treated exactly as a sample including exposure to all glassware, equipment, solvents, reagents, internal standards, and surrogates that are used with other samples. The LRB is used to determine if method analytes or other interferences are present in the laboratory environment, the reagents, or the apparatus.

2.3 Laboratory Fortified Blank (LFB)

An aliquot of reagent water or other blank matrix to which known quantities of the method analytes are added in the laboratory. The LFB is analyzed exactly like a sample, and its purpose is to determine whether the methodology is in control, and whether the laboratory is capable of making accurate and precise measurements.

2.4 Stock Standard Solution

A concentrated solution containing one or more method analytes prepared in the laboratory using assayed reference materials or purchased from a reputable commercial source.

2.5 Primary Dilution Standard Solution (PDS)

A solution of several analytes prepared in the laboratory from stock standard solutions and diluted as needed to prepare calibration solutions and other needed analyte solutions.

2.6 Calibration Standard (CAL)

A solution prepared from the primary dilution standard solution or stock standard solutions and the internal standards and surrogate analytes. The CAL solutions are used to calibrate the instrument response with respect to analyte concentration.

2.7 Quality Control Sample (QCS)

A solution of method analytes of known concentrations which is used to fortify an aliquot of LRB or sample matrix. The QCS is obtained from a source external to the laboratory and different from the source of calibration standards. It is used to check laboratory performance with externally prepared test materials.

2.8 Procedural Standard Calibration

A calibration method where aqueous calibration standards are prepared and processed (e.g., purged, extracted, and/or derivatised) in exactly the same manner as a sample. All steps in the process from addition of sampling preservatives through instrumental analyses are included in the calibration. Using procedural standard calibration compensates for any inefficiencies in the processing procedure.

3.0 INTERFERENCES

3.1 Analyses of laboratory reagent blanks provide information about the presence of contaminants. When potential interfering peaks are noted in laboratory reagent blanks, the analyst should search for the source, including instrument, glassware, reagents, or solvents. Subtracting blank values from sample results is not permitted.

3.2 Interfering contamination may occur when a sample containing low concentrations of method analytes is analyzed immediately after a sample containing relatively high concentrations of method analytes. A preventive technique is analyzing blank samples between environmental samples.

3.3 Waste water samples have to be diluted prior to analysis in order to avoid any interference during pre-concentration and analysis.

4.0 INSTRUMENTATION

4.1 HPLC system: HP 1100 from Agilent Technologies

4.2 Mass spectrometer: API 2000 from Applied Biosystems/MDS Sciex

4.3 Automated pre-concentration system: Autotrace SPE Workstations from Zymark/Caliper

4.4 Equipment and supplies:

4.4.1 Separation column 250 x 2 mm, 5 μm C18 Luna from Phenomenex

4.4.2 Brown glass bottles, nominal volume 1000 mL
4.4.3 Glass bottles with screw cap, volume: 500 mL
4.4.4 Vials: 1 mL and 10 mL from Supelco
4.4.5 Micro vial inserts from WGA, volume: 100 µL
4.4.6 Graduated cylinders, nominal volume: 100 mL, 250 mL, 500 mL, 1000 mL
4.4.7 Volumetric flasks, nominal volume: 10 mL, 50 mL
4.4.8 Syringes (5 µL, 10 µL, 25 µL, 50 µL, 100 µL, 500 µL, 1000 µL) from Hamilton or Agilent Technologies
4.4.9 Analytical balance from Sartorius
4.4.10 pH Meter from Schott instruments
4.4.11 Drying oven

5.0 CHEMICALS

5.1 Solvents
All solvents have to be of p.a. purity and shall be stored at room temperature.

5.1.1 Acetone
5.1.2 Acetonitrile
5.1.3 Methanol

5.2 Reagents
All reagents have to be of p.a. purity and shall be stored at room temperature.

5.2.1 Ammonium acetate
5.2.2 Ammonium formate
5.2.3 Caustic soda
5.2.4 Hydrochloric acid, 32%
5.2.5 Demineralised and decoct water

5.3 SPE Material

5.3.1 SDB1 from Bakerbond

5.4 Method analytes

All analytes must be purchased from a reputable commercial source and have to be of p.a. purity. The reference materials have to be stored according to the recommendations from the manufacturer.

5.4.1 Bezafibrate
5.4.2 Carbamazepine
5.4.3 Clofibric acid
5.4.4 Diazepam
5.4.5 Diclofenac (sodium salt)
5.4.6 Etofibrate
5.4.7 Fenofibrate
5.4.8 Fenofibric acid
5.4.9 Fenoprofen (calcium salt)
5.4.10 Gemfibrozil
5.4.11 Ibuprofen
5.4.12 Indometacin
5.4.13 Ketoprofen
5.4.14 Naproxen
5.4.15 Pentoxifylline

5.5 Internal Standards

As Internal Standards isotope-labeled pharmaceuticals are used. All Internal standards must be purchased from a reputable commercial source and have to be of p.a. purity. They must not contain any non-labeled compounds. The internal standards have to be stored in the refrigerator.

5.5.1 Carbamazepine-d10
5.5.2 Diclofenac-d4
5.5.3 Ibuprofen-d3

5.6 Stock standard solutions

For each of the target compounds a separate stock solution in methanol is prepared. Their concentrations depend on the solubility of the target compounds in methanol.
For the preparation of each of the stock solutions, between 1 and 10 mg of the pharmaceutical compound are added on an analytical balance to a 10 mL volumetric flask. Then, the

volumetric flask is filled up to volume with methanol. From the exact weight of the pharmaceutical and the volume of methanol, the exact concentration of the stock solution is calculated taking into account the purity of the target compounds.
The stock solutions have to be stored in the freezer and are stable for 1 year.

5.7 Primary Dilution Standard (PDS) of the target compounds; $\beta_{\textbf{single compound}}$ = 1 ng/µL

Few mL of methanol are added to a 50 mL volumetric flask. A defined volume of each stock solution of the method analytes is added with a syringe to achieve a concentration of 1 ng/µL. Tthe volumetric flask is filled up to volume with methanol. An example for the preparation of PDS of the target compounds is given in Table 3-1 in the Annex.
The PDS has to be stored in the freezer and is stable for 6 months.

5.8 Standard mixture for Quality Control Sample

The standard mixture for the QCS contains the following pharmaceuticals:

- carbamazepine
- diclofenac
- clofibric acid
- ibuprofen.

For preparation of the standard mixture for the QA/QC sample, few mL of methanol are added to a 25 mL volumetric flask. A defined volume of each stock solution of each target compound is added with a syringe to achieve a final concentration of 1 ng/µL. The volumetric flask is filled up to volume with methanol.
The QA/QC sample of the target compounds has to be stored in the freezer and is stable for 6 months.

5.9 Mixture of the internal standards (IS); β = 1 ng/µL

First, stock solutions of the internal standards carbamazepine-d10, diclofenac-d4 and ibuprofen-d3 are prepared as described in Section 5.6. These stock solutions are diluted with methanol in a 25 mL volumetric flask to a final concentration of 1 ng/µL as described in Section 5.7.
The mixture of the internal standards has to be stored in the freezer and is stable for 1 year.

5.10 Calibration samples of target compounds

Ten glass bottles (V = 1000 mL) are filled with 1000 mL tap water with a graduated cylinder. Different volumes of the standard mixture of the target compounds (see Section 5.7) and 100 µL of the standard mixture of the internal standards (see Section 5.9) are added with a syringe. The added volumes as well as the resulting concentration levels are given in Table 3-2 in the Annex.

5.11 Eluent A (positive mode): Aqueous 20 mM ammonium formate solution

An aqueous 20 mM ammonium formate solution is prepared in a 500 mL glass bottle with screw cap. This solution can not be stored and has to be prepared freshly for each day of analysis.

5.12 Eluent B (positive mode): 2:1 (v:v) mixture of acetonitrile and methanol

In a 500 mL glass bottle with screw cap, two parts per volume acetonitrile are mixed with one part per volume of methanol by using a graduated cylinder. Then a defined amount of ammonium formate is added to achieve a final concentration of 20 mM.
This solution can be stored at room temperature for a maximum of 1 week.

5.13 Eluent A (negative mode): Aqueous 20 mM ammonium acetate solution

An aqueous 20 mM ammonium acetate solution is prepared in a 500 mL glass bottle with screw cap. This solution can not be stored and has to be prepared freshly for each day of analysis.

5.14 Eluent B (negative mode): 2:1 (v:v) mixture of acetonitrile and methanol

In a 500 mL glass bottle with screw cap, two parts per volume acetonitrile are mixed with one part per volume of methanol by using a graduated cylinder. Then a defined amount of ammonium acetate is added to achieve a final concentration of 20 mM.
This solution can be stored at room temperature for a maximum of 1 week.

6.0 SAMPLE COLLECTION, PRESERVATION, AND STORAGE

6.1 Sample Collection and Dechlorination

Sampling is done in 1000 mL or 2000 mL brown glass bottles. If samples, such as finished drinking waters, are suspected to contain residual chlorine, 100 mg of sodium thiosulfate should be added for each 1000 mL of water sample.

6.2 Sample Storage

Storage of samples is done in the cold storage room at 4 °C and must not exceed a time period of 3 weeks. Extracts must be stored in the freezer and are stable for at least 2 weeks.

6.3 Sample preservation

For surface water, groundwater and drinking water samples, no sample preservation is required.

7.0 PROCEDURE

7.1 Extraction

1000 mL of water sample are adjusted to pH 3 by addition of 32% hydrochloric acid. pH is checked with a pH meter. Then, 100 µL of the mixture of the internal standards are added. Conditioning of 200 mg SDB1 bakerbond material is done automatically (Autotrace system) with 6 mL acetone and subsequently with 2 x 6 mL demineralised and decoct water. For pre-concentration of the target analytes, the pH adjusted water sample is automatically pumped through the conditioned SPE material by the Autotrace system at a flow rate of 10 mL/min. After pre-concentration, the SPE material is dried for 60 min. with nitrogen gas. Elution of the target analytes is done automatically by the Autotrace system with 10 mL acetone. After passing through the SPE material the elution solvent is collected in a 10 mL vial. The solvent is completely evaporated in a stream of nitrogen. The dry residue is reconstituted with 100 µL methanol. Then, the sample is ready for HPLC/MS-MS analysis.

7.2 HPLC/MS-MS analysis

Details on instrumentation and conditions of HPLC/MS-MS analysis are given in Tables 3-3 to 3-5 in the Annex.

7.2.1 Operation of the HPLC/MS-MS system

Operation of the HPLC/MS-MS system is done as described in the manufacturer's manual. Prior to analysis of environmental samples, a blank run is started to check the pressure conditions and to flush the column with solvent. For checking the MS detector, a standard solution is analysed. 25 µL of the standard mixture of the target compounds are evaporated to dryness in a stream of nitrogen and reconstituted with 100 µL of methanol. Subsequently, 12.5 µL are injected into the HPLC/MS-MS system. Criteria for checking the performance of the system are signal height, retention time and peak performance.

7.2.2 Calibration

Calibration is done for the overall procedure. Calibration samples are prepared as described in Section 5.10 and analysed. A new calibration is prepared for each batch of samples or whenever the QA/QC sample gives striking results.

8.0 DATA ANALYSIS AND CALCULATIONS

8.1 Complete chromatographic resolution is not necessary for accurate and precise measurements of analyte concentrations if unique ions with adequate intensities are available for quantitation. If the response for any analyte exceeds the linear range of the calibration established in Section 7.2.2, obtain and dilute a duplicate sample. Do not extrapolate beyond the calibration range.

8.2 Evaluation of the chromatograms, i.e. correlation of retention times and mass spectra to target compounds and determination of the peak areas is done by the software tool Analyst 1.2. If necessary, manual integration is done.

8.3 Linear calibration functions are established by plotting the peak area ratios of the respective target analyte and the internal standard against the concentration of the target analyte in the respective calibration solution. Linear regression of the calibration functions provides the calibration coefficients a_1 und a_0 as well as the regression coefficient r. In total, 2 linear calibration functions are established. Calibration function 1 is valid for the concentration

range from 10 to 100 ng/L, calibration function 2 for the concentration range from 100 to 1000 ng/L.

8.4 Concentrations in real samples are calculated by the software according to the following equation:

$$x = \frac{y - a_0}{a_1} \cdot C_{IS}$$

with x = concentration of the target analyte (ng/L)

y = peak area of the target analyte/peak area of the internal standard

a_1 = slope of the linear calibration function (= sensitivity)

a_0 = intercept of the linear calibration function

C_{IS} = Concentration of the internal standard (ng/L)

8.5 The recovery for the overall procedure is determined by a comparison of peak areas of pre-concentrated samples and direct injections according to the following equation:

$$R = \frac{Y_{pre-concentrated}}{Y_{direct}} \cdot 100\%$$

with $Y_{pre\text{-}concentrated}$ = peak area of a compound after pre-concentration

Y_{direct} = peak area of a compound after direct injection

The recovery is determined each time that the batch of SPE material or elution solvent is changed.

9.0 REPORT

9.1 Specification of results

Results are reported in ng/L with two significant digits.

10.0 QUALITY CONTROL

10.1 QC requirements

Quality control (QC) requirements are the initial demonstration of laboratory capability followed by regular analyses of laboratory reagent blanks, field reagent blanks, and laboratory fortified blanks. A MDL for each analyte must also be determined. Each

laboratory must maintain records to document the quality of the data generated. Additional quality control practices are recommended.

10.2 Figures of merit

Figures of merit such as LOD, LOQ, or repeatability are determined during method validation according to the German Standard Procedure DIN 32645.

10.3 Laboratory Blanks

With each batch of samples processed as a group within a work shift, analyze a LRB to determine the background system contamination.

10.4 Calibration Check

In order to check the calibration, a LFB is processed with each batch of samples. For each of the method analytes the result of the LFB must be within 30% of the spiked concentration. If the deviation between spiked and measured concentration exceeds this range, appropriate measures must be taken to improve the quality of analysis before analysis of environmental samples can be continued.

10.5 QC control chart

For QA/QC purposes control charts are used. A QA/QC sample is analysed each day of analysis.

10.5.1 The QA/QC samples are prepared by adjusting 1000 mL of tap water to a pH of 3 by addition of 32% hydrochloric acid. Then 100 µL of the mixture of the internal standards and 75 µL of the standard mixture for the QA/QC sample are added. The QA/QC sample is treated as any environmental sample according to the procedure described in Section 7.0. The results of these QA/QC measurements for carbamazepine, diclofenac, clofibric acid and ibuprofen are evaluated and documented in control charts by the SQS software from Perkin Elmer.

11.0 POLLUTION PREVENTION

11.1 No solvents are utilized in this method except the extremely small volumes of methanol and acetonitrile needed to prepare calibration standards and elution solvents. The only other chemicals used in this method are the neat materials in preparing standards and sample preservatives. All are used in extremely small amounts and pose no threat to the environment.

12.0 WASTE MANAGEMENT

12.1 SPE cartridges are only used once and are disposed with the domestic waste.

12.2 Solutions containing solvents are collected in glass bottles. Full glass bottles are shipped to a special unit for proper waste disposal.

13.0 REFERENCES

1. F. Sacher, F.T. Lange, H.-J. Brauch, I. Blankenhorn. “Pharmaceuticals in groundwaters - Analytical methods and results of a monitoring program in Baden-Württemberg, Germany.” J. Chromatogr. A 938:199-210 (2001)

ANNEX

Table 3-1. Example for the preparation of a Primary Dilution Standard (PDS) of the target compounds

Compound	Concentration of stock solution (g/L)	Volume of stock solution added to 50 mL (µL)	Concentration in mixture (ng/µL)
Bezafibrate	0.242	206.6	0.9999
Carbamazepine	0.204	245.1	1.0000
Clofibric acid	0.265	188.7	1.0001
Diazepam	1.000	50.0	1.000
Diclofenac	0.204	245.0	0.9996
Etofibrate	0.230	217.4	1.0000
Fenofibrate	0.420	119.0	0.9996
Fenofibric acid	0.284	176.1	1.0002
Fenoprofen	0.186	268.3	0.9981
Gemfibrozil	0.187	267.4	1.0001
Ibuprofen	0.203	246.3	1.0000
Indometacin	0.217	230.4	0.9999
Ketoprofen	0.213	234.7	0.9998
Naproxen	0.185	270.3	1.0001
Pentoxifylline	0.268	186.6	1.0002

Table 3-2. Added volumes for the preparation of the calibration samples

Calibration sample	Added volume (µL)	Concentration (ng/L)
1	5	5
2	10	10
3	25	25
4	50	50
5	75	75
6	100	100
7	250	250
8	500	500
9	750	750
10	1000	1000

Table 3-3. LC conditions for analysis of pharmaceuticals

Positive mode

Separation column: Luna C18, 250 mm*2 mm, 5 µm

Eluent A: Aqueous 20 mM ammonium formate solution (pH 7.0)

Eluent B: Mixture of 2 parts per volume acetonitrile and one part per volume methanol, 20 mM ammonium formate

Flow rate: 0.2 mL/min (constant flow)

Injection volume: 12.5 µL

Gradient:

time in min	A %	B %
0.00	80	20
20.0	0	100
31.0	0	100
32.0	80	20
34.0	80	20

Negative mode

Separation column: Luna C18, 250 mm*2 mm, 5 µm

Eluent A: Aqueous 20 mM ammonium acetate solution (pH 7.0)

Eluent B: Mixture of 2 parts per volume acetonitrile and one part per volume methanol, 20 mM ammonium acetate

Flow rate: 0.2 mL/min (constant flow)

Injection volume: 12.5 µL

Gradient:

time in min	A %	B %
0.00	80	20
10.0	0	100
18.0	0	100
19.0	80	20
20.0	80	20

Table 3-4. MS-MS conditions for analysis of pharmaceuticals

Positive/negative mode

System: Applied Biosystems/MDS Sciex API 2000
Interface: Electrospray
Spray position: z = 4.0 y = 3.5
Heater temperature: 200 °C
Nebuliser Gas (GS1): 45 units
Heater Gas (GS2): 65 units
Ionisation voltage: +5000 V (positive mode) and -4200 (negative mode)
Curtain gas: 45 units
CAD: 2.00

All other parameters depend on the compounds and are documented in the laboratory.

Table 3-5. Detection conditions for analysis of pharmaceuticals

All compounds are analysed in the Multiple Reaction Monitoring (MRM) mode. Details on the selection of precursor and product ions as well as on the allocation of target compounds to internal standards are summarised in the following table.

Compound	Precursor ion	Product ions	Internal standard
Bezafibrate	362.0	121.1, 138.8	Carbamazepine-d10
Carbamazepine	237.0	164.9, 193.9	Carbamazepine-d10
Clofibric acid	212.8	84.8, 126.8	Diclofenac-d4
Diclofenac	296.0	213.8, 249.8	Diclofenac-d4
Fenofibrate	361.0	138.9, 232.9	Carbamazepine-d10
Fenofibric acid	319.0	139.0, 232.9	Diclofenac-d4
Fenoprofen	240.8	93.0, 196.8	Diclofenac-d4
Gemfibrozil	268.1	83.1, 128.9	Diclofenac-d4
Ibuprofen	204.9	159.4, 160.9	Ibuprofen-d3
Indometacin	375.0	110.8, 138.8	Diclofenac-d4
Ketoprofen	254.9	105.0, 208.9	Diclofenac-d4
Pentoxifylline	278.9	99.0, 138.1	Carbamazepine-d10
Carbamazepine-d10	247.1	204.0	-
Diclofenac-d4 (positive mode)	300.2	217.9	-
Diclofenac-d4 (negative mode)	298.1	253.9	-
Ibuprofen-d3	208.1	163.9	-

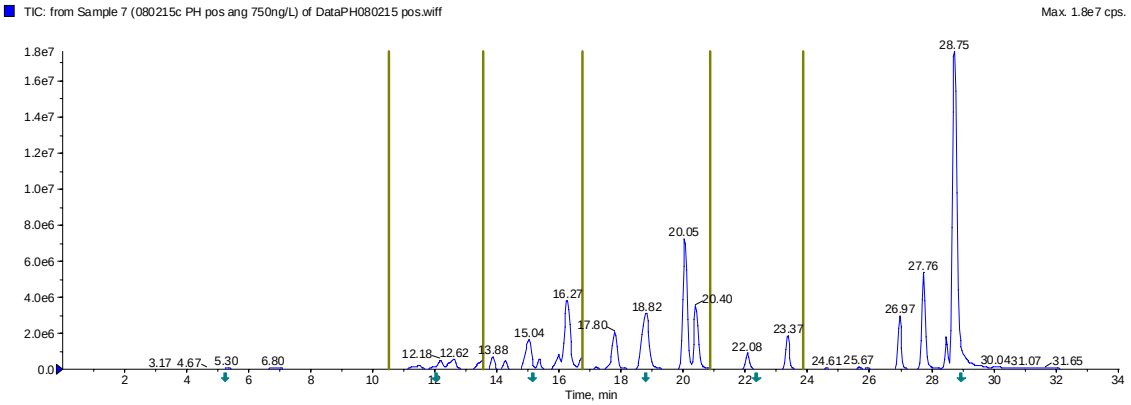

Fig. 3-1 Chromatogram of pharmaceuticals (positive mode).

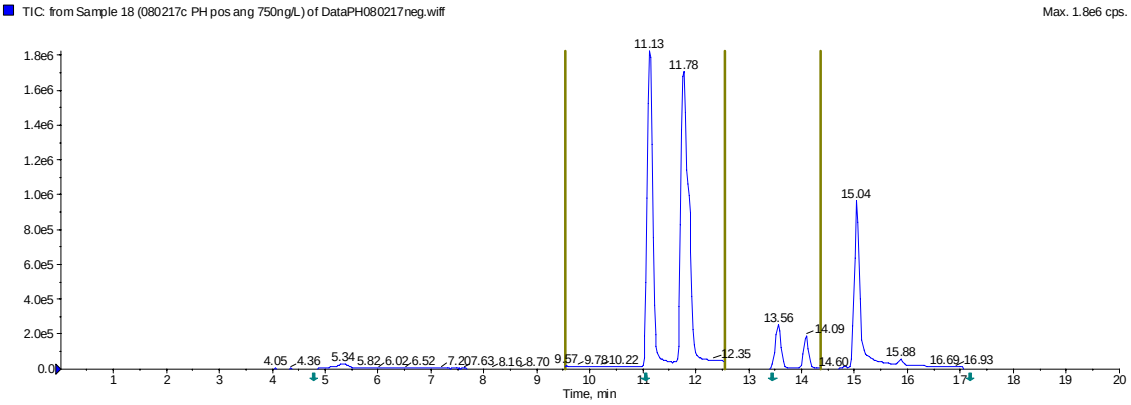

Fig. 3-2 Chromatogram of pharmaceuticals (negative mode).

DETERMINATION OF BETABLOCKERS AND OTHER PHARMACEUTICALS BY SOLID-PHASE EXTRACTION FOLLOWED BY LIQUID CHROMATOGRAPHY AND TANDEM MASS SPECTROMETRY

Edited by Prof. H.-J. Brauch and Dr. F. Sacher

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1.0 SCOPE OF APPLICATION

The procedure can be used for analyzing selected pharmaceutical residues in surface, ground and drinking waters. The application to waste waters has to be checked (see also Section 3.0). The following compounds can be determined by this method.

Analyte	CAS-No.
Atenolol	29122-68-7
Betaxolol	63659-18-7
Bisoprolol	66722-44-9
Clenbuterol	37148-27-9
Metoprolol	37350-58-6
Pindolol	13523-86-6
Propranolol	525-66-6
Salbutamol	18559-94-9
Sotalol	3930-20-9
Terbutaline	23031-25-6
Phenazone	60-80-0
Dimethylaminophenazone	58-15-1
Propyphenazone	479-82-5
Simvastatin	79902-63-9
Cyclophosphamide	50-18-0
Ifosfamide	3778-73-2

Analysis of additional pharmaceuticals with this method may be possible, but has to be checked. After pH adjustment the target compounds are pre-concentrated by solid-phase extraction onto a polymeric material (bondelut PPL). Elution of the target compounds is done by methanol. After evaporation of the solvent and reconstitution of the dry residue, an aliquot is injected into the HPLC/MS-MS system.
Method detection limits (MDLs) depend on the compound and on the matrix. For surface, ground and drinking waters the MDLs for all method analytes are in the range of 10 ng/L. The application range is approximately 10 – 1000 ng/L. Samples with higher concentrations have to be diluted before extraction.

2.0 DEFINITIONS

2.1 Laboratory Reagent Blank (LRB)
An aliquot of reagent water or other blank matrix that is treated exactly as a sample including exposure to all glassware, equipment, solvents, reagents, internal standards, and surrogates that are used with other samples. The LRB is used to determine if method analytes or other interferences are present in the laboratory environment, the reagents, or the apparatus.

2.2 Laboratory Fortified Blank (LFB)
An aliquot of reagent water or other blank matrix to which known quantities of the method analytes are added in the laboratory. The LFB is analyzed exactly like a sample, and its purpose is to determine whether the methodology is in control, and whether the laboratory is capable of making accurate and precise measurements.

2.3 Stock Standard Solution
A concentrated solution containing one or more method analytes prepared in the laboratory using assayed reference materials or purchased from a reputable commercial source.

2.4 Primary Dilution Standard Solution (PDS)
A solution of several analytes prepared in the laboratory from stock standard solutions and diluted as needed to prepare calibration solutions and other needed analyte solutions.

2.5 Calibration Standard (CAL)
A solution prepared from the primary dilution standard solution or stock standard solutions and the internal standards and surrogate analytes. The CAL solutions are used to calibrate the instrument response with respect to analyte concentration.

2.6 Quality Control Sample (QCS)
A solution of method analytes of known concentrations which is used to fortify an aliquot of LRB or sample matrix. The QCS is obtained from a source external to the laboratory and different from the source of calibration standards. It is used to check laboratory performance with externally prepared test materials.

2.7 Procedural Standard Calibration
A calibration method where aqueous calibration standards are prepared and processed (e.g., purged, extracted, and/or derivatised) in exactly the same manner as a sample. All steps in the

process from addition of sampling preservatives through instrumental analyses are included in the calibration. Using procedural standard calibration compensates for any inefficiencies in the processing procedure.

3.0 INTERFERENCES

3.1 Analyses of laboratory reagent blanks provide information about the presence of contaminants. When potential interfering peaks are noted in laboratory reagent blanks, the analyst should search for the source, including instrument, glassware, reagents, or solvents. Subtracting blank values from sample results is not permitted.

3.2 Interfering contamination may occur when a sample containing low concentrations of method analytes is analyzed immediately after a sample containing relatively high concentrations of method analytes. A preventive technique is analyzing blank samples between environmental samples.

3.3 Waste water samples have to be diluted prior to analysis in order to avoid any interference during pre-concentration and analysis.

4.0 INSTRUMENTATION

4.1 HPLC system: HP 1100 from Agilent Technologies

4.2 Mass spectrometer: API 2000 from Applied Biosystems/MDS Sciex

4.3 Automated pre-concentration system: Autotrace SPE Workstations from Zymark/Caliper

4.4 Equipment and supplies:

4.4.1 Separation column 250 x 2 mm, 3 µm Nucleosil from Bischoff
4.4.2 Brown glass bottles, nominal volume 1000 mL
4.4.3 Glass bottles with screw cap, volume: 500 mL
4.4.4 Vials: 1 mL and 5 mL from Supelco
4.4.5 Micro vial inserts from WGA, volume: 100 µL

4.4.6 Graduated cylinders, nominal volume: 100 mL, 250 mL, 500 mL, 1000 mL
4.4.7 Volumetric flasks, nominal volume: 10 mL, 50 mL
4.4.8 Syringes (5 µL, 10 µL, 25 µL, 50 µL, 100 µL, 500 µL, 1000 µL) from Hamilton or Agilent Technologies
4.4.9 Analytical balance from Sartorius
4.4.10 pH Meter from Schott instruments
4.4.11 Drying oven

5.0 CHEMICALS

5.1 Solvents
All solvents have to be of p.a. purity and shall be stored at room temperature.

5.1.1 Acetone
5.1.2 Acetonitrile
5.1.3 Methanol
5.1.4 n-Hexane

5.2 Reagents
All reagents have to be of p.a. purity and shall be stored at room temperature.

5.2.1 Ammonium acetate
5.2.2 Ammonium formate
5.2.3 Caustic soda
5.2.4 Hydrochloric acid, 32%
5.2.5 Demineralised and decoct water

5.3 SPE Material

5.3.1 Bond elute PPL from Varian

5.4 Method analytes
All analytes must be purchased from a reputable commercial source and have to be of p.a. purity. The reference materials have to be stored according to the recommendations from the manufacturer.

5.4.1 Atenolol
5.4.2 Betaxolol (adduct with hydrochloric acid)
5.4.3 Bisoprolol (as fumarate)
5.4.4 Clenbuterol (adduct with hydrochloric acid)
5.4.5 Metoprolol (as tartrate)
5.4.6 Pindolol
5.4.7 Propranolol (adduct with hydrochloric acid)
5.4.8 Salbutamol
5.4.9 Sotalol (adduct with hydrochloric acid)
5.4.10 Terbutaline (as hemisulfate)
5.4.11 Phenazone
5.4.12 Dimethylaminophenazone
5.4.13 Propyphenazone
5.4.14 Simvastatin
5.4.15 Cyclophosphamide (as monohydrate)
5.4.16 Ifosfamide

5.5 Stock standard solutions

For each of the target compounds a separate stock solution in methanol is prepared. Their concentrations depend on the solubility of the target compounds in methanol.
For the preparation of each of the stock solutions, between 1 and 10 mg of the pharmaceutical compound are added on an analytical balance to a 10 mL volumetric flask. The volumetric flask is filled up to volume with acetone (for phenazone, dimethylaminophenazone, and propyphenazone) or methanol (for all other target compounds). From the exact weight of the pharmaceutical and the volume of acetone or methanol, respectively, the exact concentration of the stock solution is calculated taking into account the purity of the target compounds.
The stock solutions have to be stored in the freezer and are stable for 1 year.

5.6 Primary Dilution Standard (PDS) of the target compounds; $\beta_{\textbf{single compound}} = 1$ ng/µL

Few mL of methanol are added to a 50 mL volumetric flask. A defined volume of each stock solution of the method analytes is added with a syringe to achieve a concentration of 1 ng/µL.

The volumetric flask is filled up to volume with methanol. An example for the preparation of this PDS is given in Table 4-1 in the Annex.
The PDS of the target compounds has to be stored in the freezer and is stable for 6 months.

5.7 Standard mixture for Quality Control Sample

The standard mixture for the QCS contains the following pharmaceuticals:

- atenolol
- sotalol
- phenazone.

For preparation of the standard mixture for the QA/QC sample, few mL of methanol are added to a 25 mL volumetric flask. A defined volume of each stock solution of each target compound is added with a syringe to achieve a final concentration of 1 ng/µL. The volumetric flask is filled up to volume with methanol.
The QA/QC sample of the target compounds has to be stored in the freezer and is stable for 6 months.

5.8 Solvent mixture for reconstitution of the dry residue

In a 100 mL graduated cylinder a mixture of 5 parts per volume acetonitrile and 95 parts per volume of an aqueous solution of 20 mM ammonium acetate is prepared.
This solution can be stored in the refrigerator for a maximum of 1 week.

5.9 Calibration samples of target compounds

Ten glass bottles (V = 1000 mL) are filled with 1000 mL tap water using a graduated cylinder. Then different volumes of the standard mixture of the target compounds (see Section 5.6) are added with a syringe. The added volumes as well as the resulting concentration levels are given in Table 4-2 in the Annex.

5.10 Eluent A: Aqueous 20 mM ammonium acetate solution

An aqueous 20 mM ammonium acetate solution is prepared in a 500 mL glass bottle with screw cap. This solution can not be stored and has to be prepared freshly for each day of analysis.

5.11 Eluent B: 2:1 (v:v) mixture of acetonitrile and methanol

In a 500 mL glass bottle with screw cap, two parts per volume acetonitrile are mixed with one part per volume of methanol by using a graduated cylinder. Then a defined amount of ammonium acetate is added to achieve a final concentration of 20 mM.
This solution can be stored at room temperature for a maximum of 1 week.

6.0 SAMPLE COLLECTION, PRESERVATION, AND STORAGE

6.1 Sample Collection and Dechlorination

Sampling is done in 1000 mL or 2000 mL brown glass bottles. If samples, such as finished drinking waters, are suspected to contain residual chlorine, 100 mg of sodium thiosulfate should be added for each 1000 mL of water sample.

6.2 Sample Storage

Storage of samples is done in the cold storage room at 4 °C and must not exceed a time period of 3 weeks. Extracts must be stored in the freezer and are stable for at least 2 weeks.

6.3 Sample preservation

For surface water, groundwater and drinking water samples, no sample preservation is required.

7.0 PROCEDURE

7.1 Extraction

1000 mL of water sample are adjusted to pH 7 by addition of 32% hydrochloric acid or caustic soda. pH is checked with a pH meter. Conditioning of 200 mg Bond elut PPL material

is done automatically (Autotrace system) with 4 mL n-hexane, 2 x 3 mL methanol, 1 mL methanol and subsequently with 2 x 3 mL demineralised and decoct water. For pre-concentration of the target analytes, the pH adjusted water sample is automatically pumped through the conditioned SPE material by the Autotrace system. Flow rate is 10 mL/min. After pre-concentration, the SPE material is dried for 60 min. with nitrogen gas. Elution of the target analytes is done automatically by the Autotrace system with 5 mL methanol. After passing through the SPE material the elution solvent is collected in a 5 mL vial. Then the solvent is completely evaporated in a stream of nitrogen. The dry residue is reconstituted with 40 µL methanol and subsequently with 60 µL of the solvent mixture (see Section 5.8). Then, the sample is ready for HPLC/MS-MS analysis.

7.2 HPLC/MS-MS analysis

Details on instrumentation and conditions of HPLC/MS-MS analysis are given in Tables 4-3 to 5 in the Annex.

7.2.1 Operation of the HPLC/MS-MS system

Operation of the HPLC/MS-MS system is done as described in the manufacturer's manual. Prior to analysis of environmental samples, a blank run is started to check the pressure conditions and to flush the column with solvent. For checking the MS detector, a standard solution is analysed. 25 µL of the standard mixture of the target compounds are evaporated to dryness in a stream of nitrogen and reconstituted with 100 µL of methanol. Subsequently, 12.5 µL are injected into the HPLC/MS-MS system. Criteria for checking the performance of the system are signal height, retention time and peak performance.

7.2.2 Calibration

Calibration is done for the overall procedure. Calibration samples are prepared as described in Section 5.9 and analysed. A new calibration is prepared for each batch of samples or whenever the QA/QC sample gives striking results.

8.0 DATA ANALYSIS AND CALCULATIONS

8.1 Complete chromatographic resolution is not necessary for accurate and precise measurements of analyte concentrations if unique ions with adequate intensities are available for quantitation. If the response for any analyte exceeds the linear range of the calibration

established in Section 7.2.2, obtain and dilute a duplicate sample. Do not extrapolate beyond the calibration range.

8.2 Evaluation of the chromatograms, i.e. correlation of retention times and mass spectra to target compounds and determination of the peak areas is done by the software tool Analyst 1.2. If necessary, manual integration is done.

8.3 Linear calibration functions are established by plotting the peak area of the respective target analyte against the concentration of the target analyte in the respective calibration solution. Linear regression of the calibration functions provides the calibration coefficients a_1 und a_0 as well as the regression coefficient r. In total, 2 linear calibration functions are established. Calibration function 1 is valid for the concentration range from 10 to 100 ng/L, calibration function 2 for the concentration range from 100 to 1000 ng/L.

8.4 Concentrations in real samples are calculated by the software according to the following equation:

$$x = \frac{y - a_0}{a_1}$$

with x = concentration of the target analyte (ng/L)
y = peak area of the target analyte
a_1 = slope of the linear calibration function (= sensitivity)
a_0 = intercept of the linear calibration function

8.5 The recovery for the overall procedure is determined by a comparison of peak areas of pre-concentrated samples and direct injections according to the following equation:

$$R = \frac{Y_{pre-concentrated}}{Y_{direct}} \cdot 100\%$$

with $Y_{pre\text{-}concentrated}$ = peak area of a compound after pre-concentration
Y_{direct} = peak area of a compound after direct injection

The recovery is determined each time that the batch of SPE material or elution solvent is changed.

9.0 REPORT

9.1 Specification of results
Results are reported in ng/L with two significant digits.

10.0 QUALITY CONTROL

10.1 QC requirements
Quality control (QC) requirements are the initial demonstration of laboratory capability followed by regular analyses of laboratory reagent blanks, field reagent blanks, and laboratory fortified blanks. A MDL for each analyte must also be determined. Each laboratory must maintain records to document the quality of the data generated. Additional quality control practices are recommended.

10.2 Figures of merit
Figures of merit such as LOD, LOQ, or repeatability are determined during method validation according to the German Standard Procedure DIN 32645.

10.3 Laboratory Blanks
With each batch of samples processed as a group within a work shift, analyze a LRB to determine the background system contamination.

10.4 Calibration Check
In order to check the calibration, a LFB is processed with each batch of samples. For each of the method analytes the result of the LFB must be within 30% of the spiked concentration. If the deviation between spiked and measured concentration exceeds this range, appropriate measures must be taken to improve the quality of analysis before analysis of environmental samples can be continued.

10.5 QC control chart
For QA/QC purposes control charts are used. A QA/QC sample is analysed each day of analysis.

10.5.1 The QA/QC samples are prepared by adjusting 1000 mL of tap water to a pH of 7 by addition of 32% hydrochloric acid or caustic soda. Then 75 µL of the standard mixture for the QA/QC sample are added. The QA/QC sample is treated as any environmental

sample according to the procedure described in Section 7.0. The results of these QA/QC measurements for atenolol, sotalol and phenazone are evaluated and documented in control charts by the SQS software from
Perkin Elmer.

11.0 POLLUTION PREVENTION

11.1 No solvents are utilized in this method except the extremely small volumes of methanol and acetonitrile needed to prepare calibration standards and elution solvents. The only other chemicals used in this method are the neat materials in preparing standards and sample preservatives. All are used in extremely small amounts and pose no threat to the environment.

12.0 WASTE MANAGEMENT

12.1 SPE cartridges are only used once and are disposed with the domestic waste.

12.2 Solutions containing solvents are collected in glass bottles. Full glass bottles are shipped to a special unit for proper waste disposal.

13.0 REFERENCES

1. F. Sacher, F.T. Lange, H.-J. Brauch, I. Blankenhorn. “Pharmaceuticals in groundwaters - Analytical methods and results of a monitoring program in Baden-Württemberg, Germany.” J. Chromatogr. A 938:199-210 (2001)

ANNEX

Table 4-1. Example for the preparation of a standard mixture of the target compounds

Compound	Concentration of stock solution (g/L)	Volume of stock solution added to 50 mL (µL)	Concentration in mixture (ng/µL)
Atenolol	0.144	347.2	1.00
Betaxolol	0.120	416.9	1.00
Bisoprolol	0.145	344.4	1.00
Clenbuterol	0.246	203.2	1.00
Metoprolol	0.129	388.2	1.00
Pindolol	0.165	303.0	1.00
Propranolol	0.368	135.9	1.00
Salbutamol	0.105	476.2	1.00
Sotalol	0.372	134.5	1.00
Terbutaline	0.127	392.8	1.00
Phenazone	0.627	79.7	1.00
Dimethylamino-phenazone	0.487	102.7	1.00
Propyphenazone	0.379	131.9	1.00
Simvastatin	0.157	318.5	1.00
Cyclophosphamide	0.380	131.7	1.00
Ifosfamide	0.206	242.7	1.00

Table 4-2. Added volumes for the preparation of the calibration samples

Calibration sample	Added volume (µL)	Concentration (ng/L)
1	5	5
2	10	10
3	25	25
4	50	50
5	75	75
6	100	100
7	250	250
8	500	500
9	750	750
10	1000	1000

Table 4-3. LC conditions for analysis of betablockers and other pharmaceuticals

Separation column: Nucleosil, 250 mm*2 mm, 3 µm
Eluent A: Aqueous 20 mM ammonium acetate solution (pH 7.0)
Eluent B: Mixture of 2 parts per volume acetonitrile and one part per volume methanol, 20 mM ammonium acetate
Flow rate: 0.2 mL/min (constant flow)
Injection volume: 12.5 µL
Gradient:

time in min	A %	B %
0.00	98	2
1.0	98	2
6.0	90	10
20.0	0	100
31.0	0	100

Table 4-4. MS-MS conditions for analysis of betablockers and other pharmaceuticals

System: Applied Biosystems/MDS Sciex API 2000
Interface: Electrospray
Spray position: z = 4.0 y = 3.5
Heater temperature: 200 °C
Nebuliser Gas (GS1): 45 units
Heater Gas (GS2): 45 units
Ionisation mode: positive
Ionisation voltage: +5500 V
Curtain gas: 20 units
CAD: 3.00

All other parameters depend on the compounds and are documented in the laboratory.

Table 4-5. Detection conditions for analysis of pharmaceuticals

All compounds are analysed in the Multiple Reaction Monitoring (MRM) mode. Details on the selection of precursor and product ions are summarised in the following table.

Compound	Precursor ion	Product ions
Atenolol	267.1	74.1, 145.0
Betaxolol	308.1	55.1, 116.0
Bisoprolol	326.1	116.0, 107.0
Clenbuterol	277.0	203.0, 132.0
Metoprolol	268.1	116.1, 56.2
Pindolol	249.1	116.1, 171.9
Propranolol	260.1	56.0, 115.9
Salbutamol	240.0	147.9, 165.9
Sotalol	273.0	213.0, 133.1
Terbutaline	226.0	151.9
Phenazone	188.9	104.0, 56.2
Dimethylaminophenazone	231.9	112.9, 56.2
Propyphenazone	231.0	188.9, 56.1
Simvastatin	419.3	224.9, 199.0
Cyclophosphamide	260.8	119.9, 106.0
Ifosfamide	260.8	91.9, 63.0

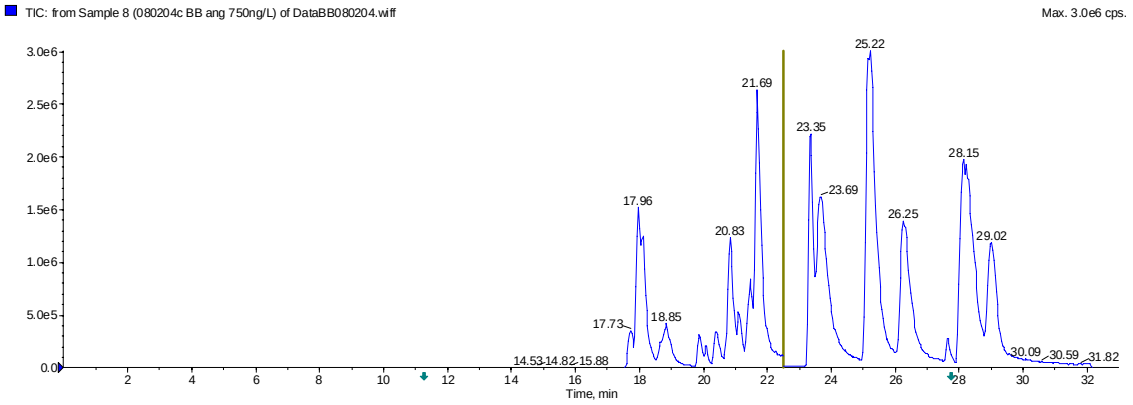

Fig. 4-1 Chromatogram of betablockers and other pharmaceuticals.

DETERMINATION OF X-RAY CONTRAST AGENTS BY SOLID-PHASE EXTRACTION FOLLOWED BY LIQUID CHROMATOGRAPHY AND TANDEM MASS SPECTROMETRY

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1.0 SCOPE OF APPLICATION

The procedure can be used for analyzing selected X-ray contrast agents in surface, ground and drinking waters. The application to waste waters has to be checked (see also Section 3.0). The following compounds can be determined by this method.

Analyte	CAS-No.
Iopamidol	60166-93-0
Iopromide	73334-07-3
Iomeprol	78649-41-9
Amidotrizoic acid (Ditrizoate)	131-49-7
Ioxitalamic acid	28179-44-4
Ioxaglic acid	59017-64-0
Iohexol	66108-95-0
Iotalamic acid	2276-90-6

Analysis of additional X-ray contrast agents with this method may be possible, but has to be checked. After pH adjustment the target compounds are pre-concentrated by solid-phase extraction onto a styrene-divinylbenzene co-polymer (SDB). Elution of the target compounds is done by methanol and acetonitrile. After evaporation of the solvent and reconstitution of the dry residue, an aliquot is injected into the HPLC/MS-MS system.
Method detection limits (MDLs) depend on the compound and on the matrix. For surface, ground and drinking waters the MDLs for all method analytes are in the range of 10 ng/L. The application range is approximately 10 – 1000 ng/L. Samples with higher concentrations have to be diluted before extraction.

2.0 DEFINITIONS

2.1 Laboratory Reagent Blank (LRB)
An aliquot of reagent water or other blank matrix that is treated exactly as a sample including exposure to all glassware, equipment, solvents, reagents, internal standards, and surrogates that are used with other samples. The LRB is used to determine if method analytes or other interferences are present in the laboratory environment, the reagents, or the apparatus.
2.2 Laboratory Fortified Blank (LFB)
An aliquot of reagent water or other blank matrix to which known quantities of the method

analytes are added in the laboratory. The LFB is analyzed exactly like a sample, and its purpose is to determine whether the methodology is in control, and whether the laboratory is capable of making accurate and precise measurements.

2.3 Stock Standard Solution
A concentrated solution containing one or more method analytes prepared in the laboratory using assayed reference materials or purchased from a reputable commercial source.

2.4 Primary Dilution Standard Solution (PDS)
A solution of several analytes prepared in the laboratory from stock standard solutions and diluted as needed to prepare calibration solutions and other needed analyte solutions.

2.5 Calibration Standard (CAL)
A solution prepared from the primary dilution standard solution or stock standard solutions and the internal standards and surrogate analytes. The CAL solutions are used to calibrate the instrument response with respect to analyte concentration.

2.6 Quality Control Sample (QCS)
A solution of method analytes of known concentrations which is used to fortify an aliquot of LRB or sample matrix. The QCS is obtained from a source external to the laboratory and different from the source of calibration standards. It is used to check laboratory performance with externally prepared test materials.

2.7 Procedural Standard Calibration
A calibration method where aqueous calibration standards are prepared and processed (e.g., purged, extracted, and/or derivatised) in exactly the same manner as a sample. All steps in the process from addition of sampling preservatives through instrumental analyses are included in the calibration. Using procedural standard calibration compensates for any inefficiencies in the processing procedure.

3.0 INTERFERENCES

3.1 Analyses of laboratory reagent blanks provide information about the presence of contaminants. When potential interfering peaks are noted in laboratory reagent blanks, the analyst should search for the source, including instrument, glassware, reagents, or solvents.

Subtracting blank values from sample results is not permitted.

3.2 Interfering contamination may occur when a sample containing low concentrations of method analytes is analyzed immediately after a sample containing relatively high concentrations of method analytes. A preventive technique is analyzing blank samples between environmental samples.

3.3 Waste water samples have to be diluted prior to analysis in order to avoid any interference during pre-concentration and analysis.

4.0 INSTRUMENTATION

4.1 HPLC system: HP 1090 II from Agilent Technologies

4.2 Mass spectrometer: API 2000 from Applied Biosystems/MDS Sciex

4.3 Automated pre-concentration system: Autotrace SPE Workstations from Zymark/Caliper

4.4 Equipment and supplies:

4.4.1 Separation column 250 x 2 mm, 3 µm Nucleosil from Phenomenex
4.4.2 Brown glass bottles, nominal volume 1000 mL
4.4.3 Glass bottles with screw cap, volume: 500 mL
4.4.4 Vials: 1 mL 5 mL and 10 mL from Supelco
4.4.5 Micro vial inserts from WGA, volume: 100 µL
4.4.6 Graduated cylinders, nominal volume: 100 mL, 250 mL, 500 mL, 1000 mL
4.4.7 Volumetric flasks, nominal volume: 10 mL, 50 mL
4.4.8 Syringes (5 µL, 10 µL, 25 µL, 50 µL, 100 µL, 500 µL) from Hamilton or Agilent Technologies
4.4.9 Syringe driven filters (0.45 µm) from Millex LH
4.4.10 Analytical balance from Sartorius
4.4.11 pH Meter from Schott instruments
4.4.12 Drying oven

5.0 CHEMICALS

5.1 Solvents

All solvents have to be of p.a. purity and shall be stored at room temperature.

5.1.1 Acetonitrile
5.1.2 Methanol

5.2 Reagents

All reagents have to be of p.a. purity and shall be stored at room temperature.

5.2.1 Ammonium formate
5.2.2 Caustic soda
5.2.3 Hydrochloric acid, 32%
5.2.4 Demineralised and decoct water

5.3 SPE Material

5.3.1 SPE material SDB1 from Bakerbond

5.4 Method analytes

All analytes must be purchased from a reputable commercial source and have to be of p.a. purity. The reference materials have to be stored according to the recommendations from the manufacturer.

5.4.1 Iopamidol
5.4.2 Iopromide
5.4.3 Iomeprol
5.4.4 Amidotrizoic acid
5.4.5 Ioxitalamic acid
5.4.6 Ioxaglic acid
5.4.7 Iohexol
5.4.8 Iotalamic acid

5.5 Stock standard solutions

For each of the target compounds a separate stock solution in methanol is prepared. Their concentrations depend on the solubility of the target compounds in methanol.
For the preparation of each of the stock solutions few mL of methanol are added to a 10 mL volumetric flask. Between 1 and 10 mg of the X-ray contrast agents are added on an analytical balance. The volumetric flask is filled up to volume with methanol. From the exact weight of the X-ray contrast agent and the volume of methanol, the exact concentration of the stock solution is calculated taking into account the purity of the target compounds.
The stock solutions have to be stored in the freezer and are stable for 6 months.

5.6 Primary Dilution Standard (PDS) of the target compounds; $\beta_{\textbf{single compound}} = 1$ ng/µL

Few mL of methanol are added to a 25 mL volumetric flask. A defined volume of each stock solution of the method analytes is added with a syringe to achieve a concentration of 1 ng/µL. Then, the volumetric flask is filled up to volume with methanol.
The PDS has to be stored in the freezer and is stable for 3 months.

5.7 Standard mixture for Quality Control Sample

The standard mixture for the QCS contains the following pharmaceuticals:

- iopamidol
- iopromide
- iomeprol
- amidotrizoic acid
- iohexol.

For preparation of the standard mixture for the QA/QC sample, few mL of methanol are added to a 25 mL volumetric flask. A defined volume of each stock solution of the target compounds is added with a syringe to achieve a final concentration of 1 ng/µL. The volumetric flask is filled up to volume with methanol.
The QA/QC sample of the target compounds has to be stored in the freezer and is stable for 3 months.

5.8 Solvent mixture for reconstitution of the dry residue

In a 100 mL graduated cylinder a mixture of 5 parts per volume acetonitrile and 95 parts per volume of an aqueous solution of 2 mM ammonium formate is prepared.

This solution can be stored in the refrigerator for a maximum of 1 week.

5.9 Calibration samples of target compounds

Ten glass bottles (V = 1000 mL) are filled with 1000 mL tap water with a graduated cylinder. Different volumes of the standard mixture of the target compounds (see Section 5.6) are added with a syringe. The added volumes as well as the resulting concentration levels are given in Table 5-1 in the Annex.

5.10 Eluent A: Aqueous 2 mM ammonium formate solution

An aqueous 2 mM ammonium formate solution is prepared in a 500 mL glass bottle with screw cap. This solution can not be stored and has to be prepared freshly for each day of analysis.

5.11 Eluent B: 2:1 (v:v) mixture of acetonitrile and methanol

In a 500 mL glass bottle with screw cap, two parts per volume acetonitrile are mixed with one part per volume of methanol by using a graduated cylinder. Then a defined amount of ammonium formate is added to achieve a final concentration of 2 mM.
This solution can be stored at room temperature for a maximum of 1 week.

6.0 SAMPLE COLLECTION, PRESERVATION, AND STORAGE

6.1 Sample Collection and Dechlorination

Sampling is done in 1000 mL or 2000 mL brown glass bottles. If samples, such as finished drinking waters, are suspected to contain residual chlorine, 100 mg of sodium thiosulfate should be added for each 1000 mL of water sample.

6.2 Sample Storage

Storage of samples is done in the cold storage room at 4 °C and must not exceed a time period of 3 weeks. Extracts must be stored in the freezer and are stable for at least 2 weeks.

6.3 Sample preservation

For surface water, groundwater and drinking water samples, no sample preservation is required.

7.0 PROCEDURE

7.1 Extraction

1000 mL of water sample are adjusted to pH 3 by addition of 32% hydrochloric acid. pH is checked with a pH meter. Conditioning of 200 mg SDB1 bakerbond material is done automatically (Autotrace system) with 5 mL acetone and subsequently with 5 mL demineralised and decoct water which has been adjusted to pH 3 by addition of 32% hydrochloric acid. For pre-concentration of the target analytes, the pH adjusted water sample is automatically pumped through the conditioned SPE material by the Autotrace system. Flow rate is 10 mL/min. After pre-concentration, the SPE material is dried for 60 min. with nitrogen gas. Elution of the target analytes is done automatically by the Autotrace system with 5 mL methanol followed by 5 mL acetonitrile. After passing through the SPE material the elution solvent is collected in a 10 mL vial. The solvent is completely evaporated in a stream of nitrogen. The dry residue is reconstituted with 100 µL of the solvent mixture (see Section 5.8). After filtration of the extract with a 0.45 µm syringe driven filter, the sample is ready for HPLC/MS-MS analysis.

7.2 HPLC/MS-MS analysis

Details on instrumentation and conditions of HPLC/MS-MS analysis are given in Tables 5-2 to 5-4 in the Annex.

7.2.1 Operation of the HPLC/MS-MS system

Operation of the HPLC/MS-MS system is done as described in the manufacturer's manual. Prior to analysis of environmental samples, a blank run is started to check the pressure conditions and to flush the column with solvent. For checking the MS detector, a standard solution is analysed. 50 µL of the PDS of the target compounds are evaporated to dryness in a stream of nitrogen and reconstituted with 100 µL of eluent A. Subsequently, 12.5 µL are injected into the HPLC/MS-MS system. Criteria for checking

the performance of the system are signal height, retention time and peak performance.

7.2.2 Calibration

Calibration is done for the overall procedure. Calibration samples are prepared as described in Section 5.9 and analysed. A new calibration is prepared for each batch of samples or whenever the QA/QC sample gives striking results.

8.0 DATA ANALYSIS AND CALCULATIONS

8.1 Complete chromatographic resolution is not necessary for accurate and precise measurements of analyte concentrations if unique ions with adequate intensities are available for quantitation. If the response for any analyte exceeds the linear range of the calibration established in Section 7.2.2, obtain and dilute a duplicate sample. Do not extrapolate beyond the calibration range.

8.2 Evaluation of the chromatograms, i.e. correlation of retention times and mass spectra to target compounds and determination of the peak areas is done by the software tool Analyst 1.2. If necessary, manual integration is done.

8.3 Linear calibration functions are established by plotting the peak area of the respective target analyte against the concentration of the target analyte in the respective calibration solution. Linear regression of the calibration functions provides the calibration coefficients a_1 und a_0 as well as the regression coefficient r.

8.4 Concentrations in real samples are calculated by the software according to the following equation:

$$x = \frac{y - a_0}{a_1}$$

with x = concentration of the target analyte (ng/L)

y = peak area of the target analyte

a_1 = slope of the linear calibration function (= sensitivity)

a_0 = intercept of the linear calibration function

8.5 The recovery for the overall procedure is determined by a comparison of peak areas of pre-concentrated samples and direct injections according to the following equation:

$$R = \frac{Y_{pre-concentrated}}{Y_{direct}} \cdot 100\%$$

with $Y_{pre\text{-}concentrated}$ = peak area of a compound after pre-concentration

Y_{direct} = peak area of a compound after direct injection

The recovery is determined each time that the batch of SPE material or elution solvent is changed.

9.0 REPORT

9.1 Specification of results

Results are reported in ng/L with two significant digits.

10.0 QUALITY CONTROL

10.1 QC requirements

Quality control (QC) requirements are the initial demonstration of laboratory capability followed by regular analyses of laboratory reagent blanks, field reagent blanks, and laboratory fortified blanks. A MDL for each analyte must also be determined. Each laboratory must maintain records to document the quality of the data generated. Additional quality control practices are recommended.

10.2 Figures of merit

Figures of merit such as LOD, LOQ, or repeatability are determined during method validation according to the German Standard Procedure DIN 32645.

10.3 Laboratory Blanks

With each batch of samples processed as a group within a work shift, analyze a LRB to determine the background system contamination.

10.4 Calibration Check

In order to check the calibration, a LFB is processed with each batch of samples. For each of the method analytes the result of the LFB must be within 30% of the spiked concentration. If the deviation between spiked and measured concentration exceeds this range, appropriate

measures must be taken to improve the quality of analysis before analysis of environmental samples can be continued.

10.5 QC control chart
For QA/QC purposes control charts are used. A QA/QC sample is analysed each day of analysis.

10.5.1 The QA/QC samples are prepared by adjusting 1000 mL of tap water to a pH of 3 by addition of 32% hydrochloric acid. Then 75 µL of the standard mixture for the QA/QC sample are added. The QA/QC sample is treated as any environmental sample according to the procedure described in Section 7.0. The results of these QA/QC measurements for iopamidol, iopromide, iomeprol, amidotrizoic acid and iohexol are evaluated and documented in control charts by the SQS software from Perkin Elmer.

11.0 POLLUTION PREVENTION

11.1 No solvents are utilized in this method except the extremely small volumes of methanol and acetone needed to prepare calibration standards and elution solvents. The only other chemicals used in this method are the neat materials in preparing standards and sample preservatives. All are used in extremely small amounts and pose no threat to the environment.

12.0 WASTE MANAGEMENT

12.1 SPE cartridges are only used once and are disposed with the domestic waste.

12.2 Solutions containing solvents are collected in glass bottles. Full glass bottles are shipped to a special unit for proper waste disposal.

13.0 REFERENCES

1. F. Sacher, F.T. Lange, H.-J. Brauch, I. Blankenhorn. “Pharmaceuticals in groundwaters

- Analytical methods and results of a monitoring program in Baden-Württemberg, Germany.” J. Chromatogr. A 938:199-210 (2001)

ANNEX

Table 5-1. Added volumes for the preparation of the calibration samples

Calibration sample	Added volume (µL)	Concentration (ng/L)
1	5	5
2	10	10
3	25	25
4	50	50
5	75	75
6	100	100
7	250	250
8	500	500
9	750	750
10	1000	1000

Table 5-2. LC conditions for analysis of X-ray contrast agents

Separation column: Nucleosil, 250 mm*2 mm, 3 µm
Eluent A: Aqueous 2 mM ammonium formate solution (pH 7.0)
Eluent B: Mixture of 2 parts per volume acetonitrile and one part per volume methanol, 2 mM ammonium formate
Flow rate: 0.2 mL/min (constant flow)
Injection volume: 12.5 µL
Gradient:

time in min	A %	B %
0.00	95	5
22.0	50	50
23.0	0	100
29.0	0	100
29.5	95	5

Table 5-3. MS-MS conditions for analysis of X-ray contrast agents

System:	Applied Biosystems/MDS Sciex API 2000
Interface:	Electrospray
Spray position:	z = 1.0 y = 1.5
Heater temperature:	200 °C
Nebuliser Gas (GS1):	45 units
Heater Gas (GS2):	45 units
Ionisation mode:	positive
Ionisation voltage:	+5500 V
Curtain gas:	25 units
CAD:	3.00

All other parameters depend on the compounds and are documented in the laboratory.

Table 5-4. Detection conditions for analysis of pharmaceuticals

All compounds are analysed in the Multiple Reaction Monitoring (MRM) mode. Details on the selection of precursor and product ions are summarised in the following table.

Compound	Precursor ion	Product ions
Iopamidol	777.9	558.9, 387.0
Iopromide	791.8	573.0, 300.0
Iomeprol	778.0	405.2, 531.9
Amidotrizoic acid	631.7	233.0, 148.1
Ioxitalamic acid	614.7	177.2, 486.8
Ioxaglic acid	1286.5	1269.2, 328.9
Iohexol	821.7	803.8, 374.8
Iotalamic acid	644.6	583.0, 301.9
Iopamidol	777.9	558.9, 387.0

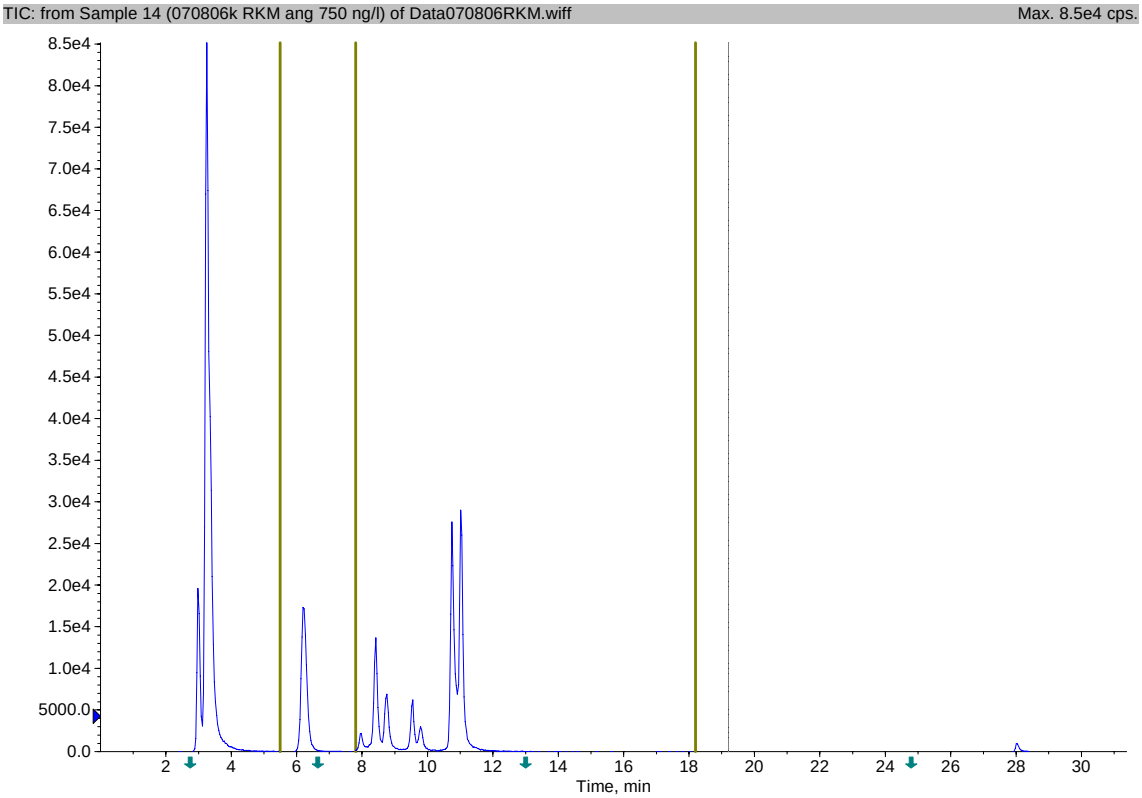

Fig. 5-1 Chromatogram of X-ray contrast agents.

DETERMINATION OF MACROLIDE ANTIBIOTICS AND OTHER ANTIBIOTICS BY SOLID-PHASE EXTRACTION FOLLOWED BY LIQUID CHROMATOGRAPHY AND TANDEM MASS SPECTROMETRY

Edited by Prof. H.-J. Brauch and Dr. F. Sacher

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1.0 SCOPE OF APPLICATION

The procedure can be used for analyzing selected antibiotics (and metabolites) in surface, ground and drinking waters. The application to waste waters has to be checked (see also Section 3.0). The following compounds can be determined by this method.

Analyte	CAS-No.
Chloramphenicol	56-75-7
Oleandomycin	3922-90-5
Erythromycin	114-07-8
Anhydro-Erythromycin	15307-79-6
Roxithromycin	80214-83-1
Clarithromycin	81103-11-9
Spiramycin	8025-81-8
Tylosin	1401-69-0

Analysis of additional antibiotics or metabolites with this method may be possible, but has to be checked. After pH adjustment the target compounds are pre-concentrated by solid-phase extraction onto a hydroxylated styrene-divinylbenzene polymer. Elution of the target compounds is done by a mixture of acetonitrile, water and triethylamine. After evaporation of the solvent and reconstitution of the dry residue, an aliquot is injected into the HPLC/MS-MS system.

Method detection limits (MDLs) depend on the compound and on the matrix. For surface, ground and drinking waters the MDLs for all method analytes are in the range of 10 ng/L. The application range is approximately 10 – 500 ng/L. Samples with higher concentrations have to be diluted before extraction.

2.0 DEFINITIONS

2.1 Laboratory Reagent Blank (LRB)

An aliquot of reagent water or other blank matrix that is treated exactly as a sample including exposure to all glassware, equipment, solvents, reagents, internal standards, and surrogates that are used with other samples. The LRB is used to determine if method analytes or other interferences are present in the laboratory environment, the reagents, or the apparatus.

2.2 Laboratory Fortified Blank (LFB)
An aliquot of reagent water or other blank matrix to which known quantities of the method analytes are added in the laboratory. The LFB is analyzed exactly like a sample, and its purpose is to determine whether the methodology is in control, and whether the laboratory is capable of making accurate and precise measurements.

2.3 Stock Standard Solution
A concentrated solution containing one or more method analytes prepared in the laboratory using assayed reference materials or purchased from a reputable commercial source.

2.4 Primary Dilution Standard Solution (PDS)
A solution of several analytes prepared in the laboratory from stock standard solutions and diluted as needed to prepare calibration solutions and other needed analyte solutions.

2.5 Calibration Standard (CAL)
A solution prepared from the primary dilution standard solution or stock standard solutions and the internal standards and surrogate analytes. The CAL solutions are used to calibrate the instrument response with respect to analyte concentration.

2.6 Quality Control Sample (QCS)
A solution of method analytes of known concentrations which is used to fortify an aliquot of LRB or sample matrix. The QCS is obtained from a source external to the laboratory and different from the source of calibration standards. It is used to check laboratory performance with externally prepared test materials.

2.7 Procedural Standard Calibration
A calibration method where aqueous calibration standards are prepared and processed (e.g., purged, extracted, and/or derivatised) in exactly the same manner as a sample. All steps in the process from addition of sampling preservatives through instrumental analyses are included in the calibration. Using procedural standard calibration compensates for any inefficiencies in the processing procedure.

3.0 INTERFERENCES

3.1 Analyses of laboratory reagent blanks provide information about the presence of

contaminants. When potential interfering peaks are noted in laboratory reagent blanks, the analyst should search for the source, including instrument, glassware, reagents, or solvents. Subtracting blank values from sample results is not permitted.

3.2 Interfering contamination may occur when a sample containing low concentrations of method analytes is analyzed immediately after a sample containing relatively high concentrations of method analytes. A preventive technique is analyzing blank samples between environmental samples.

3.3 Waste water samples have to be diluted prior to analysis in order to avoid any interference during pre-concentration and analysis.

4.0 INSTRUMENTATION

4.1 HPLC system: HP 1100 from Agilent Technologies

4.2 Mass spectrometer: API 2000 from Applied Biosystems/MDS Sciex

4.3 Automated pre-concentration system: Autotrace SPE Workstations from Zymark/Caliper

4.4 Equipment and supplies:

4.4.1 Separation column 250 x 2.1 mm, 3 µm MZ-Aqua perfect from MZ-Analysentechnik
4.4.2 Brown glass bottles, nominal volume 1000 mL
4.4.3 Glass bottles with screw cap, volume: 500 mL
4.4.4 Vials: 1 mL and 10 mL from Supelco
4.4.5 Micro vial inserts from WGA, volume: 100 µL
4.4.6 Graduated cylinders, nominal volume: 100 mL, 250 mL, 500 mL, 1000 mL
4.4.7 Volumetric flasks, nominal volume: 10 mL, 25 mL
4.4.8 Syringes (5 µL, 10 µL, 25 µL, 50 µL, 100 µL, 500 µL) from Hamilton or Agilent Technologies
4.4.9 Analytical balance from Sartorius
4.4.10 pH Meter from Schott instruments
4.4.11 Drying oven

5.0 CHEMICALS

5.1 Solvents
All solvents have to be of p.a. purity and shall be stored at room temperature.

5.1.1 Acetonitrile
5.1.2 Methanol

5.2 Reagents
All reagents have to be of p.a. purity and shall be stored at room temperature.

5.2.1 Ammonium acetate
5.2.2 Triethylamine
5.2.3 1 M Caustic soda
5.2.4 Hydrochloric acid, 32%
5.2.5 Demineralised and decoct water
5.2.6 Ethylenediaminetetraacetic acid (EDTA disodium salt)
5.2.7 Sodium azide

5.3 SPE Material

5.3.1 Isolute ENV+ from Separtis

5.4 Method analytes
All analytes must be purchased from a reputable commercial source and have to be of p.a. purity. The reference materials have to be stored according to the recommendations from the manufacturer.

5.4.1 Chloroamphenicol
5.4.2 Oleandomycin
5.4.3 Erythromycin
5.4.4 Anhydro-Erythromycin
5.4.5 Roxithromycin
5.4.6 Clarithromycin
5.4.7 Spiramycin
5.4.8 Tylosin

5.5 Stock standard solutions

For each of the target compounds a separate stock solution in methanol is prepared. Their concentrations depend on the solubility of the target compounds in methanol.
For the preparation of each of the stock solutions, between 1 and 10 mg of the pharmaceutical compound are added on an analytical balance to a 10 mL volumetric flask. Then, the volumetric flask is filled up to volume with methanol. From the exact weight of the antibiotic and the volume of methanol, the exact concentration of the stock solution is calculated taking into account the purity of the target compounds.
The stock solutions have to be stored in the freezer and are stable for 1 year.

5.6 Primary Dilution Standard (PDS) of the target compounds; $\beta_{\textbf{single compound}} = 1$ ng/µL

Few mL of methanol are added to a 25 mL volumetric flask. A defined volume of each stock solution of the method analytes is added with a syringe to achieve a concentration of 1 ng/µL. Then, the volumetric flask is filled up to volume with methanol.
The PDS of the target compounds has to be stored in the freezer and is stable for 3 months.

5.7 Standard mixture for Quality Control Sample

The standard mixture for the QCS contains the following antibiotics:

- chloroamphenicol
- roxithromycin
- clarithromycin.

For preparation of the standard mixture for the QA/QC sample, few mL of methanol are added to a 25 mL volumetric flask. A defined volume of each stock solution of each target compound is added with a syringe to achieve a final concentration of 1 ng/µL. The volumetric flask is filled up to volume with methanol.
The QA/QC sample of the target compounds has to be stored in the freezer and is stable for 6 months.

5.8 Solvent mixture for reconstitution of the dry residue

In a 100 mL graduated cylinder a mixture of 5 parts per volume acetonitrile and 95 parts per

volume of an aqueous solution of 20 mM ammonium acetate is prepared.
This solution can be stored in the refrigerator for a maximum of 1 week.

5.9 Calibration samples of target compounds

Eight glass bottles (V = 1000 mL) are filled with 500 mL tap water with a graduated cylinder. Then different volumes of the standard mixture of the target compounds (see Section 5.6) are added with a syringe. The added volumes as well as the resulting concentration levels are given in Table 6-1 in the Annex.

5.10 Eluent A: Aqueous 20 mM ammonium acetate solution

An aqueous 20 mM ammonium acetate solution is prepared in a 500 mL glass bottle with screw cap. This solution can not be stored and has to be prepared freshly fot each day of analysis.

5.11 Eluent B: 2:1 (v:v) mixture of acetonitrile and methanol

In a 500 mL glass bottle with screw cap, two parts per volume acetonitrile are mixed with one part per volume of methanol by using a graduated cylinder. Then a defined amount of ammonium acetate is added to achieve a final concentration of 20 mM.
This solution can be stored at room temperature for a maximum of 1 week.

5.12 Elution solvent

In a 100 mL graduated cylinder a mixture of 90 parts per volume acetonitrile, 10 parts per volume of demineralised and decoct water and 0.5 parts per volume of triethylamine is prepared.
This solution can not be stored and has to be prepared anew each day of analysis.

6.0 SAMPLE COLLECTION, PRESERVATION, AND STORAGE

6.1 Sample Collection and Dechlorination

Sampling is done in 1000 mL brown glass bottles. If samples, such as finished drinking

waters, are suspected to contain residual chlorine, 100 mg of sodium thiosulfate should be added for each 1000 mL of water sample.

6.2 Sample Storage

Storage of samples is done in the cold storage room at 4 °C and must not exceed a time period of 3 weeks. Extracts must be stored in the freezer and are stable for at least 2 weeks.

6.3 Sample preservation

For preservation of surface water, groundwater and drinking water samples 100 mg/L sodium azide are added immediately after sampling.

7.0 PROCEDURE

7.1 Extraction

First, 1.3 g of ethylendiaminetetraacetic acid are added to 500 mL of water sample. Then the sample is adjusted to pH 5 by addition of 32% hydrochloric acid or 1 M caustic soda. pH is checked with a pH meter. Conditioning of 100 mg Isolute ENV+ material is done automatically (Autotrace system) with 5 mL acetonitrile and subsequently with 5 mL demineralised and decoct water. For pre-concentration of the target analytes, the pH adjusted water sample is automatically pumped through the conditioned SPE material by the Autotrace system. Flow rate is 7.0 mL/min. After pre-concentration, the SPE material is dried for 30 min. with nitrogen gas. Elution of the target analytes is done automatically by the Autotrace system with 5 mL acetonitrile and 5 mL of elution solvent (see Section 5.12). After passing through the SPE material the elution solvent is collected in a 10 mL vial. The solvent is completely evaporated in a stream of nitrogen. The dry residue is reconstituted with 100 µL of the solvent mixture (see Section 5.8). Then, the sample is ready for HPLC/MS-MS analysis.

7.2 HPLC/MS-MS analysis

Details on instrumentation and conditions of HPLC/MS-MS analysis are given in Tables 6-2 to 6-4 in the Annex.

7.2.1 Operation of the HPLC/MS-MS system

Operation of the HPLC/MS-MS system is done as described in the manufacturer's manual. Prior to analysis of environmental samples, a blank run is started to check the pressure conditions and to flush the column with solvent. For checking the MS detector, a standard solution is analysed. 25 µL of the standard mixture of the target compounds are evaporated to dryness in a stream of nitrogen and reconstituted with 100 µL of the solvent mixture (see Section 5.8). Subsequently, 12.5 µL are injected into the HPLC/MS-MS system. Criteria for checking the performance of the system are signal height, retention time and peak performance.

7.2.2 Calibration

Calibration is done for the overall procedure. Calibration samples are prepared as described in Section 5.9 and analysed. A new calibration is prepared for each batch of samples or whenever the QA/QC sample gives striking results.

8.0 DATA ANALYSIS AND CALCULATIONS

8.1 Complete chromatographic resolution is not necessary for accurate and precise measurements of analyte concentrations if unique ions with adequate intensities are available for quantitation. If the response for any analyte exceeds the linear range of the calibration established in Section 7.2.2, obtain and dilute a duplicate sample. Do not extrapolate beyond the calibration range.

8.2 Evaluation of the chromatograms, i.e. correlation of retention times and mass spectra to target compounds and determination of the peak areas is done by the software tool Analyst 1.2. If necessary, manual integration is done.

8.3 Linear calibration functions are established by plotting the peak area of the respective target analyte against the concentration of the target analyte in the respective calibration solution. Linear regression of the calibration functions provides the calibration coefficients a_1 und a_0 as well as the regression coefficient r.

8.4 Concentrations in real samples are calculated by the software according to the following equation:

$$x = \frac{y - a_0}{a_1}$$

with x = concentration of the target analyte (ng/L)
y = peak area of the target analyte
a_1 = slope of the linear calibration function (= sensitivity)
a_0 = intercept of the linear calibration function

8.5 The recovery for the overall procedure is determined by a comparison of peak areas of pre-concentrated samples and direct injections according to the following equation:

$$R = \frac{Y_{pre-concentrated}}{Y_{direct}} \cdot 100\%$$

with $Y_{pre\text{-}concentrated}$ = peak area of a compound after pre-concentration
Y_{direct} = peak area of a compound after direct injection

The recovery is determined each time that the batch of SPE material or elution solvent is changed.

9.0 REPORT

9.1 Specification of results
Results are reported in ng/L with two significant digits.

10.0 QUALITY CONTROL

10.1 QC requirements
Quality control (QC) requirements are the initial demonstration of laboratory capability followed by regular analyses of laboratory reagent blanks, field reagent blanks, and laboratory fortified blanks. A MDL for each analyte must also be determined. Each laboratory must maintain records to document the quality of the data generated. Additional quality control practices are recommended.

10.2 Figures of merit
Figures of merit such as LOD, LOQ, or repeatability are determined during method validation according to the German Standard Procedure DIN 32645.

10.3 Laboratory Blanks
With each batch of samples processed as a group within a work shift, analyze a LRB to determine the background system contamination.

10.4 Calibration Check
In order to check the calibration, a LFB is processed with each batch of samples. For each of the method analytes the result of the LFB must be within 30% of the spiked concentration. If the deviation between spiked and measured concentration exceeds this range, appropriate measures must be taken to improve the quality of analysis before analysis of environmental samples can be continued.

10.5 QC control chart
For QA/QC purposes control charts are used. A QA/QC sample is analysed each day of analysis.

10.5.1 The QA/QC samples are prepared by adjusting 500 mL of tap water to a pH of 5 by addition of 32% hydrochloric acid or 1 M caustic soda. Then 100 µL of the standard mixture for the QA/QC sample are added. The QA/QC sample is treated as any environmental sample according to the procedure described in Section 7.0. The results of these QA/QC measurements for chloroamphenicol, roxithromycin and clarithromycin are evaluated and documented in control charts by the SQS software from Perkin Elmer.

11.0 POLLUTION PREVENTION

11.1 No solvents are utilized in this method except the extremely small volumes of methanol and acetonitrile needed to prepare calibration standards and elution solvents. The only other chemicals used in this method are the neat materials in preparing standards and sample preservatives. All are used in extremely small amounts and pose no threat to the environment.

12.0 WASTE MANAGEMENT

12.1 SPE cartridges are only used once and are disposed with the domestic waste.

12.2 Solutions containing solvents are collected in glass bottles. Full glass bottles are shipped to a special unit for proper waste disposal.

13.0 REFERENCES

1. F. Sacher, F.T. Lange, H.-J. Brauch, I. Blankenhorn. “Pharmaceuticals in groundwaters - Analytical methods and results of a monitoring program in Baden-Württemberg, Germany.” J. Chromatogr. A 938:199-210 (2001)

ANNEX

Table 6-1. Added volumes for the preparation of the calibration samples

Calibration sample	Added volume (µL)	Concentration (ng/L)
1	5	5
2	10	10
3	25	25
4	50	50
5	75	75
6	100	100
7	250	250
8	500	500

Table 6-2. LC conditions for analysis of macrolides and other antibiotics

Separation column: AquaPerfect, 250 mm*2.1 mm, 3 µm
Eluent A: Aqueous 20 mM ammonium acetate solution (pH 6.8)
Eluent B: Mixture of 2 parts per volume acetonitrile and one part per volume methanol, 20 mM ammonium acetate
Flow rate: 0.2 mL/min (constant flow)
Injection volume: 12.5 µL
Gradient:

time in min	A %	B %
0.00	80	20
3.0	80	20
13.0	20	80
18.0	0	100
26.0	0	100
26.5	80	20
28.0	80	20

Table 6-3. MS-MS conditions for analysis of macrolides and other antibiotics

System: Applied Biosystems/MDS Sciex API 2000
Interface: Electrospray
Spray position: z = 4.0 y = 3.5
Heater temperature: 200 °C (300 °C for analysis of chloroamphenicol)
Nebuliser Gas (GS1): 45 units
Heater Gas (GS2): 45 units
Ionisation mode: positive (negative for analysis of chloroamphenicol)
Ionisation voltage: +5500 V (-4200 V for analysis of chloroamphenicol)
Curtain gas: 20 units
CAD: 3.00

All other parameters depend on the compounds and are documented in the laboratory.

Table 6-4. Detection conditions for analysis of macrolides and other antibiotics

All compounds are analysed in the Multiple Reaction Monitoring (MRM) mode. Details on the selection of precursor and product ions are summarised in the following table.

Compound	Precursor ion	Product ions
Chloroamphenicol	381.0	320.1, 151.9
Oleandomycin	688.5	116.2, 158.1
Erythromycin	734.6	158.1, 83.2
Anhydro-Erythromycin	716.5	83.2, 158.1
Roxithromycin	837.5	116.1, 158.1
Clarithromycin	748.3	83.2, 158.2
Spiramycin	843.5	174.1, 101.1

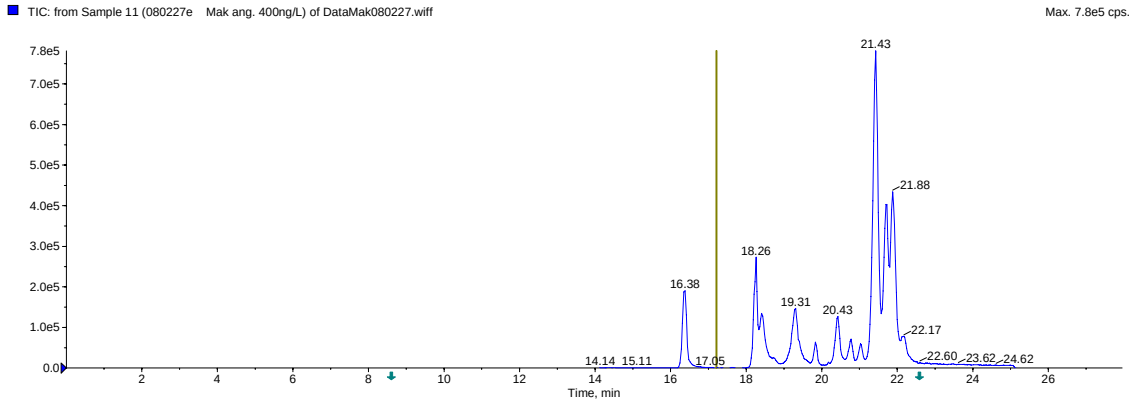

Fig. 6-1 Chromatogram of antibiotics (macrolides and others).

DETERMINATION OF SULFONAMIDE ANTIBIOTICS AND OTHER ANTIBIOTICS BY SOLID-PHASE EXTRACTION FOLLOWED BY LIQUID CHROMATOGRAPHY AND TANDEM MASS SPECTROMETRY

Edited by Prof. H.-J. Brauch and Dr. F. Sacher

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1.0 SCOPE OF APPLICATION

The procedure can be used for analyzing selected antibiotics in surface, ground and drinking waters. The application to waste waters has to be checked (see also Section 3.0). The following compounds can be determined by this method.

Analyte	CAS-No.
Sulfadiazine	68-35-9
Sulfadimidine (Sulfamethazine)	57-68-1
Sulfamerazine	127-79-7
Sulfamethoxazole	723-46-6
Trimethoprim	738-70-5
Metronidazole	443-48-1
Ronidazole	7681-76-7

Analysis of additional antibiotics with this method may be possible, but has to be checked. After pH adjustment the target compounds are pre-concentrated by solid-phase extraction onto a hydroxylated styrene-divinylbenzene polymer. Elution of the target compounds is done by a mixture of acetonitrile, water and triethylamine. After evaporation of the solvent and reconstitution of the dry residue, an aliquot is injected into the HPLC/MS-MS system.
Method detection limits (MDLs) depend on the compound and on the matrix. For surface, ground and drinking waters the MDLs for all method analytes are in the range of 10 ng/L. The application range is approximately 10 – 500 ng/L. Samples with higher concentrations have to be diluted before extraction.

2.0 DEFINITIONS

2.1 Laboratory Reagent Blank (LRB)
An aliquot of reagent water or other blank matrix that is treated exactly as a sample including exposure to all glassware, equipment, solvents, reagents, internal standards, and surrogates that are used with other samples. The LRB is used to determine if method analytes or other interferences are present in the laboratory environment, the reagents, or the apparatus.

2.2 Laboratory Fortified Blank (LFB)
An aliquot of reagent water or other blank matrix to which known quantities of the method

analytes are added in the laboratory. The LFB is analyzed exactly like a sample, and its purpose is to determine whether the methodology is in control, and whether the laboratory is capable of making accurate and precise measurements.

2.3 Stock Standard Solution
A concentrated solution containing one or more method analytes prepared in the laboratory using assayed reference materials or purchased from a reputable commercial source.

2.4 Primary Dilution Standard Solution (PDS)
A solution of several analytes prepared in the laboratory from stock standard solutions and diluted as needed to prepare calibration solutions and other needed analyte solutions.

2.5 Quality Control Sample (QCS)
A solution of method analytes of known concentrations which is used to fortify an aliquot of LRB or sample matrix. The QCS is obtained from a source external to the laboratory and different from the source of calibration standards. It is used to check laboratory performance with externally prepared test materials.

2.6 Calibration Standard (CAL)
A solution prepared from the primary dilution standard solution or stock standard solutions and the internal standards and surrogate analytes. The CAL solutions are used to calibrate the instrument response with respect to analyte concentration.

2.7 Procedural Standard Calibration
A calibration method where aqueous calibration standards are prepared and processed (e.g., purged, extracted, and/or derivatised) in exactly the same manner as a sample. All steps in the process from addition of sampling preservatives through instrumental analyses are included in the calibration. Using procedural standard calibration compensates for any inefficiencies in the processing procedure.

3.0 INTERFERENCES

3.1 Analyses of laboratory reagent blanks provide information about the presence of contaminants. When potential interfering peaks are noted in laboratory reagent blanks, the analyst should search for the source, including instrument, glassware, reagents, or solvents.

Subtracting blank values from sample results is not permitted.

3.2 Interfering contamination may occur when a sample containing low concentrations of method analytes is analyzed immediately after a sample containing relatively high concentrations of method analytes. A preventive technique is analyzing blank samples between environmental samples.

3.3 Waste water samples have to be diluted prior to analysis in order to avoid any interference during pre-concentration and analysis.

4.0 INSTRUMENTATION

4.1 HPLC system: HP 1100 from Agilent Technologies

4.2 Mass spectrometer: API 2000 from Applied Biosystems/MDS Sciex

4.3 Automated pre-concentration system: Autotrace SPE Workstations from Zymark/Caliper

4.4 Equipment and supplies:

4.4.1 Separation column 250 x 2 mm, 3 µm Nucleosil from Bischoff Analysentechnik
4.4.2 Brown glass bottles, nominal volume 1000 mL
4.4.3 Glass bottles with screw cap, volume: 500 mL
4.4.4 Vials: 1 mL and 10 mL from Supelco
4.4.5 Micro vial inserts from WGA, volume: 100 µL
4.4.6 Graduated cylinders, nominal volume: 100 mL, 250 mL, 500 mL, 1000 mL
4.4.7 Volumetric flasks, nominal volume: 10 mL, 25 mL
4.4.8 Syringes (5 µL, 10 µL, 25 µL, 50 µL, 100 µL, 500 µL) from Hamilton or Agilent Technologies
4.4.9 Analytical balance from Sartorius
4.4.10 pH Meter from Schott instruments
4.4.11 Drying oven

5.0 CHEMICALS

5.1 Solvents

All solvents have to be of p.a. purity and shall be stored at room temperature.

5.1.1 Acetonitrile
5.1.2 Methanol

5.2 Reagents

All reagents have to be of p.a. purity and shall be stored at room temperature.

5.2.1 Ammonium acetate
5.2.2 Triethylamine
5.2.3 1 M Caustic soda
5.2.4 Hydrochloric acid, 32%
5.2.5 Acetic acid, conc.
5.2.6 Demineralised and decoct water
5.2.7 Ethylenediaminetetraacetic acid (EDTA disodium salt)
5.2.8 Sodium azide

5.3 SPE Material

5.3.1 Isolute ENV+ from Separtis

5.4 Method analytes

All analytes must be purchased from a reputable commercial source and have to be of p.a. purity. The reference materials have to be stored according to the recommendations from the manufacturer.

5.4.1 Sulfadiazine
5.4.2 Sulfadimidine
5.4.3 Sulfamerazine
5.4.4 Sulfamethoxazole
5.4.5 Trimethoprim
5.4.6 Metronidazole
5.4.7 Ronidazole

5.5 Stock standard solutions

For each of the target compounds a separate stock solution in methanol is prepared. Their concentrations depend on the solubility of the target compounds in methanol.
For the preparation of each of the stock solutions, between 1 and 10 mg of the pharmaceutical compound are added on an analytical balance to a 10 mL volumetric flask. The volumetric flask is filled up to volume with methanol. From the exact weight of the antibiotic and the volume of methanol, the exact concentration of the stock solution is calculated taking into account the purity of the target compounds.
The stock solutions have to be stored in the freezer and are stable for 1 year.

5.6 Primary Dilution Standard (PDS) of the target compounds; $\beta_{\textbf{single compound}} = 1$ ng/µL

Few mL of methanol are added to a 25 mL volumetric flask. Then, a defined volume of each stock solution of the method analytes is added with a syringe to achieve a concentration of 1 ng/µL. The volumetric flask is filled up to volume with methanol.
The PDS of the target compounds has to be stored in the freezer and is stable for 3 months.

5.7 Standard mixture for Quality Control Sample

The standard mixture for the QCS contains the following antibiotics:

- chloroamphenicol
- roxithromycin
- clarithromycin.

For preparation of the standard mixture for the QA/QC sample, few mL of methanol are added to a 25 mL volumetric flask. A defined volume of each stock solution of the target compounds is added with a syringe to achieve a final concentration of 1 ng/µL. Then, the volumetric flask is filled up to volume with methanol.
The QA/QC sample of the target compounds has to be stored in the freezer and is stable for 6 months.

5.8 Solvent mixture for reconstitution of the dry residue

In a 100 mL graduated cylinder a mixture of 5 parts per volume acetonitrile and 95 parts per

volume of an aqueous solution of 20 mM ammonium acetate is prepared.
This solution can be stored in the refrigerator for a maximum of 1 week.

5.9 Calibration samples of target compounds

Eight glass bottles (V = 1000 mL) are filled with 500 mL tap water with a graduated cylinder. Then different volumes of the standard mixture of the target compounds (see Section 5.6) are added with a syringe. The added volumes as well as the resulting concentration levels are given in Table 7-1 in the Annex.

5.10 Eluent A: Aqueous 20 mM ammonium acetate solution

An aqueous 20 mM ammonium acetate solution is prepared in a 500 mL glass bottle with screw cap. This solution can not be stored and has to be prepared freshly for each day of analysis.

5.11 Eluent B: 2:1 (v:v) mixture of acetonitrile and methanol

In a 500 mL glass bottle with screw cap, two parts per volume acetonitrile are mixed with one part per volume of methanol by using a graduated cylinder. Then a defined amount of ammonium acetate is added to achieve a final concentration of 20 mM.
This solution can be stored at room temperature for a maximum of 1 week.

5.12 Elution solvent

In a 100 mL graduated cylinder a mixture of 90 parts per volume acetonitrile, 10 parts per volume of demineralised and decoct water and 0.5 parts per volume of triethylamine is prepared.
This solution can not be stored and has to be prepared anew each day of analysis.

6.0 SAMPLE COLLECTION, PRESERVATION, AND STORAGE

6.1 Sample Collection and Dechlorination

Sampling is done in 1000 mL brown glass bottles. If samples, such as finished drinking

waters, are suspected to contain residual chlorine, 100 mg of sodium thiosulfate should be added for each 1000 mL of water sample.

6.2 Sample Storage

Storage of samples is done in the cold storage room at 4 °C and must not exceed a time period of 3 weeks. Extracts must be stored in the freezer and are stable for at least 2 weeks.

6.3 Sample preservation

For preservation of surface water, groundwater and drinking water samples 100 mg/L sodium azide are added immediately after sampling.

7.0 PROCEDURE

7.1 Extraction

First, 1.3 g of ethylendiaminetetraacetic acid are added to 500 mL of water sample. Then the sample is adjusted to pH 5 by addition of 32% hydrochloric acid or 1 M caustic soda. pH is checked with a pH meter. Conditioning of 100 mg Isolute ENV+ material is done automatically (Autotrace system) with 5 mL acetonitrile and subsequently with 5 mL demineralised and decoct water. For pre-concentration of the target analytes, the pH adjusted water sample is automatically pumped through the conditioned SPE material by the Autotrace system. Flow rate is 7.0 mL/min. After pre-concentration, the SPE material is dried for 30 min. with nitrogen gas. Elution of the target analytes is done automatically by the Autotrace system with 5 mL acetonitrile and 5 mL of elution solvent (see Section 5.12). After passing through the SPE material the elution solvent is collected in a 10 mL vial. The solvent is completely evaporated in a stream of nitrogen. The dry residue is reconstituted with 100 µL of the solvent mixture (see Section 5.8). Then, the sample is ready for HPLC/MS-MS analysis.

7.2 HPLC/MS-MS analysis

Details on instrumentation and conditions of HPLC/MS-MS analysis are given in Tables 7-2 to 7-4 in the Annex.

7.2.1 Operation of the HPLC/MS-MS system

Operation of the HPLC/MS-MS system is done as described in the manufacturer's manual. Prior to analysis of environmental samples, a blank run is started to check the pressure conditions and to flush the column with solvent. For checking the MS detector, a standard solution is analysed. 25 µL of the standard mixture of the target compounds are evaporated to dryness in a stream of nitrogen and reconstituted with 100 µL of the solvent mixture (see Section 5.8). Subsequently, 12.5 µL are injected into the HPLC/MS-MS system. Criteria for checking the performance of the system are signal height, retention time and peak performance.

7.2.2 Calibration

Calibration is done for the overall procedure. Calibration samples are prepared as described in Section 5.9 and analysed. A new calibration is prepared for each batch of samples or whenever the QA/QC sample gives striking results.

8.0 DATA ANALYSIS AND CALCULATIONS

8.1 Complete chromatographic resolution is not necessary for accurate and precise measurements of analyte concentrations if unique ions with adequate intensities are available for quantitation. If the response for any analyte exceeds the linear range of the calibration established in Section 7.2.2, obtain and dilute a duplicate sample. Do not extrapolate beyond the calibration range.

8.2 Evaluation of the chromatograms, i.e. correlation of retention times and mass spectra to target compounds and determination of the peak areas is done by the software tool Analyst 1.2. If necessary, manual integration is done.

8.3 Linear calibration functions are established by plotting the peak area of the respective target analyte against the concentration of the target analyte in the respective calibration solution. Linear regression of the calibration functions provides the calibration coefficients a_1 and a_0 as well as the regression coefficient r.

8.4 Concentrations in real samples are calculated by the software according to the following equation:

$$x = \frac{y - a_0}{a_1}$$

with x = concentration of the target analyte (ng/L)
y = peak area of the target analyte
a_1 = slope of the linear calibration function (= sensitivity)
a_0 = intercept of the linear calibration function

8.5 The recovery for the overall procedure is determined by a comparison of peak areas of pre-concentrated samples and direct injections according to the following equation:

$$R = \frac{Y_{pre-concentrated}}{Y_{direct}} \cdot 100\%$$

with $Y_{pre\text{-}concentrated}$ = peak area of a compound after pre-concentration
Y_{direct} = peak area of a compound after direct injection

The recovery is determined each time that the batch of SPE material or elution solvent is changed.

9.0 REPORT

9.1 Specification of results
Results are reported in ng/L with two significant digits.

10.0 QUALITY CONTROL

10.1 QC requirements
Quality control (QC) requirements are the initial demonstration of laboratory capability followed by regular analyses of laboratory reagent blanks, field reagent blanks, and laboratory fortified blanks. A MDL for each analyte must also be determined. Each laboratory must maintain records to document the quality of the data generated. Additional quality control practices are recommended.

10.2 Figures of merit
Figures of merit such as LOD, LOQ, or repeatability are determined during method validation according to the German Standard Procedure DIN 32645.

10.3 Laboratory Blanks

With each batch of samples processed as a group within a work shift, analyze a LRB to determine the background system contamination.

10.4 Calibration Check

In order to check the calibration, a LFB is processed with each batch of samples. For each of the method analytes the result of the LFB must be within 30% of the spiked concentration. If the deviation between spiked and measured concentration exceeds this range, appropriate measures must be taken to improve the quality of analysis before analysis of environmental samples can be continued.

10.5 QC control chart

For QA/QC purposes control charts are used. A QA/QC sample is analysed each day of analysis.

10.5.1 The QA/QC samples are prepared by adjusting 500 mL of tap water to a pH of 5 by addition of 32% hydrochloric acid or 1 M caustic soda. Then 100 µL of the standard mixture for the QA/QC sample are added. The QA/QC sample is treated as any environmental sample according to the procedure described in Section 7.0. The results of these QA/QC measurements for sulfamethoxazole, trimethoprim and ronidazole are evaluated and documented in control charts by the SQS software from Perkin Elmer.

11.0 POLLUTION PREVENTION

11.1 No solvents are utilized in this method except the extremely small volumes of methanol and acetonitrile needed to prepare calibration standards and elution solvents. The only other chemicals used in this method are the neat materials in preparing standards and sample preservatives. All are used in extremely small amounts and pose no threat to the environment.

12.0 WASTE MANAGEMENT

12.1 SPE cartridges are only used once and are disposed with the domestic waste.

12.2 Solutions containing solvents are collected in glass bottles. Full glass bottles are shipped to a special unit for proper waste disposal.

13.0 REFERENCES

1. F. Sacher, F.T. Lange, H.-J. Brauch, I. Blankenhorn. “Pharmaceuticals in groundwaters - Analytical methods and results of a monitoring program in Baden-Württemberg, Germany.” J. Chromatogr. A 938:199-210 (2001)

ANNEX

Table 7-1. Added volumes for the preparation of the calibration samples

Calibration sample	Added volume (µL)	Concentration (ng/L)
1	5	5
2	10	10
3	25	25
4	50	50
5	75	75
6	100	100
7	250	250
8	500	500

Table 7-2. LC conditions for analysis of sulfonamides and other antibiotics

Separation column: Nucleosil, 250 mm*2 mm, 3 µm
Eluent A: Aqueous 20 mM ammonium acetate solution (pH 5.1)
Eluent B: Mixture of 2 parts per volume acetonitrile and one part per volume methanol, 20 mM ammonium acetate
Flow rate: 0.2 mL/min (constant flow)
Injection volume: 15 µL
Gradient:

time in min	A %	B %
0.00	98	2
1.0	98	2
10.0	0	100
22.0	0	100
23.0	98	2
24.0	98	2

Table 7-3. MS-MS conditions for analysis of sulfonamides and other antibiotics

System: Applied Biosystems/MDS Sciex API 2000
Interface: Electrospray
Spray position: z = 4.0 y = 3.5
Heater temperature: 300 °C
Nebuliser Gas (GS1): 45 units
Heater Gas (GS2): 45 units
Ionisation mode: positive
Ionisation voltage: +5500 V
Curtain gas: 20 units
CAD: 3.00

All other parameters depend on the compounds and are documented in the laboratory.

Table 7-4. Detection conditions for analysis of sulfonamides and other antibiotics

All compounds are analysed in the Multiple Reaction Monitoring (MRM) mode. Details on the selection of precursor and product ions are summarised in the following table.

Compound	Precursor ion	Product ions
Sulfadiazine	251.0	155.9, 92.1
Sulfadimidine	279.0	185.9, 124.1
Sulfamerazine	265.0	65.1, 156.0
Sulfamethoxazole	271.0	155.8, 254.0
Trimethoprim	291.0	229.9, 123.0
Metronidazole	171.9	128.0, 82.0
Ronidazole	201.0	140.0, 55.1

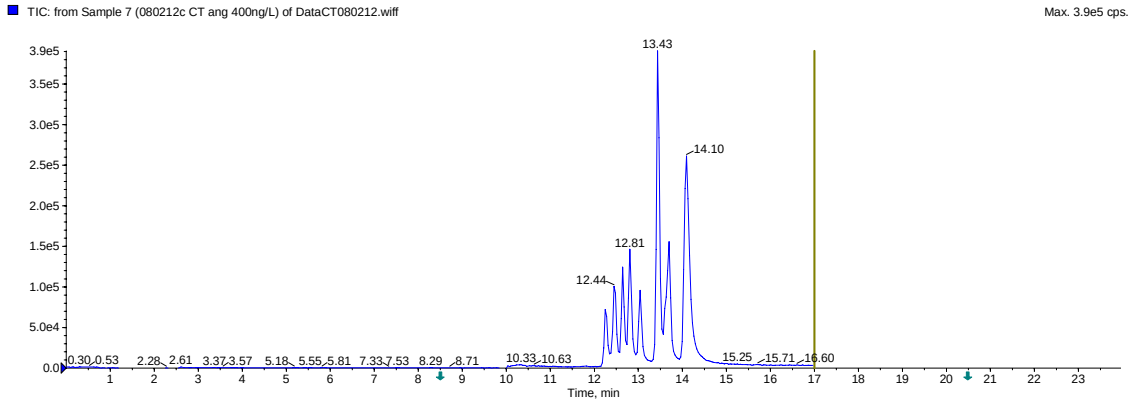

Fig. 7-1 Chromatogram of antibiotics (sulfonamides and others).

DETERMINATION OF PENICILLIN ANTIBIOTICS AND OTHER ANTIBIOTICS BY SOLID-PHASE EXTRACTION FOLLOWED BY LIQUID CHROMATOGRAPHY AND TANDEM MASS SPECTROMETRY

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1.0 SCOPE OF APPLICATION

The procedure can be used for analyzing selected antibiotics in surface, ground and drinking waters. The application to waste waters has to be checked (see also Section 3.0). The following compounds can be determined by this method.

Analyte	CAS-No.
Amoxicillin	61336-70-7
Oxacillin	66-79-5
Cloxacillin	61-72-3
Dicloxacillin	3116-76-5
Nafcillin	147-52-4
Penicillin G	61-33-6
Penicillin V	87-08-1

Analysis of additional antibiotics with this method may be possible, but has to be checked. After pH adjustment the target compounds are pre-concentrated by solid-phase extraction onto a hydroxylated styrene-divinylbenzene co-polymer. Elution of the target compounds is done by a mixture of acetonitrile, water and triethylamine. After evaporation of the solvent and reconstitution of the dry residue, an aliquot is injected into the HPLC/MS-MS system.
Method detection limits (MDLs) depend on the compound and on the matrix. For surface, ground and drinking waters the MDLs for all method analytes are in the range of 20 ng/L. The application range is approximately 20 – 500 ng/L. Samples with higher concentrations have to be diluted before extraction.

2.0 DEFINITIONS

2.1 Laboratory Reagent Blank (LRB)
An aliquot of reagent water or other blank matrix that is treated exactly as a sample including exposure to all glassware, equipment, solvents, reagents, internal standards, and surrogates that are used with other samples. The LRB is used to determine if method analytes or other interferences are present in the laboratory environment, the reagents, or the apparatus.

2.2 Laboratory Fortified Blank (LFB)
An aliquot of reagent water or other blank matrix to which known quantities of the method

analytes are added in the laboratory. The LFB is analyzed exactly like a sample, and its purpose is to determine whether the methodology is in control, and whether the laboratory is capable of making accurate and precise measurements.

2.3 Stock Standard Solution
A concentrated solution containing one or more method analytes prepared in the laboratory using assayed reference materials or purchased from a reputable commercial source.

2.4 Primary Dilution Standard Solution (PDS)
A solution of several analytes prepared in the laboratory from stock standard solutions and diluted as needed to prepare calibration solutions and other needed analyte solutions.

2.5 Calibration Standard (CAL)
A solution prepared from the primary dilution standard solution or stock standard solutions and the internal standards and surrogate analytes. The CAL solutions are used to calibrate the instrument response with respect to analyte concentration.

2.6 Quality Control Sample (QCS)
A solution of method analytes of known concentrations which is used to fortify an aliquot of LRB or sample matrix. The QCS is obtained from a source external to the laboratory and different from the source of calibration standards. It is used to check laboratory performance with externally prepared test materials.

2.7 Procedural Standard Calibration
A calibration method where aqueous calibration standards are prepared and processed (e.g., purged, extracted, and/or derivatised) in exactly the same manner as a sample. All steps in the process from addition of sampling preservatives through instrumental analyses are included in the calibration. Using procedural standard calibration compensates for any inefficiencies in the processing procedure.

3.0 INTERFERENCES

3.1 Analyses of laboratory reagent blanks provide information about the presence of contaminants. When potential interfering peaks are noted in laboratory reagent blanks, the

analyst should search for the source, including instrument, glassware, reagents, or solvents. Subtracting blank values from sample results is not permitted.

3.2 Interfering contamination may occur when a sample containing low concentrations of method analytes is analyzed immediately after a sample containing relatively high concentrations of method analytes. A preventive technique is analyzing blank samples between environmental samples.

3.3 Waste water samples have to be diluted prior to analysis in order to avoid any interference during pre-concentration and analysis.

4.0 INSTRUMENTATION

4.1 HPLC system: HP 1100 from Agilent Technologies

4.2 Mass spectrometer: API 2000 from Applied Biosystems/MDS Sciex

4.3 Automated pre-concentration system: Autotrace SPE Workstations from Zymark/Caliper

4.4 Equipment and supplies:

4.4.1 Separation column 250 x 2 mm, 3 µm Nucleosil from Bischoff Analysentechnik
4.4.2 Brown glass bottles, nominal volume 1000 mL
4.4.3 Glass bottles with screw cap, volume: 500 mL
4.4.4 Vials: 1 mL and 10 mL from Supelco
4.4.5 Micro vial inserts from WGA, volume: 100 µL
4.4.6 Graduated cylinders, nominal volume: 100 mL, 250 mL, 500 mL, 1000 mL
4.4.7 Volumetric flasks, nominal volume: 10 mL, 25 mL
4.4.8 Syringes (5 µL, 10 µL, 25 µL, 50 µL, 100 µL, 500 µL) from Hamilton or Agilent Technologies
4.4.9 Analytical balance from Sartorius
4.4.10 pH Meter from Schott instruments
4.4.11 Drying oven

5.0 CHEMICALS

5.1 Solvents

All solvents have to be of p.a. purity and shall be stored at room temperature.

5.1.1 Acetonitrile
5.1.2 Methanol
5.1.3 Acetone

5.2 Reagents

All reagents have to be of p.a. purity and shall be stored at room temperature.

5.2.1 Ammonium acetate
5.2.2 Ammonium formate
5.2.3 Triethylamine
5.2.4 1 M Caustic soda
5.2.5 Hydrochloric acid, 32%
5.2.6 Demineralised and decoct water
5.2.7 Ethylenediaminetetraacetic acid (EDTA disodium salt)
5.2.8 Sodium azide

5.3 SPE Material

5.3.1 Isolute ENV+ from Separtis

5.4 Method analytes

All analytes must be purchased from a reputable commercial source and have to be of p.a. purity. The reference materials have to be stored according to the recommendations from the manufacturer.

5.4.1 Amoxicillin
5.4.2 Oxacillin
5.4.3 Cloxacillin
5.4.4 Dicloxacillin
5.4.5 Nafcillin
5.4.6 Penicillin G

5.4.7 Penicillin V

5.5 Stock standard solutions

Caution: Not all penicillins are stable in methanol. Thus, stock solutions are prepared in acetone.
For each of the target compounds a separate stock solution in acetone is prepared. Their concentrations depend on the solubility of the target compounds in acetone.
For the preparation of each of the stock solutions, between 1 and 10 mg of the antibiotic are added on an analytical balance to a 10 mL volumetric flask. Then, the volumetric flask is filled up to volume with acetone. From the exact weight of the antibiotic and the volume of acetone, the exact concentration of the stock solution is calculated taking into account the purity of the target compounds.
The stock solutions have to be stored in the freezer and are stable for 2 months.

5.6 Primary Dilution Standard (PDS) of the target compounds; $\beta_{\textbf{single compound}} = 1$ ng/µL

Few mL of acetone are added to a 25 mL volumetric flask. A defined volume of each stock solution of the method analytes is added with a syringe to achieve a concentration of 1 ng/µL. The volumetric flask is filled up to volume with acetone.
The PDS of the target compounds has to be stored in the freezer and is stable for 1 month.

5.7 Standard mixture for Quality Control Sample

The standard mixture for the QCS contains the following antibiotics:

- nafcillin
- penicillin G.

For preparation of the standard mixture for the QA/QC sample, few mL of acetone are added to a 25 mL volumetric flask. A defined volume of each stock solution of each target compound is added with a syringe to achieve a final concentration of 1 ng/µL. The volumetric flask is filled up to volume with acetone.
The QA/QC sample of the target compounds has to be stored in the freezer and is stable for 3 months.

5.8 Solvent mixture for reconstitution of the dry residue

In a 100 mL graduated cylinder a mixture of 5 parts per volume acetonitrile and 95 parts per volume of an aqueous solution of 20 mM ammonium acetate is prepared.
This solution can be stored in the refrigerator for a maximum of 1 week.

5.9 Calibration samples of target compounds

Eight glass bottles (V = 1000 mL) are filled with 500 mL tap water with a graduated cylinder. Then different volumes of the standard mixture of the target compounds (see Section 5.6) are added with a syringe. The added volumes as well as the resulting concentration levels are given in Table 8-1 in the Annex.

5.10 Eluent A: Aqueous 2 mM ammonium formate solution

An aqueous 2 mM ammonium formate solution is prepared in a 500 mL glass bottle with screw cap. This solution can not be stored and has to be prepared anew each day of analysis.

5.11 Eluent B: 2:1 (v:v) mixture of acetonitrile and methanol

In a 500 mL glass bottle with screw cap, two parts per volume acetonitrile are mixed with one part per volume of methanol by using a graduated cylinder. Then a defined amount of ammonium formate is added to achieve a final concentration of 2 mM.
This solution can be stored at room temperature for a maximum of 1 week.

5.12 Elution solvent

In a 100 mL graduated cylinder a mixture of 90 parts per volume acetonitrile, 10 parts per volume of demineralised and decoct water and 0.5 parts per volume of triethylamine is prepared.
This solution can not be stored and has to be prepared freshly for each day of analysis.

6.0 SAMPLE COLLECTION, PRESERVATION, AND STORAGE

6.1 Sample Collection and Dechlorination

Sampling is done in 1000 mL brown glass bottles. If samples, such as finished drinking waters, are suspected to contain residual chlorine, 100 mg of sodium thiosulfate should be added for each 1000 mL of water sample.

6.2 Sample Storage

Storage of samples is done in the cold storage room at 4 °C and must not exceed a time period of 3 weeks. Extracts must be stored in the freezer and are stable for at least 2 weeks.

6.3 Sample preservation

For preservation of surface water, groundwater and drinking water samples 100 mg/L sodium azide are added immediately after sampling.

7.0 PROCEDURE

7.1 Extraction

First, 1.3 g of ethylendiaminetetraacetic acid are added to 500 mL of water sample. The sample is adjusted to pH 5 by addition of 32% hydrochloric acid or 1 M caustic soda. pH is checked with a pH meter. Conditioning of 100 mg Isolute ENV+ material is done automatically (Autotrace system) with 5 mL acetonitrile and subsequently with 5 mL demineralised and decoct water. For pre-concentration of the target analytes, the pH adjusted water sample is automatically pumped through the conditioned SPE material by the Autotrace system. Flow rate is 7.0 mL/min. After pre-concentration, the SPE material is dried for 30 min. with nitrogen gas. Elution of the target analytes is done automatically by the Autotrace system with 5 mL acetonitrile and 5 mL of elution solvent (see Section 5.12). After passing through the SPE material the elution solvent is collected in a 10 mL vial. Then the solvent is completely evaporated in a stream of nitrogen. The dry residue is reconstituted with 100 µL of the solvent mixture (see Section 5.8). Then, the sample is ready for HPLC/MS-MS analysis.

7.2 HPLC/MS-MS analysis

Details on instrumentation and conditions of HPLC/MS-MS analysis are given in Tables 8-2

to 8-4 in the Annex.

7.2.1 Operation of the HPLC/MS-MS system

Operation of the HPLC/MS-MS system is done as described in the manufacturer's manual. Prior to analysis of environmental samples, a blank run is started to check the pressure conditions and to flush the column with solvent. For checking the MS detector, a standard solution is analysed. 25 µL of the standard mixture of the target compounds are evaporated to dryness in a stream of nitrogen and reconstituted with 100 µL of the solvent mixture (see Section 5.8). Subsequently, 12.5 µL are injected into the HPLC/MS-MS system. Criteria for checking the performance of the system are signal height, retention time and peak performance.

7.2.2 Calibration

Calibration is done for the overall procedure. Calibration samples are prepared as described in Section 5.9 and analysed. A new calibration is prepared for each batch of samples or whenever the QA/QC sample gives striking results.

8.0 DATA ANALYSIS AND CALCULATIONS

8.1 Complete chromatographic resolution is not necessary for accurate and precise measurements of analyte concentrations if unique ions with adequate intensities are available for quantitation. If the response for any analyte exceeds the linear range of the calibration established in Section 7.2.2, obtain and dilute a duplicate sample. Do not extrapolate beyond the calibration range.

8.2 Evaluation of the chromatograms, i.e. correlation of retention times and mass spectra to target compounds and determination of the peak areas is done by the software tool Analyst 1.2. If necessary, manual integration is done.

8.3 Linear calibration functions are established by plotting the peak area of the respective target analyte against the concentration of the target analyte in the respective calibration solution. Linear regression of the calibration functions provides the calibration coefficients a_1 und a_0 as well as the regression coefficient r.

8.4 Concentrations in real samples are calculated by the software according to the following equation:

$$x = \frac{y - a_0}{a_1}$$

with x = concentration of the target analyte (ng/L)
y = peak area of the target analyte
a_1 = slope of the linear calibration function (= sensitivity)
a_0 = intercept of the linear calibration function

8.5 The recovery for the overall procedure is determined by a comparison of peak areas of pre-concentrated samples and direct injections according to the following equation:

$$R = \frac{Y_{pre-concentrated}}{Y_{direct}} \cdot 100\%$$

with $Y_{pre\text{-}concentrated}$ = peak area of a compound after pre-concentration
Y_{direct} = peak area of a compound after direct injection

The recovery is determined each time that the batch of SPE material or elution solvent is changed.

9.0 REPORT

9.1 Specification of results
Results are reported in ng/L with two significant digits.

10.0 QUALITY CONTROL

10.1 QC requirements
Quality control (QC) requirements are the initial demonstration of laboratory capability followed by regular analyses of laboratory reagent blanks, field reagent blanks, and laboratory fortified blanks. A MDL for each analyte must also be determined. Each laboratory must maintain records to document the quality of the data generated. Additional quality control practices are recommended.

10.2 Figures of merit

Figures of merit such as LOD, LOQ, or repeatability are determined during method validation according to the German Standard Procedure DIN 32645.

10.3 Laboratory Blanks
With each batch of samples processed as a group within a work shift, analyze a LRB to determine the background system contamination.

10.4 Calibration Check
In order to check the calibration, a LFB is processed with each batch of samples. For each of the method analytes the result of the LFB must be within 30% of the spiked concentration. If the deviation between spiked and measured concentration exceeds this range, appropriate measures must be taken to improve the quality of analysis before analysis of environmental samples can be continued.

10.5 QC control chart
For QA/QC purposes control charts are used. A QA/QC sample is analysed each day of analysis.

10.5.1 The QA/QC samples are prepared by adjusting 500 mL of tap water to a pH of 5 by addition of 32% hydrochloric acid or 1 M caustic soda. Then 100 µL of the standard mixture for the QA/QC sample are added. The QA/QC sample is treated as any environmental sample according to the procedure described in Section 7.0. The results of these QA/QC measurements for nafcillin and penicillin G are evaluated and documented in control charts by the SQS software from Perkin Elmer.

11.0 POLLUTION PREVENTION

11.1 No solvents are utilized in this method except the extremely small volumes of methanol and acetonitrile needed to prepare calibration standards and elution solvents. The only other chemicals used in this method are the neat materials in preparing standards and sample preservatives. All are used in extremely small amounts and pose no threat to the environment.

12.0 WASTE MANAGEMENT

12.1 SPE cartridges are only used once and are disposed with the domestic waste.

12.2 Solutions containing solvents are collected in glass bottles. Full glass bottles are shipped to a special unit for proper waste disposal.

13.0 REFERENCES

1. F. Sacher, F.T. Lange, H.-J. Brauch, I. Blankenhorn. “Pharmaceuticals in groundwaters - Analytical methods and results of a monitoring program in Baden-Württemberg, Germany.” J. Chromatogr. A 938:199-210 (2001)

ANNEX

Table 8-1. Added volumes for the preparation of the calibration samples

Calibration sample	Added volume (µL)	Concentration (ng/L)
1	5	5
2	10	10
3	25	25
4	50	50
5	75	75
6	100	100
7	250	250
8	500	500

Table 8-2. LC conditions for analysis of penicillins and other antibiotics

Separation column: Nucleosil, 250 mm*2 mm, 3 µm
Eluent A: Aqueous 2 mM ammonium formate solution (pH 7.0)
Eluent B: Mixture of 2 parts per volume acetonitrile and one part per volume methanol, 2 mM ammonium formate
Flow rate: 0.2 mL/min (constant flow)
Injection volume: 10 µL
Gradient:

time in min	A %	B %
0.00	95	5
1.0	95	5
17.0	50	50
18.0	0	100
27.0	0	100
28.0	95	5
30.0	95	5

Table 8-3. MS-MS conditions for analysis of penicillins and other antibiotics

System: Applied Biosystems/MDS Sciex API 2000
Interface: Electrospray
Spray position: z = 4.0 y = 3.5
Heater temperature: 300 °C
Nebuliser Gas (GS1): 45 units
Heater Gas (GS2): 65 units
Ionisation mode: positive
Ionisation voltage: +4200 V
Curtain gas: 45 units
CAD: 2.00

All other parameters depend on the compounds and are documented in the laboratory.

Table 8-4. Detection conditions for analysis of penicillins and other antibiotics

All compounds are analysed in the Multiple Reaction Monitoring (MRM) mode. Details on the selection of precursor and product ions are summarised in the following table.

Compound	Precursor ion	Product ions
Amoxicillin	366.1	114.0, 86.1
Oxacillin	419.1	243.0, 160.0
Cloxacillin	453.0	276.9, 160.0
Dicloxacillin	470.0	310.9, 160.0
Nafcillin	415.1	199.1, 171.1
Penicillin G	352.1	176.1, 160.0
Penicillin V	368.1	114.1, 160.1

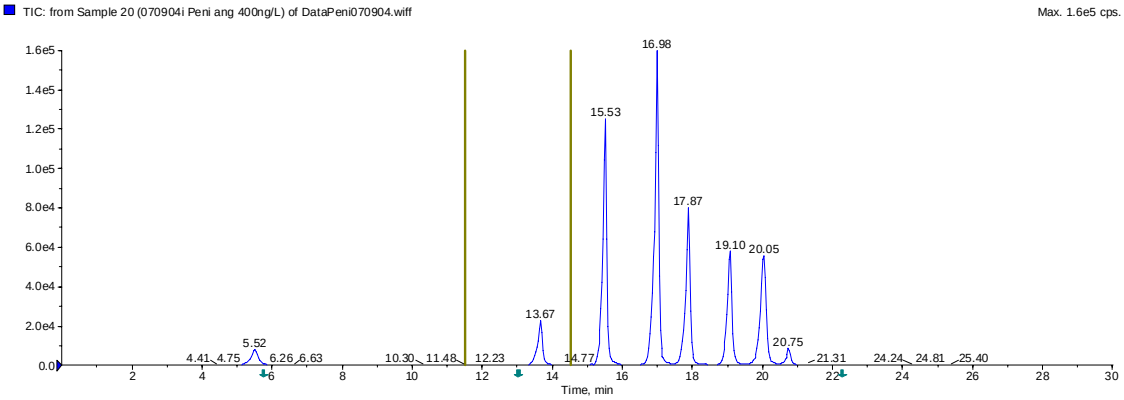

Fig. 8-1 Chromatogram of antibiotics (penicillins and others).

DETERMINATION OF CEPHALOSPORIN ANTIBIOTICS BY SOLID-PHASE EXTRACTION FOLLOWED BY LIQUID CHROMATOGRAPHY AND TANDEM MASS SPECTROMETRY

Edited by Prof. H.-J. Brauch and Dr. F. Sacher

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1.0 SCOPE OF APPLICATION

The procedure can be used for analyzing selected cephalosporin antibiotics in surface, ground and drinking waters. The application to waste waters has to be checked (see also Section 3.0). The following compounds can be determined by this method.

Analyte	CAS-No.
Cefaclor	53994-73-3
Cefadroxil	66592-87-8
Cefalexin	15686-71-2
Cefixime	79350-37-1
Ceftiofur	80370-57-6
Ceftriaxone	63527-52-6
Cefuroxime	55268-75-2

Analysis of additional cephalosporins with this method may be possible, but has to be checked. After pH adjustment the target compounds are pre-concentrated by solid-phase extraction onto a hydroxylated styrene-divinylbenzene polymer. Elution of the target compounds is done by methanol and a mixture of acetonitrile, water and triethylamine. After evaporation of the solvent and reconstitution of the dry residue, an aliquot is injected into the HPLC/MS-MS system.
Method detection limits (MDLs) depend on the compound and on the matrix. For surface, ground and drinking waters the MDLs for all method analytes are in the range of 20 ng/L. The application range is approximately 20 – 500 ng/L. Samples with higher concentrations have to be diluted before extraction.

2.0 DEFINITIONS

2.1 Laboratory Reagent Blank (LRB)
An aliquot of reagent water or other blank matrix that is treated exactly as a sample including exposure to all glassware, equipment, solvents, reagents, internal standards, and surrogates that are used with other samples. The LRB is used to determine if method analytes or other interferences are present in the laboratory environment, the reagents, or the apparatus.

2.2 Laboratory Fortified Blank (LFB)

An aliquot of reagent water or other blank matrix to which known quantities of the method analytes are added in the laboratory. The LFB is analyzed exactly like a sample, and its purpose is to determine whether the methodology is in control, and whether the laboratory is capable of making accurate and precise measurements.

2.3 Stock Standard Solution
A concentrated solution containing one or more method analytes prepared in the laboratory using assayed reference materials or purchased from a reputable commercial source.

2.4 Primary Dilution Standard Solution (PDS)
A solution of several analytes prepared in the laboratory from stock standard solutions and diluted as needed to prepare calibration solutions and other needed analyte solutions.

2.5 Calibration Standard (CAL)
A solution prepared from the primary dilution standard solution or stock standard solutions and the internal standards and surrogate analytes. The CAL solutions are used to calibrate the instrument response with respect to analyte concentration.

2.6 Quality Control Sample (QCS)
A solution of method analytes of known concentrations which is used to fortify an aliquot of LRB or sample matrix. The QCS is obtained from a source external to the laboratory and different from the source of calibration standards. It is used to check laboratory performance with externally prepared test materials.

2.7 Procedural Standard Calibration
A calibration method where aqueous calibration standards are prepared and processed (e.g., purged, extracted, and/or derivatised) in exactly the same manner as a sample. All steps in the process from addition of sampling preservatives through instrumental analyses are included in the calibration. Using procedural standard calibration compensates for any inefficiencies in the processing procedure.

3.0 INTERFERENCES

3.1 Analyses of laboratory reagent blanks provide information about the presence of contaminants. When potential interfering peaks are noted in laboratory reagent blanks, the

analyst should search for the source, including instrument, glassware, reagents, or solvents. Subtracting blank values from sample results is not permitted.

3.2 Interfering contamination may occur when a sample containing low concentrations of method analytes is analyzed immediately after a sample containing relatively high concentrations of method analytes. A preventive technique is analyzing blank samples between environmental samples.

3.3 Waste water samples have to be diluted prior to analysis in order to avoid any interference during pre-concentration and analysis.

4.0 INSTRUMENTATION

4.1 HPLC system: HP 1100 from Agilent Technologies

4.2 Mass spectrometer: API 2000 from Applied Biosystems/MDS Sciex

4.3 Automated pre-concentration system: Autotrace SPE Workstations from Zymark/Caliper

4.4 Equipment and supplies:

4.4.1 Separation column 250 x 2 mm, 5 µm Luna from Phenomenex
4.4.2 Brown glass bottles, nominal volume 1000 mL
4.4.3 Glass bottles with screw cap, volume: 500 mL
4.4.4 Vials: 1 mL and 10 mL from Supelco
4.4.5 Micro vial inserts from WGA, volume: 100 µL
4.4.6 Graduated cylinders, nominal volume: 100 mL, 250 mL, 500 mL, 1000 mL
4.4.7 Volumetric flasks, nominal volume: 10 mL, 25 mL
4.4.8 Syringes (5 µL, 10 µL, 25 µL, 50 µL, 100 µL, 500 µL) from Hamilton or Agilent Technologies
4.4.9 Analytical balance from Sartorius
4.4.10 pH Meter from Schott instruments
4.4.11 Drying oven

5.0 CHEMICALS

5.1 Solvents
All solvents have to be of p.a. purity and shall be stored at room temperature.

5.1.1 Acetonitrile
5.1.2 Methanol
5.1.3 Acetone

5.2 Reagents
All reagents have to be of p.a. purity and shall be stored at room temperature.

5.2.1 Ammonium acetate
5.2.2 Ammonium formate
5.2.3 Triethylamine
5.2.4 1 M Caustic soda
5.2.5 Hydrochloric acid, 32%
5.2.6 Formic acid, conc.
5.2.7 Demineralised and decoct water
5.2.8 Ethylenediaminetetraacetic acid (EDTA disodium salt)
5.2.9 Sodium azide

5.3 SPE Material

5.3.1 Isolute ENV+ from Separtis

5.4 Method analytes
All analytes must be purchased from a reputable commercial source and have to be of p.a. purity. The reference materials have to be stored according to the recommendations from the manufacturer.

5.4.1 Cefaclor
5.4.2 Cefadroxil
5.4.3 Cefalexin
5.4.4 Cefixime
5.4.5 Ceftiofur

5.4.6 Ceftriaxone
5.4.7 Cefuroxime

5.5 Stock standard solutions

For each of the target compounds a separate stock solution in water is prepared. Their concentrations depend on the solubility of the target compounds in water.
For the preparation of each of the stock solutions, between 1 and 10 mg of the antibiotic are added on an analytical balance to a 10 mL volumetric flask. Then, the volumetric flask is filled up to volume with water. From the exact weight of the antibiotic and the volume of water, the exact concentration of the stock solution is calculated taking into account the purity of the target compounds.
The stock solutions have to be stored in the freezer and are stable for 2 months.

5.6 Primary Dilution Standard (PDS) of the target compounds; $\beta_{\textbf{single compound}} = 1$ ng/µL

Few mL of water are added to a 25 mL volumetric flask. Then, a defined volume of each stock solution of the method analytes is added with a syringe to achieve a concentration of 1 ng/µL. Then, the volumetric flask is filled up to volume with water.
The PDS of the target compounds has to be stored in the freezer and is stable for 1 month.

5.7 Standard mixture for Quality Control Sample

The standard mixture for the QCS contains the following antibiotics:

- cefuroxime
- cefaclor.

For preparation of the standard mixture for the QA/QC sample, few mL of water are added to a 25 mL volumetric flask. A defined volume of each stock solution of the target compounds is added with a syringe to achieve a final concentration of 1 ng/µL. The volumetric flask is filled up to volume with water.
The QA/QC sample of the target compounds has to be stored in the freezer and is stable for 3 months.

5.8 Solvent mixture for reconstitution of the dry residue

In a 100 mL graduated cylinder a mixture of 90 parts per volume demineralised and decoct water and 10 parts per volume acetonitrile is prepared.
This solution can be stored in the refrigerator for a maximum of 1 week.

5.9 Calibration samples of target compounds

Eight glass bottles (V = 1000 mL) are filled with 500 mL tap water with a graduated cylinder. Different volumes of the standard mixture of the target compounds (see Section 5.6) are added with a syringe. The added volumes as well as the resulting concentration levels are given in Table 9-1 in the Annex.

5.10 Eluent A: 0.1 % Formic acid

A 0.1% formic acid is prepared in a 500 mL glass bottle with screw cap. This solution can not be stored and has to be prepared anew each day of analysis.

5.11 Eluent B: 2:1 (v:v) mixture of acetonitrile and methanol

In a 500 mL glass bottle with screw cap, two parts per volume acetonitrile are mixed with one part per volume of methanol by using a graduated cylinder. Then formic acid is added to achieve a final concentration of 0.1%.
This solution can be stored at room temperature for a maximum of 1 week.

5.12 Elution solvent

In a 100 mL graduated cylinder a mixture of 90 parts per volume acetonitrile, 10 parts per volume of demineralised and decoct water and 0.5 parts per volume of triethylamine is prepared.
This solution can not be stored and has to be prepared anew each day of analysis.

6.0 SAMPLE COLLECTION, PRESERVATION, AND STORAGE

6.1 Sample Collection and Dechlorination

Sampling is done in 1000 mL brown glass bottles. If samples, such as finished drinking

waters, are suspected to contain residual chlorine, 100 mg of sodium thiosulfate should be added for each 1000 mL of water sample.

6.2 Sample Storage

Storage of samples is done in the cold storage room at 4 °C and must not exceed a time period of 3 weeks. Extracts must be stored in the freezer and are stable for at least 2 weeks.

6.3 Sample preservation

For preservation of surface water, groundwater and drinking water samples 100 mg/L sodium azide are added immediately after sampling.

7.0 PROCEDURE

7.1 Extraction

First, 1.3 g of ethylendiaminetetraacetic acid are added to 1000 mL of water sample. Then the sample is adjusted to pH 5 by addition of 32% hydrochloric acid or 1 M caustic soda. pH is checked with a pH meter. Conditioning of 100 mg Isolute ENV+ material is done automatically (Autotrace system) with 5 mL methanol and subsequently with 5 mL 0.1% formic acid. For pre-concentration of the target analytes, the pH adjusted water sample is automatically pumped through the conditioned SPE material by the Autotrace system. Flow rate is 10 mL/min. After pre-concentration, the SPE material is dried for 45 min. with nitrogen gas. Elution of the target analytes is done automatically by the Autotrace system with 5 mL methanol and 5 mL of elution solvent (see Section 5.12). After passing through the SPE material the elution solvent is collected in a 10 mL vial. Then the solvent is completely evaporated in a stream of nitrogen. The dry residue is reconstituted with 100 µL of the solvent mixture (see Section 5.8). Then, the sample is ready for HPLC/MS-MS analysis.

7.2 HPLC/MS-MS analysis

Details on instrumentation and conditions of HPLC/MS-MS analysis are given in Tables 9-2 to 9-4 in the Annex.

7.2.1 Operation of the HPLC/MS-MS system

Operation of the HPLC/MS-MS system is done as described in the manufacturer's manual. Prior to analysis of environmental samples, a blank run is started to check the pressure conditions and to flush the column with solvent. For checking the MS detector, a standard solution is analysed. 25 µL of the standard mixture of the target compounds are evaporated to dryness in a stream of nitrogen and reconstituted with 100 µL of the solvent mixture (see Section 5.8). Subsequently, 12.5 µL are injected into the HPLC/MS-MS system. Criteria for checking the performance of the system are signal height, retention time and peak performance.

7.2.2 Calibration

Calibration is done for the overall procedure. Calibration samples are prepared as described in Section 5.9 and analysed. A new calibration is prepared for each batch of samples or whenever the QA/QC sample gives striking results.

8.0 DATA ANALYSIS AND CALCULATIONS

8.1 Complete chromatographic resolution is not necessary for accurate and precise measurements of analyte concentrations if unique ions with adequate intensities are available for quantitation. If the response for any analyte exceeds the linear range of the calibration established in Section 7.2.2, obtain and dilute a duplicate sample. Do not extrapolate beyond the calibration range.

8.2 Evaluation of the chromatograms, i.e. correlation of retention times and mass spectra to target compounds and determination of the peak areas is done by the software tool Analyst 1.2. If necessary, manual integration is done.

8.3 Linear calibration functions are established by plotting the peak area of the respective target analyte against the concentration of the target analyte in the respective calibration solution. Linear regression of the calibration functions provides the calibration coefficients a_1 und a_0 as well as the regression coefficient r.

8.4 Concentrations in real samples are calculated by the software according to the following equation:

$$x = \frac{y - a_0}{a_1}$$

with x = concentration of the target analyte (ng/L)
y = peak area of the target analyte
a_1 = slope of the linear calibration function (= sensitivity)
a_0 = intercept of the linear calibration function

8.5 The recovery for the overall procedure is determined by a comparison of peak areas of pre-concentrated samples and direct injections according to the following equation:

$$R = \frac{Y_{pre-concentrated}}{Y_{direct}} \cdot 100\%$$

with $Y_{pre\text{-}concentrated}$ = peak area of a compound after pre-concentration
Y_{direct} = peak area of a compound after direct injection

The recovery is determined each time that the batch of SPE material or elution solvent is changed.

9.0 REPORT

9.1 Specification of results
Results are reported in ng/L with two significant digits.

10.0 QUALITY CONTROL

10.1 QC requirements
Quality control (QC) requirements are the initial demonstration of laboratory capability followed by regular analyses of laboratory reagent blanks, field reagent blanks, and laboratory fortified blanks. A MDL for each analyte must also be determined. Each laboratory must maintain records to document the quality of the data generated. Additional quality control practices are recommended.

10.2 Figures of merit
Figures of merit such as LOD, LOQ, or repeatability are determined during method validation according to the German Standard Procedure DIN 32645.

10.3 Laboratory Blanks
With each batch of samples processed as a group within a work shift, analyze a LRB to determine the background system contamination.

10.4 Calibration Check
In order to check the calibration, a LFB is processed with each batch of samples. For each of the method analytes the result of the LFB must be within 30% of the spiked concentration. If the deviation between spiked and measured concentration exceeds this range, appropriate measures must be taken to improve the quality of analysis before analysis of environmental samples can be continued.

10.5 QC control chart
For QA/QC purposes control charts are used. A QA/QC sample is analysed each day of analysis.

10.5.1 The QA/QC samples are prepared by adjusting 1000 mL of tap water to a pH of 5 by addition of 32% hydrochloric acid or 1 M caustic soda. Then 100 µL of the standard mixture for the QA/QC sample are added. The QA/QC sample is treated as any environmental sample according to the procedure described in Section 7.0. The results of these QA/QC measurements for cefuroxim and cefaclor are evaluated and documented in control charts by the SQS software from Perkin Elmer.

11.0 POLLUTION PREVENTION

11.1 No solvents are utilized in this method except the extremely small volumes of methanol and acetonitrile needed to prepare calibration standards and elution solvents. The only other chemicals used in this method are the neat materials in preparing standards and sample preservatives. All are used in extremely small amounts and pose no threat to the environment.

12.0 WASTE MANAGEMENT

12.1 SPE cartridges are only used once and are disposed with the domestic waste.

12.2 Solutions containing solvents are collected in glass bottles. Full glass bottles are shipped to a special unit for proper waste disposal.

ANNEX

Table 9-1. Added volumes for the preparation of the calibration samples

Calibration sample	Added volume (µL)	Concentration (ng/L)
1	5	5
2	10	10
3	25	25
4	50	50
5	75	75
6	100	100
7	250	250
8	500	500

Table 9-2. LC conditions for analysis of cephalosporin antibiotics

Separation column: Luna, 250 mm*2 mm, 5 µm
Eluent A: Aqueous 0.1% formic acid
Eluent B: Mixture of 2 parts per volume acetonitrile and one part per volume methanol and 0.1% formic acid
Flow rate: 0.2 mL/min (constant flow)
Injection volume: 12.5 µL
Gradient:

time in min	A %	B %
0.00	98	2
1.0	98	2
15.0	0	100
24.0	0	100
25.0	98	2
26.0	98	2

Table 9-3. MS-MS conditions for analysis of cephalosporin antibiotics

System: Applied Biosystems/MDS Sciex API 2000
Interface: Electrospray
Spray position: z = 4.0 y = 3.5
Heater temperature: 200 °C
Nebuliser Gas (GS1): 20 units
Heater Gas (GS2): 0 units
Ionisation mode: positive
Ionisation voltage: +5000 V
Curtain gas: 20 units
CAD: 5.00

All other parameters depend on the compounds and are documented in the laboratory.

Table 9-4. Detection conditions for analysis of cephalosporin antibiotics

All compounds are analysed in the Multiple Reaction Monitoring (MRM) mode. Details on the selection of precursor and product ions are summarised in the following table.

Compound	Precursor ion	Product ions
Cefaclor	368.3	174.1, 106.1
Cefadroxil	364.0	113.8, 85.9
Cefalexin	347.9	158.0, 106.0
Cefixime	454.5	285.1, 125.9
Ceftiofur	524.1	240.9, 94.9
Ceftriaxone	555.1	124.9, 323.9
Cefuroxime	442.3	336.0, 93.0

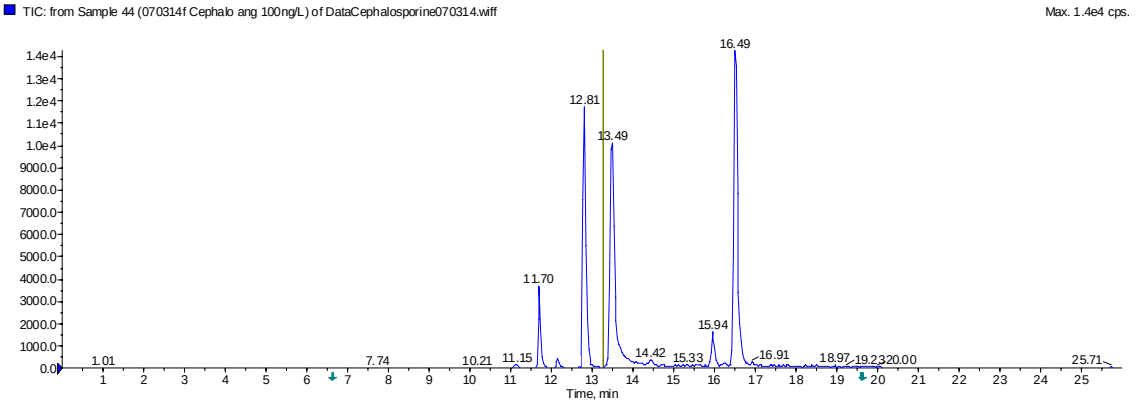

Fig. 9-1 Chromatogram of cephalosporin antibiotics.

MULTI-RESIDUAL ANALYTICAL METHODS OF PERSISTENT ORGANIC POLLUTANTS (POPS) IN WATER BY HIGH RESOLUTION GAS CHROMATOGRAPHY/ HIGH RESOLUTION MASS SPECTROMETRY

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ABBREVIATIONS

POPs	Persistent organic pollutants
HRGC	High-resolution gas chromatography
HRMS	High-resolution mass spectrometry
LRB	Laboratory Reagent Blank
LFB	Laboratory Fortified Blank
PDS	Primary Dilution Standard Solution
QCS	Quality Control Sample
DCM	Dichloromethane
LOD	Limit of detection
LOQ	Limit of quantification
RRF	Relative Response Factor
QA	Quality assurance
QC	Quality control
SPE	Solid-phase extraction
SIM	Selected ion monitoring

Units

pg	picogram
mL	milliliter
μL	microliter

1.0 SCOPE OF APPLICATION

The Persistent Organic Pollutants (POPs) are chemicals that remain intact in the environment for long periods, bio-accumulated in the living organisms and cause adverse effects on human health and environment. This is a general purpose method for the identification and simultaneous measurement of POPs in surface water, ground water and drinking water in any stage of treatment. The following compounds can be determined by this method.

POPs	Cas no.	Principle uses
Hexachlorobenzene (HCB)	118-74-1	Fungicide; industrial intermediate
Aldrin	309-00-2	Insecticide
Dieldrin	60-57-1	Insecticide; aldrin metabolite
Endrin	72-20-8	Insecticide; isodrin metabolite
p,p'-DDT	50-29-3	Insecticide
o,p'-DDT	789-02-6	*p,p'*-DDT impurity
p,p'-DDD	72-54-8	Insecticide; DDT-metabolite
o,p'-DDD	789-02-6	*o,p'*-DDT metabolite
p,p'-DDE	72-55-9	DDT-metabolite
o,p'-DDE	3424-82-6	*o,p'*-DDT metabolite
trans-Chlordane	5103-74-2	Technical chlordane constituent
cis-Chlordane	5103-71-9	Insecticide; technical chlordane constituent
trans-Nonachlor	39765-80-5	Technical chlordane constituent
cis-Nonachlor	5103-73-1	Technical chlordane constituent
Oxychlordane	27304-13-8	*cis*-Chlordane metabolite
Heptachlor	76-44-8	Insecticide
Heptachlor epoxide	1024-57-3	Heptachlor metabolite; technical chlordane constituent/metabolite

alpha-Hexachlorocyclohexane (α-HCH)	319-84-6	Constituent of insecticide mixture containing various HCH isomers; also known as a-benzene hexachloride (BHC)
beta-Hexachlorocyclohexane (β-HCH)	319-85-7	Technical HCH (BHC) constituent
gamma-Hexachlorocyclohexane (δ-HCH) (Lindane)	58-89-9	Technical HCH (BHC) constituent
delta-Hexachlorocyclohexane (ɣ-HCH)	319-86-8	Insecticide; technical HCH (BHC) constituent
Mirex	2385-85-5	Insecticide; fire retardant

As with most organic contaminants at trace levels in water, extraction/concentration is required as part of the analytical procedure in order to increase the analyte concentration to a level where it can be detected by the analytical technique used. This also allows for sample cleanup in that analytical interferences in the original sample can be removed. The method for determination of POPs in water is by isotope dilution high-resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS). After cleanup, the extract is concentrated to near dryness. Immediately prior to injection, internal standards were added to each extract, and an aliquot of the extract was injected into the gas chromatograph. The POPs were separated by the HRGC, and then detected by a high resolution (≥10,000) mass spectrometer. The quality of the analysis was assured through reproducible calibration and testing of the extraction, cleanup, and HRGC/HRMS systems. Method detection limits (MDLs) depend on the compound and on the matrix. For surface, ground and drinking waters the MDLs for all method analytes are in the range of 0.1-0.4 pg/L. The application range is approximately 0.1 – 1000 pg/L. Samples with higher concentrations have to be diluted before extraction.

2.0 DEFINITIONS

2.1 Laboratory Reagent Blank (LRB)

An aliquot of reagent water or other blank matrix that is treated exactly as a sample including exposure to all glassware, equipment, solvents, reagents, internal standards, and surrogates that are used with other samples. The LRB is used to determine if method analytes or other

interferences are present in the laboratory environment, the reagents, or the apparatus.

2.2 Laboratory Fortified Blank (LFB)
An aliquot of reagent water or other blank matrix to which known quantities of the method analytes are added in the laboratory. The LFB is analyzed exactly like a sample, and its purpose is to determine whether the methodology is in control, and whether the laboratory is capable of making accurate and precise measurements.

2.3 Stock Standard Solution
A concentrated solution containing one or more method analytes prepared in the laboratory using assayed reference materials or purchased from a reputable commercial source.

2.4 Primary Dilution Standard Solution (PDS)
A solution of several analytes prepared in the laboratory from stock standard solutions and diluted as needed to prepare calibration solutions and other needed analyte solutions.

2.5 Calibration Standard (CAL)
A solution prepared from the primary dilution standard solution or stock standard solutions and the internal standards and surrogate analytes. The CAL solutions are used to calibrate the instrument response with respect to analyte concentration.

2.6 Quality Control Sample (QCS)
A solution of method analytes of known concentrations which is used to fortify an aliquot of LRB or sample matrix. The QCS is obtained from a source external to the laboratory and different from the source of calibration standards. It is used to check laboratory performance with externally prepared test materials.

2.7 Procedural Standard Calibration
A calibration method where aqueous calibration standards are prepared and processed (e.g., purged, extracted, and/or derivatised) in exactly the same manner as a sample. All steps in the process from addition of sampling preservatives through instrumental analyses are included in the calibration. Using procedural standard calibration compensates for any inefficiencies in the processing procedure.

3.0 INTERFERENCES

3.1 Analyses of laboratory reagent blanks provide information about the presence of contaminants. When potential interfering peaks are noted in laboratory reagent blanks, the analyst should search for the source, including instrument, glassware, reagents, or solvents. Subtracting blank values from sample results are not permitted.

3.2 Interfering contamination may occur when a sample containing low concentrations of method analytes is analyzed immediately after a sample containing relatively high concentrations of method analytes. A preventive technique is analyzing blank samples between environmental samples.

3.3 Waste water samples have to be diluted prior to analysis in order to avoid any interference during pre-concentration and analysis.

4.0 INSTRUMENTATION

4.1 GC system: Agilent 6890 GC from Agilent Technologies (Palo Alto, CA, USA)

4.2 Mass spectrometer: AutoSpec Ultima from Micromass Ltd.(Manchester,. UK)

4.3 Elution pump system: DPU-8B from GL Science Inc. (Tokyo, Japan)

4.4 Solid-phase extraction system: KS-90UH and DV-20 from Advantec Ltd. (Tokyo, Japan)

4.5 Equipment and supplies:

4.5.1 Separation ENV-8MS, 60m * 0.25mm i.d., film thickness 0.25μm from Kanto Chemical Co.
4.5.2 Glass bottles, nominal volume 1000 mL
4.5.3 Glass bottles with screw cap, volume: 500 mL
4.5.4 Vials: interlock vial with fused glass insert, volume 300 μL
4.5.5 Soxhlet extraction unit (Shibata, Japan)
4.5.6 Graduated cylinders, nominal volume: 100 mL, 250 mL, 500 mL, 1000 mL
4.5.7 Volumetric flask, nominal volume: 10 mL

4.5.8 Round-bottom flasks, nominal volume: 100 mL, 200 mL
4.5.9 Syringes, volume 10 μL, 100 μL, 500 μL
4.5.10 Analytical balance

5.0 CHEMICALS

5.1 Solvents and Reagent
All solvents and reagent have to be of PCB analytical grade and shall be stored at room temperature.
5.1.1 Acetone
5.1.2 Dichloromethane
5.1.3 Hexane
5.1.4 Sodium sulfate

5.2 SPE material

5.2.1 Empore^{TM} Disk from 3M, polyurethane foam plug (PUF)) supplemented with active carbon fiber felt (ACF)

5.3 Filter
5.3.1 Glass microfibre filters GMF150 from Whatman

5.4 Purification cartridge
5.4.1 Bond Elut® MEGA BE-FL, 5 g 200 mL from Varian
5.4.2 Supelclean™ ENVI-Carb™ SPE Tube bed wt. 500 mg, volume 6 mL from Supelco

5.5 POPs standard (native compounds and labeled internal standards)
The standards for calibration, quantification and determination of recovery of POPs can be purchased from Cambridge Isotope Laboratories and Wellingtone Isotope Laboratories.

5.6 Stock standard solutions
The POPs are stable in decane. Thus, stock solutions are prepared in decane. The stock solutions have to be stored in the freezer.

6.0 SAMPLE COLLECTION AND STORAGE

6.1 Sample Collection
Sampling is done in 1000 mL brown glass bottles.

6.2 Sample Storage
Storage of samples is done in the cold storage room at 4 °C and must not exceed a time period of 3 weeks. Extracts must be stored in the freezer and are stable for at least 2 weeks.

7.0 PROCEDURE

7.1 Extraction
For water samples, the stable isotopically-labeled analogs for POPs are spiked into a 10 L sample. Water samples with no visible particles are vacuum-filtered through a glass-fiber filter on top of a solid-phase extraction (SPE) disk at flow rate of 100 mL/min. Water samples containing visible particles are vacuum-filtered through a glass-fiber filter. The glass-fiber filter and the SPE disk are extracted in a Soxhlet extractor with acetone for 16-24 h. The extract is concentrated for clean-up.

7.2 Clean-up
The extract is applied to an hexane prewashed Florisil cartridge and is eluted with 100 ml of DCM:hexane (1:3). Flow rate is 2.5 mL/min. The eluate is concentrated with a rotary evaporator. If the eluate is still viscous and color, it is necessary to pass through a cartridge of active carbon (ENVI-Carb) with 10 ml of hexane. Flow rate is 2.0 mL/min.
This final eluate is concentrated under vacuum and the recovery standard was added and then further concentrated using clean dry nitrogen to a final volume of 100 μL prior to HRGC/HRMS analysis.

7.3 HRGC/HRMS analysis
Details on instrumentation and conditions of HRGC/HRMS analysis are given in Tables 10-1 to 10-3 in the Annex.

7.3.1 Operation of the HRGC/HRMS system
Operation of the HRGC/HRMS system is done as described in the manufacturer's manual.

Prior to analysis of environmental samples, a blank run is started to check the pressure conditions and to flush the column with solvent. The selective ion monitoring (SIM) mode is constituted by tracing M^+ and (M+2)+ ions of each compound. Two mass methods for determination of POPs are accomplished with its most intensive ions. The range from m/z 210 to 300 for low mass compounds, hexachlorocyclohexane, aldrin, dieldrin, endrin, DDTs, heptachlor, hexachlorobenzene, mirex and m/z 350–420 for high mass compounds, heptachlor epoxide, chlordane, nonachlor and oxy-chlordane is used for the identification and quantification. Quantification of the individual compound of POPs in the samples is conducted by the internal standard method using a RRF. Two surrogate standards of ^{13}C-labeled PCB, #81 and #153 are spiked into the final eluate to calculate recovery as internal standard. The HRMS is operated in an electron impact and SIM mode at resolution more than 10,000 (10% valley). Criteria for checking the performance of the system are signal height, retention time and peak performance.

7.3.2 Calibration

Calibration is done for the overall procedure. A new calibration is prepared for each batch of samples or whenever the QA/QC sample gives striking results.

8.0 IDENTIFICATION AND CALCULATIONS

8.1 Identification

Retention time should match between sample and internal standard. For positive identification with MS detection, the retention time of the labeled internal standard to the native compound should be within 3 seconds. Positive identification should be done on isotopic ratios within 20 % of theoretical value for SIM.;

8.2 Relative Response Factor, RRF

Relative Response Factors (RRF) are determined by the analysis of standards and are used to calculate the concentrations of analytes in samples. The RRF can be calculated using the following equation:

$$RRF = \frac{A_s \times C_{is}}{A_{is} \times C_s}$$

Where:

A_s = SIM response (Peak area) for the native quantitation ions at m/z given in Table 10-4 for POPs.

C_{is} = Concentration of the appropriate internal standard in standard solution

A_{is} = SIM response (Peak area) of the quantitation ions of the appropriate internal standard at the m/z given in Table 10-4 for POPs

C_s = Concentration of the target analyte in standard solution

If the RRF value over the working range is a constant (<10% relative standard deviation, RSD), the RRF can be assumed to be invariant and the average RRF can be used for calculations. Alternatively, the results can be used to plot a calibration curve of response ratios, A_s/A_{is}, vs. concentration ratios C_s/C_{is}

8.3 Percent Recovery of Internal Standard, R_{is}

Calculate the percent recovery, R_{is} for each internal standard in the sample extract. The R_{is} can be calculated using the following equation:

$$R_{is} = \frac{A_{is} \times Q_{rs}}{A_{rs} \times RF_r \times Q_{is}} \times 100$$

Where:

A_{rs} = SIM response (Peak area) of the quantitation ions of the recovery standard
Q_{is} = Amount of internal standard added to each sample
Q_{rs} = ng of recovery standard
RFr = Relative response factor of the sampling spike to the cleanup spike

8.4 Concentration of POPs in Water

Determine the concentration of POPs in water according to following equation

$$C_w = \frac{Q_{is} \times A_s}{A_{is} \times RRF} \times \frac{1}{W}$$

Where:

Q_{is} = Amount of internal standard added to each sample
A_S = SIM response for native quantitation ions at the m/z
A_{is} = SIM response for the quantitation ions of the internal standard
RRF = Relative response factor calculated as required by Section 8.2
W = Volume of water sample

9.0 REPORT

9.1 Specification of results
The basis of reporting for primary and quality control samples is as follows:

- pg/L with two significant digits
- Data were corrected for recovery of ^{13}C surrogate standards
- For all samples, data for quantified analytes were reported to 2 or 3 significant figures
- Limit of detection data for non-quantified analytes were reported to 1 significant figure

10.0 QUALITY CONTROL

10.1 QC requirements
Quality control (QC) process includes those activities required during analytical data collection to produce data of known and documented quality. The analytical data acquired from QC procedures are used to estimate and evaluate the analytical results and to determine the necessity for, or the effect of, corrective action procedures. A MDL for each analyte must also be determined. Each laboratory must maintain records to document the quality of the data generated.

10.2 Laboratory Blanks
With each batch of samples processed as a group within a work shift, analyze a LRB to determine the background system contamination.

10.3 Calibration Check
In order to check the calibration, a LFB is processed with each batch of samples. For each of

the method analytes the result of the LFB must be within 30 % of the spiked concentration. If the deviation between spiked and measured concentration exceeds this range, appropriate measures must be taken to improve the quality of analysis before analysis of environmental samples can be continued.

10.4 QC control chart
For QA/QC purposes control charts are used. A QA/QC sample is analysed each day of analysis.

11.0 WASTE MANAGEMENT

11.1 SPE cartridges are only used once and are disposed with the domestic waste.

11.2 Solutions containing solvents are collected in glass bottles. Full glass bottles are shipped to a special unit for proper waste disposal.

12.0 REFERENCES

1. Yun-Seok Kim, Heesoo Eun, Takao Katase, Hideshi Fujiwara “Vertical distributions of persistent organic pollutants (POPs) caused from organochlorine pesticides in a sediment core taken from Ariake bay, Japan” Chemosphere 67 (2007) 456–463.
2. The Ministry of the Environment, Japan, “Monitoring Manual for POPs” http://www.env.go.jp/chemi/kurohon/http2003/02moni-manu/000moni-manu.htm
3. UNEP (2007): Draft Guidance on the Global Monitoring Plan for Persistent Organic Pollutants. UNEP, February 2007

13.0 ACKNOWLEDGEMENT

The author thank to Dr. Yun-Seok Kim and Ms. Emiko Iizumi, K water, South Korea and NIAES, Japan, respectively, for their help in chemical experiment.

ANNEX

Table 10-1. GC conditions for analysis of POPs

Capillary column:	ENV-8MS, 60m x0.25mm i.d., film thickness 0.25μm
Carrier Gas	Helium
Mode	Constant Flow
Maximum Oven Temp	360.00
Equilibrium Time	0.00
Initial Temperature (°C)	120.00
Time at initial Temp (min.)	0.50
Injection mode	1μL splitless injection
Initial Pressure (kpa)	1.00
Purge Pressure (kPa)	45.00
Purge Time (min.)	1.00

Oven:	Time(min.)	Rate(°C/min)	Temp(°C)
	0.00	10.0	180
	1.0	4.0	210
	17.0	10.0	300

Table 10-2. HRMS conditions for analysis of POPs

System:	Micromass Auto-Spec Ultima
Ion source temperature	240 °C
Transfer line temperature	280°C
Ionisation energy	45eV
Filament current	0.7mA
Resolution	>10,000 (10 % valley definition)
Mode	EI+

All other parameters depend on the compounds and are documented in the laboratory.

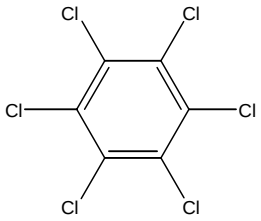
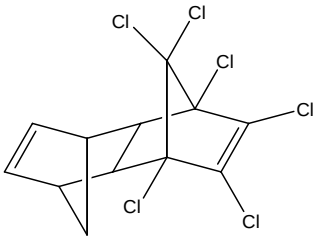
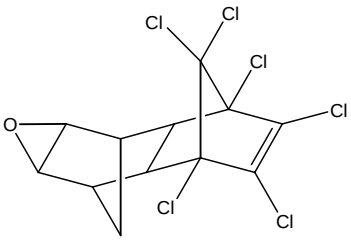
Table 10-3. Detection conditions for analysis of POPs

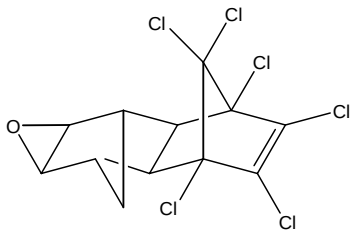
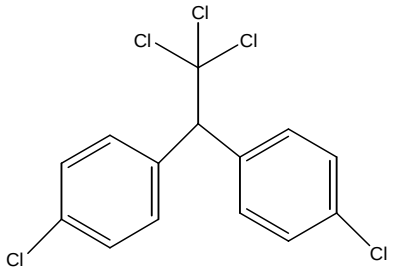
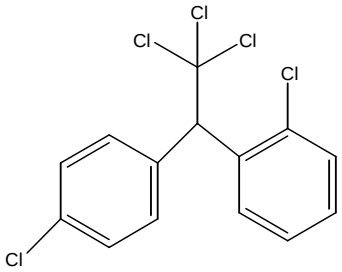
All compounds are analysed in the selective ion monitoring (SIM) mode. Details on the selection of quantitative and ratio mass are summarized in the following table.

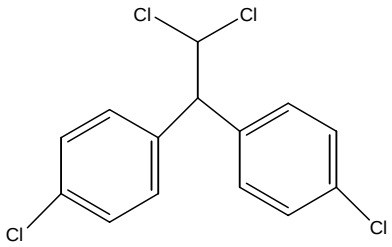
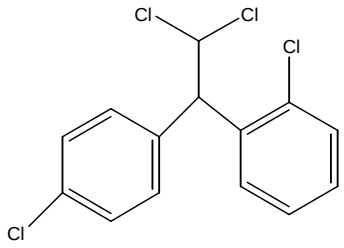
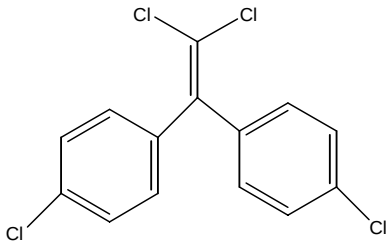
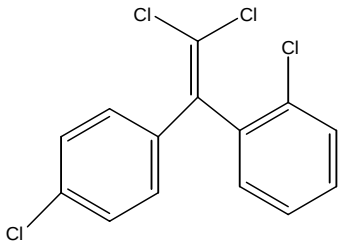
Compound	Quan. Mass	Ratio Mass
Hexachlorocyclohexane	218.9116	216.9145
^{13}C Hexachlorocyclohexane	224.9317	222.9347
DDT, DDD	235.0081	237.0052
^{13}C DDT, DDD	247.0484	249.0454
DDE	246.0003	247.9974
^{13}C DDE	258.0406	260.0473
Aldrin, Dieldrin, Endrin	262.8570	264.8540
^{13}C Aldrin, Dieldrin, Endrin	269.8804	271.8775
Mirex, Heptachlor	271.8102	273.8072
^{13}C Mirex, Heptachlor	276.8269	278.824
Hexachlorobenzene	283.8102	285.8072
^{13}C Hexachlorobenzene	289.8303	291.8273
^{13}C PCB (Syringe Spike)	243.0583	245.0553
Lock mass	280.9824	

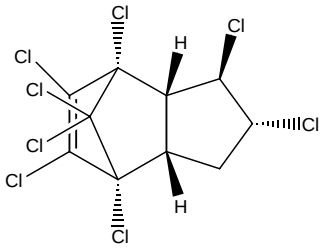
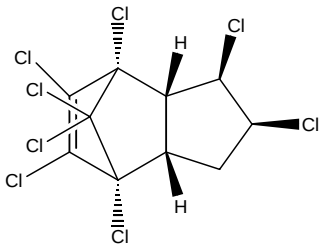
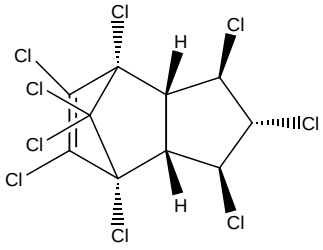
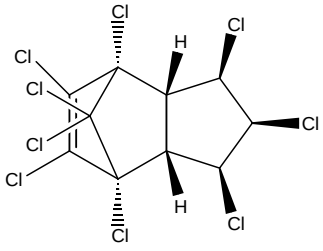
Compound	Quan. Mass	Ratio Mass
trans,cis-Heptachlorepoxide	352.8442	354.8413
^{13}C trans,cis-Heptachlorepoxide	362.8778	364.8748
trans,cis-Chlordane	372.8260	374.8230
^{13}C trans,cis-Chlordane	382.8595	384.8566
oxy-Chlordane	386.8052	388.8023
^{13}C oxy-Chlordane	396.8388	398.8358
trans,cis-Nonachlor	406.7870	408.7840
^{13}C trans,cis-Nonachlor	416.8205	418.8176
^{13}C PCB (Syringe Spike)	371.8817	373.8788
Lock mass	380.9760	

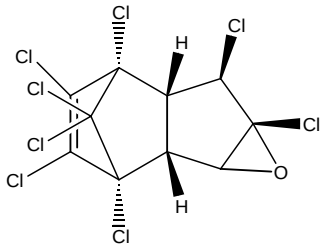
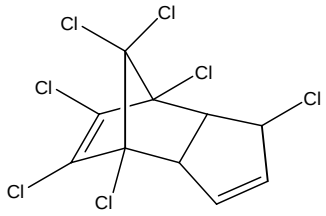
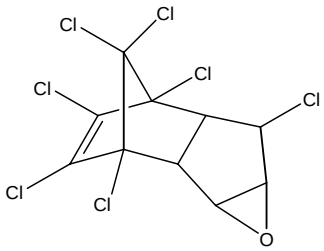
Table 10-4. Properties and Structure of POPs

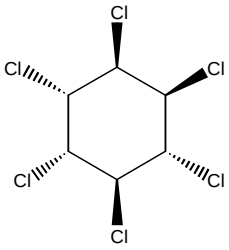
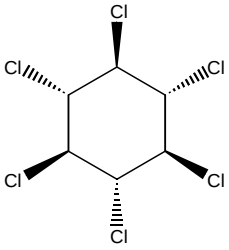
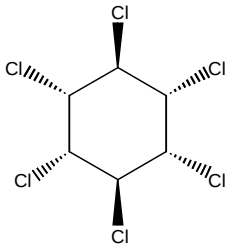
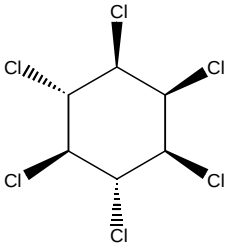
Hexachlorobenzene	
	Formula; C_6Cl_6 IUPAC Name; 1,2,3,4,5,6-hexachlorobenzene CAS; 118-74-1 M.W.; 284.78 b.p.; 323 ~ 326 °C LogPow; 5.23 ~ 6.18
Aldrin	
	Formula; $C_{12}H_8Cl_6$ IUPAC Name; (IR,4S,5S,8R)-1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-1,4:5,8-dimethanonaphthalene CAS; 309-00-2 M.W.; 364.9 b.p.; 145 °C LogPow; 3.01 ~ 6.75
Dieldrin	
	Formula; $C_{12}H_8Cl_6O$ IUPAC Name; (IR,4S,5S,8R)-1,2,3,4,10,10-hexachloro-1,4,4a,5,6,7,8,8a-octahydro-6,7-epoxy-1,4:5,8-dimethanonaphthalene CAS; 60-57-1 M.W.; 380.91 b.p.; 385 °C LogPow; 4.7 ~ 5.61

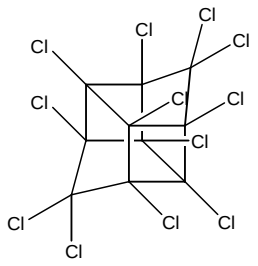
Endrin	
	Formula; $C_{12}H_8Cl_6O$ IUPAC Name; (1R,4S,4aS,5S,6S,7R,8R,8aR)-1,2,3,4,10,10-hexachloro-1,4,4a,5,6,7,8,8a-octahydro-6,7-epoxy-1,4:5,8-dimethanonaphthalene CAS; 72-20-8 M.W.; 380.91 b.p.; 245 °C LogPow; 5.22
***p,p'*-DDT**	
	Formula; $C_{14}H_9Cl_5$ IUPAC Name; 1-chloro-4-[2,2,2-trichloro-1-(4-chlorophenyl)ethyl]benzene CAS; 50-29-3 M.W.; 354.5 b.p.; 260 °C LogPow; 6.19 ~ 6.38
***o,p'*-DDT**	
	Formula; $C_{14}H_9Cl_5$ IUPAC Name; 1-chloro-2-[2,2,2-trichloro-1-(4-chlorophenyl)ethyl]benzene CAS; 789-02-6 M.W.; 354.49 b.p.; - LogPow; 5.98
***p,p'*-DDD**	

	Formula; $C_{14}H_{10}Cl_4$ IUPAC Name; 1-chloro-4-[2,2-dichloro-1-(4-chlorophenyl)ethyl]benzene CAS; 72-54-8 M.W.; 320.04 b.p.; 193 °C LogPow; 6.02
***o,p'*-DDD**	
	Formula; $C_{14}H_9Cl_5$ IUPAC Name; 1-chloro-2-[2,2,2-trichloro-1-(4-chlorophenyl)ethyl]benzene CAS; 789-02-6 M.W.; 354.49 b.p.; - LogPow; 5.98
***p,p'*-DDE**	
	Formula; $C_{14}H_8Cl_4$ IUPAC Name; 1-chloro-4-[2,2-dichloro-1-(4-chlorophenyl)ethenyl]benzene CAS; 72-55-9 M.W.; 284.78 b.p.; 323 ~ 326 °C LogPow; 5.23 ~ 6.18
***o,p'*-DDE**	
	Formula; $C_{14}H_8Cl_4$ IUPAC Name; 1-chloro-2-[2,2-dichloro-1-(4-chlorophenyl)ethenyl]benzene CAS; 3424-82-6 M.W.; 318.03 b.p.; - LogPow; -
***trans*-Chlordane**	

	Formula; $C_{10}H_6Cl_8$ Alternate Names; 1,2,4,5,6,7,8,8-octachloro-2,3,3a,4,7,7ahexahydro-4,7-methano-1H-indene CAS; 5103-74-2 M.W.; 409.78 b.p.; 175 °C LogPow; -
***cis*-Chlordane**	
	Formula; $C_{10}H_6Cl_8$ Alternate Names; 1,2,4,5,6,7,8,8-octachloro-2,3,3a,4,7,7ahexahydro-4,7-methano-1H-indene CAS; 5103-71-9 M.W.; 409.78 b.p.; - LogPow; -
***trans*-Nonachlor**	
	Formula; $C_{10}H_5Cl_9$ Alternate Names; 1,2,3,4,5,6,7,8,8-nonachloro-2,3,3a,4,7,7a-hexahydro-4,7-methano-1H-indene CAS; 39765-80-5 M.W.; 444.23 b.p.; - LogPow; -
***cis*-Nonachlor**	
	Formula; $C_{10}H_5Cl_9$ Alternate Names; 1,2,3,4,5,6,7,8,8-nonachloro-2,3,3a,4,7,7a-hexahydro-4,7-methano-1H-indene CAS; 5103-73-1 M.W.; 444.23 b.p.; - LogPow; -
Oxychlordane	

	Formula; $C_{10}H_4Cl_8O$ IUPAC Name; 27304-13-8 CAS; 2,3,4,5,6,6a,7,7-octachloro-1a,1b,5,5a,6,6a-hexahydro-2,5-methano-2H-indeno(1,2,b)oxirene M.W.; 423.76 b.p.; - LogPow; -
Heptachlor	
	Formula; $C_{10}H_5Cl_7$ IUPAC Name; 1,4,5,6,7,8,8-heptachloro-3a,4,7,7a-tetrahydro-4,7-methanoindene CAS; 76-44-8 M.W.; 373.32 b.p.; 145 °C LogPow; 3.87 ~ 6.13
Heptachlor epoxide	
	Formula; $C_{10}H_5Cl_7O$ Alternate Names; 2,3,4,5,6,7,7-heptachloro-1a,1b,5,5a,6,6ahexahydro-2,5-methano-2H-indeno=(1,2-b)oxirene CAS; 1024-57-3 M.W.; 545.54 b.p.; 323.4 °C LogPow; 6.89

α-HCH	
	Formula; $C_6H_6Cl_6$ Synonyms; 1a,2a,3b,4a,5b,6b-hexachlorocyclohexane CAS; 319-84-6 M.W.; 290.83 b.p.; 288 °C LogPow; 3.8
β-HCH	
	Formula; $C_6H_6Cl_6$ Synonyms;1a,2b,3a,4b,5a,6b-hexachlorocyclohexane CAS; 319-85-7 M.W.; 290.83 b.p.; 60 °C LogPow; 3.78
γ-HCH	
	Formula; $C_6H_6Cl_6$ IUPAC Name; 1α,2α,3β,4α,5α,6β-hexachlorocyclohexane CAS; 58-89-9 M.W.; 290.83 b.p.; 323.4 °C LogPow; 3.72
δ-HCH	
	Formula; $C_6H_6Cl_6$ Synonyms; 1a,2a,3a,4b,5a,6b-hexachlorocyclohexane CAS; 319-86-8 M.W.; 290.83 b.p.; 60 °C LogPow; 4.14
Mirex	



Formula; $C_{10}Cl_{12}$

IUPAC Name;

dodecachloropentacyclo[5.3.0.$0^{2,6}$.$0^{3,9}$.$0^{4,8}$]decane

CAS; 2385-85-5

M.W.; 545.54

b.p.; 323.4 °C

LogPow; 6.89

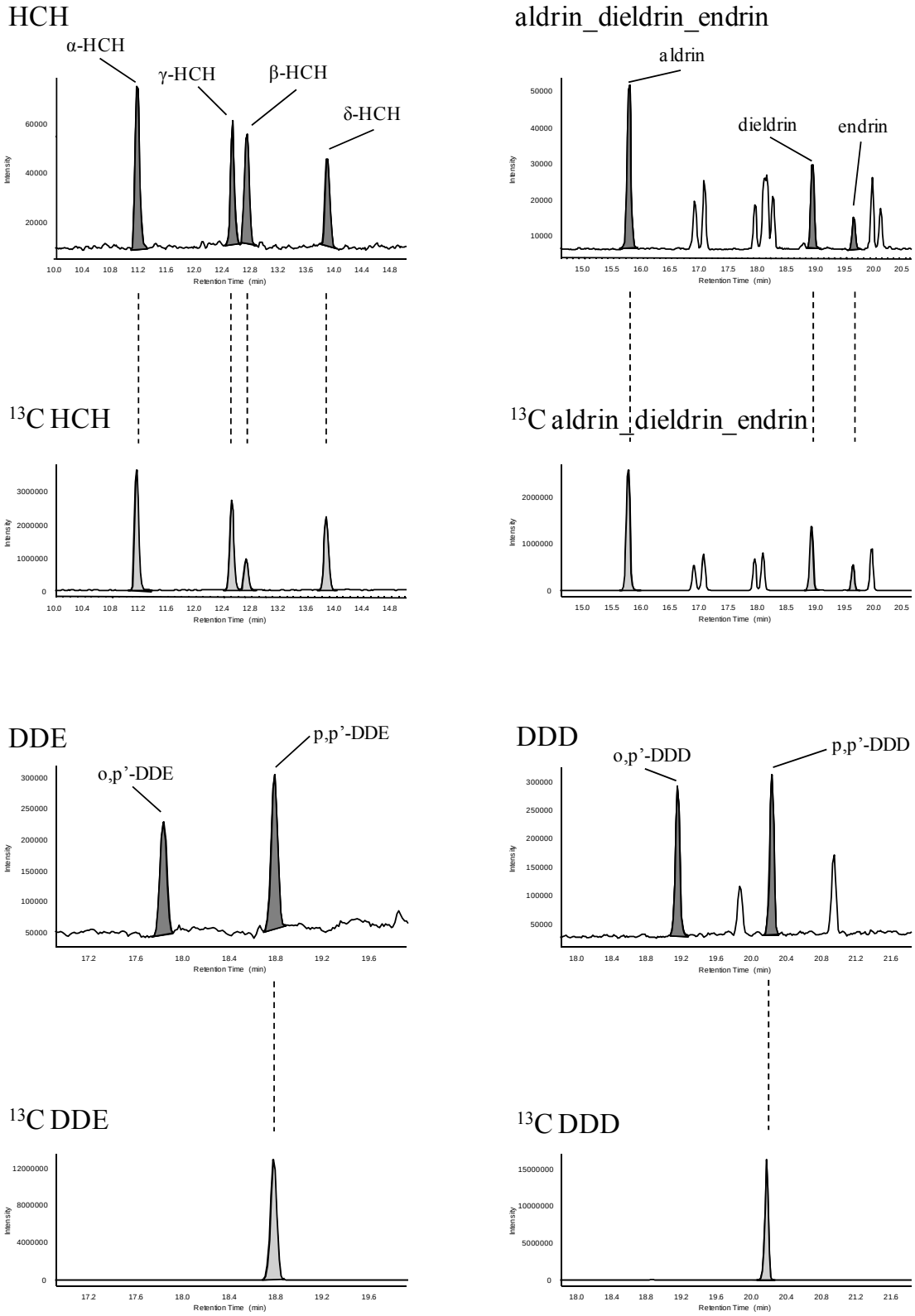


Fig. 10-1 Chromatogram of POPs for native and labeled compounds (continue).

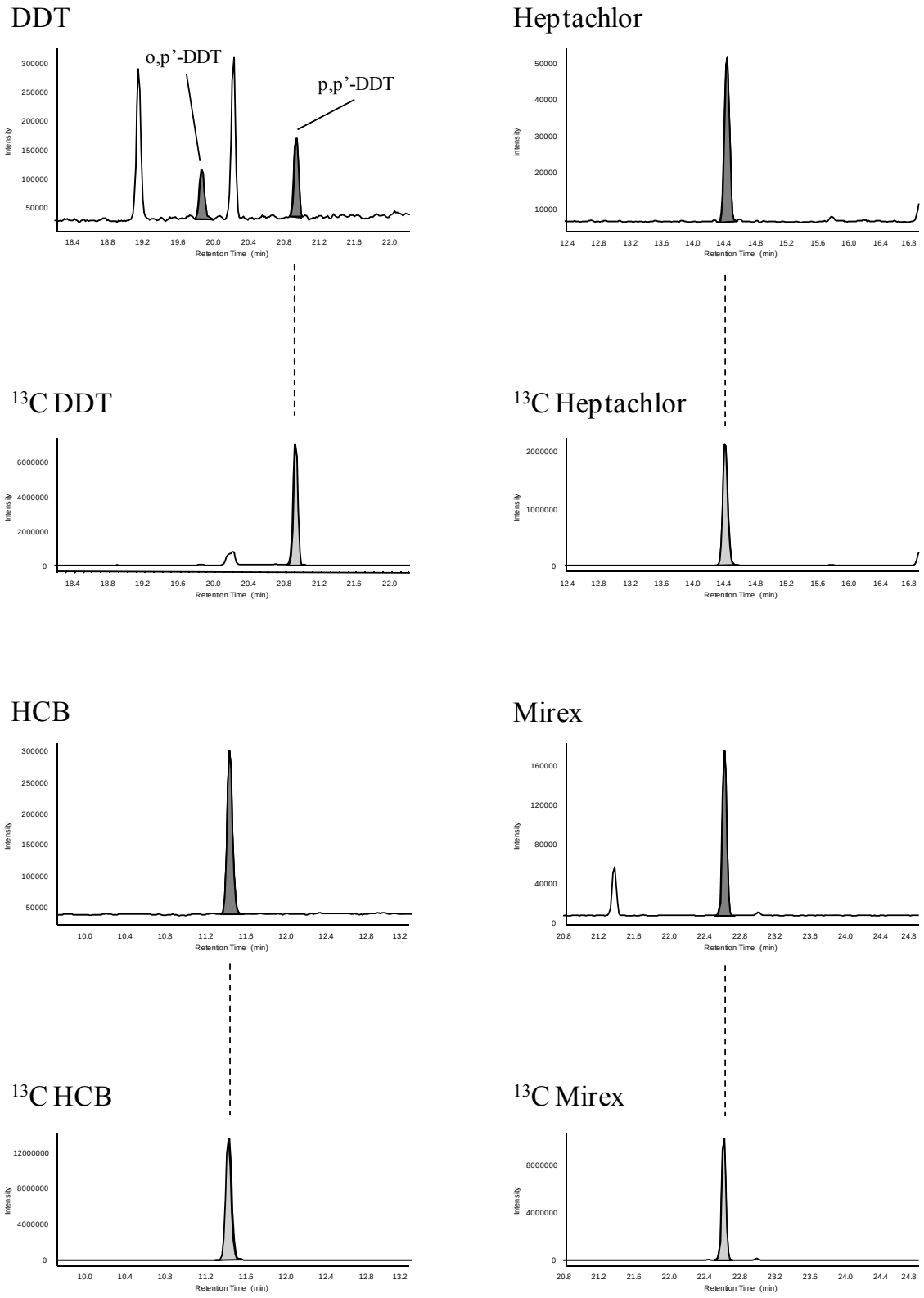


Fig. 10-1 Chromatogram of POPs for native and labeled compounds (continue).

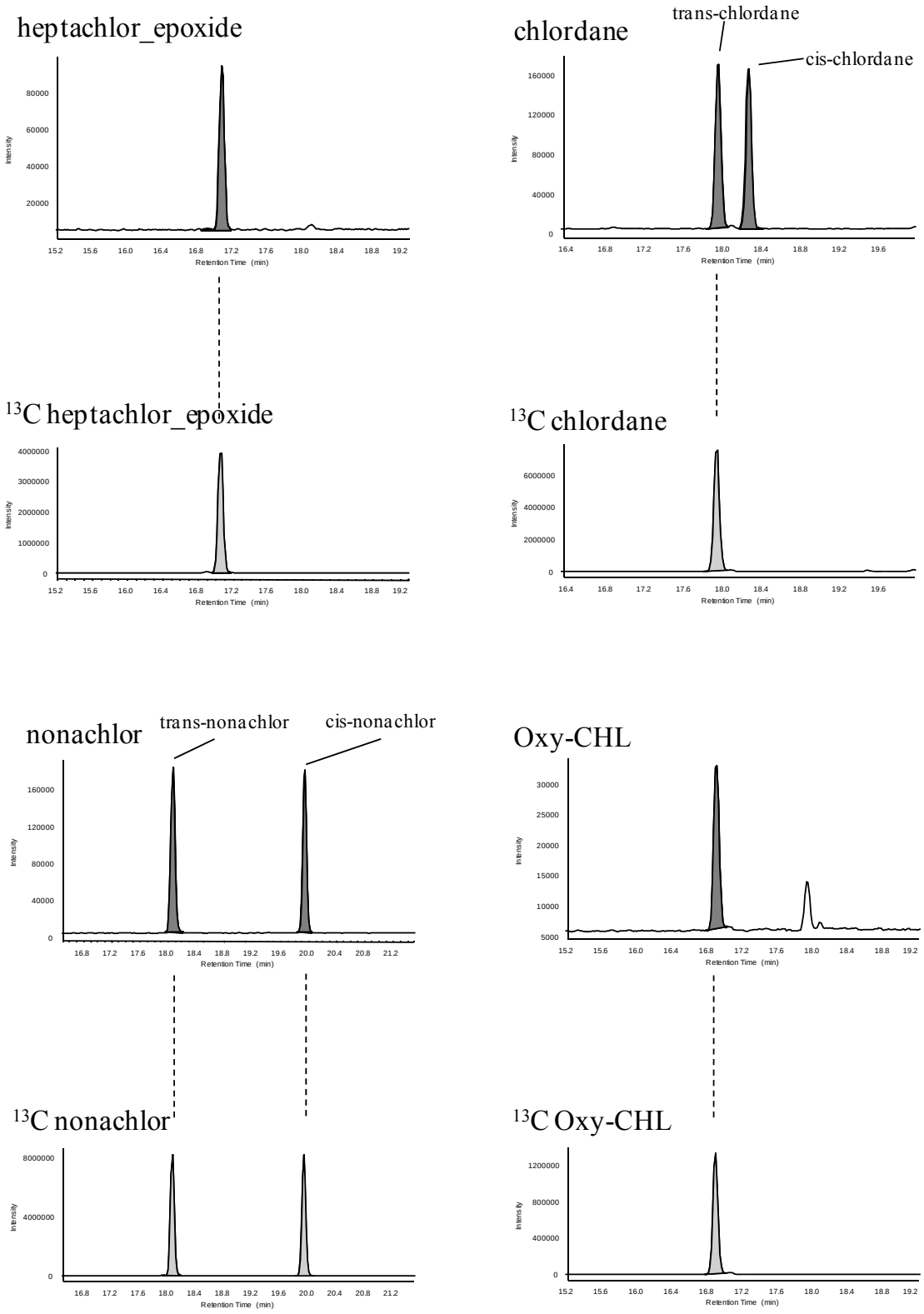


Fig. 10-1 Chromatogram of POPs for native and labeled compounds.

ANALYSIS OF ESTROGENS/HORMONES USING ESI/LC/MS COUPLED WITH UNIVERSAL RESIN EXTRACTION TECHNOLOGY

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1.0 ABSTRACT

The occurrence of estrogens/hormones in drinking water, surface water and groundwater is largely dependent on location and treatment technology. High concentrations of estrogens/hormones are commonly present in the effluents of wastewater and sewage treatment plants. It has become more important to monitor these compounds in drinking water as well as the effluents of sewage treatment plants. It is open required for the trace analysis of these compounds, especially for human hormones/estrogens present at sub- to low part-per-trillion levels in waters. GC/MS/MS can provide sensitive and selective detection of these compounds, but it requires a time-consuming and labor-intensive sample derivatization process to be suitable for gas chromatographic analysis. Recently, LC/MS and LC/MS/MS have become more attractive because they are able to provide high sensitivity and selectivity without the need of derivatization.

Orange County Water District (OCWD) has developed very sensitive and reliable detection method for estrogens/hormones from the different matrices of water samples using ESI/LC/MS coupled with new type of universal resin extraction disk. The system was optimized for each target compounds with single ion monitoring and varied cone voltages to maximize the response and reproducibility. Also the LC gradient method for the separation of these compounds was examined with different size particles of C18 columns from different manufactures.

The universal resin extraction technique generated excellent recoveries for the target compounds with 90% labor and solvent reduction. The method detection limit for estrogens/hormones using ESI/LC/MS are sub parts per trillion for drinking water, surface water and reclaimed water samples. The established method was effectively applied for the monitoring sub parts per trillion levels of estrogens/hormones from the wastewater treatment plant using medium pressure UV and micro membrane technology.

2.0 INTRODUCTION

The demand of water is increasing tremendously as the population move to urban area, where the growth of housing and business is a lot higher than rural area. The reclamation of wastewater is very important to supply enough water to the community right now in Southern California and it will be more crucial in the future due to the uncertain policy for the imported water from

Northern California and Colorado river. The wastewater has possibly polluted with more endocrine disrupting compounds (EDC's) than the imported water supply.

There is growing evidence that a number of man-made chemicals may disrupt the endocrine system of wildlife and humans. These endocrine or "hormone" disruptors may cause a variety of problems with development, behavior, and reproduction. The implications for wildlife and human populations and, in particular, for unborn children, who may be at greatest risk from these chemicals, are seriously examined by US EPA.

US EPA is very concerned about EDC's and is investigating significant resources into leaning how and to what extent these chemicals may be adversely affecting human health and wildlife. Although the evidence is growing, considerable scientific uncertainty remains. US EPA has taken steps over the last few years to begin to assess how and to what extent certain chemicals may disrupt the endocrine system.

The need of the monitoring for the EDC's from the reclaimed water is the another costly requirement to ensure quality of the highly purified wastewater. Among the EDC's, estrogens/hormones could be detected by ESI/LC/MS from the wastewater, reclaimed water and ground water. The advanced treatment technology such as micro-filteration and Ultra violet (UV) system would be the choice of technology to remove the EDC's in the reclaimed water to ensure the safety of the treated wastewater.

Orange County Water District (OCWD) has developed a solid phase extraction (SPE) procedure followed by ESI/LC/MS for the determination of estrogens/hormones. The SPE method uses 47 mm of a universal resin or C-18 disk, which are formulated with Teflon webbing allows fast flow and minimum labor for processing 1-2 liter samples less than 20 minutes instead of 2-3 hours of filteration with a cartridge type SPE system. The elution was done 10 –15 ml of methylene chloride while 250 ml of methylene chloride was used with the conventional liquid-liquid extraction method. Initially, six-position manual extraction was introduced and the automated SPE method will be established with a 47 mm universal resin or C-18 disk.

This study is establishing the cost-effective and sensitive analytical method, which could determine sub part per trillion concentrations of the target compounds using ESI/LC/MS, which could be easily applicable for the determination of 9 estrogens /hormones from different matrix samples such as a reclaimed water, ground water and surface water.

Table 11-1. Target Compounds of estrogens/hormones by ESI/LC/MS

Targets	MW	CAS #	LIMS ID
Estrone	270	53-16-7	ESTRON
Epitestosterone (cis-Testosterone)	288	481-30-1	EPITES
Testosterone (trans-)	288	58-22-0	TESTOR
Estriol	288	50-27-1	ESTRIO
17-alpha-Estradiol	272	57-91-0	aESTRA
17-beta-Estradiol	272	50-28-2	bESTRA
17-alpha-Ethynylestradiol	296	57-63-6	aETEST
Progesterone	314.5	57-83-0	PRGSTR
Diethylstilbestrol	268	56-53-1	DESTBL

3.0 EXPERIMENTAL SECTION

3.1 APPARATUS AND EQUIPMENT

3.1.1 Sample Bottles – 2.5 liter amber glass bottles fitted with a screw cap lined with teflon

3.1.2 Autosampler vials - 2 mL amber glass, screw cap with Teflon septa

3.1.3 Concentrator Tube - Zymark 250 mL tubes used with the Zymark Turbo-Vap

3.1.4 Analytical Balance- Capable of weighing accurately to nearest 10^{-4} gm

3.1.5 Zymark Turbo-Vap - used to concentrate extracts

3.1.6 Waters - Micromass ZQ Liquid Chromatography, quaternary pump, autosampler, column oven and Millennium 4.0 data workstation

3.1.7 Column: Waters – Xterra MS C18 (2.0 x 150 mm, 3.5 um)

3.1.8 Guard Column: Varian - MetaGuard 2.0 mm Pursuit 3 um C18

3.1.9 Disposable pasteur pipets, graduated cylinders (1000 ml), 1ml volumetric flasks, and Hamilton micro syringes - 10 ul to 100 ul

3.2 REAGENTS AND CONSUMABLE MATERIALS

3.2.1 Reagent Water - Millipore Milli-Q System or equivalent

3.2.2 Methylene Chloride – GC2 quality

3.2.3 Acetonitrile - HPLC quality

3.2.4 Methanol - GC2 quality

3.2.5 Empore disks (C-18): 0.5 grams of 8 um octadecyl bonded silica in 47 mm * 0.5 mm disk

3.2.6 Nitrogen Generator -- Domnick Hunter

3.3 SAMPLE COLLECTION, PRESERVATION AND HANDLING

3.3.1 Samples are collected in amber 2.5 liter bottles, fitted with a screw cap lined with teflon. Keep samples sealed from collection time until analysis. Sampling equipment must be free of plastic tubing, gaskets, and other parts that may leach interfering analytes into the water.

3.3.2 All samples should be iced or refrigerated at 4 ℃ and kept in the dark from the time of collection until extraction.

3.4 EXTRACTION

3.4.1 Liquid - Solid phase extraction (SPE):

Assemble C-18 Empore disk and filtration apparatus. Add 10 mL of CH_3CN, allow it to soak into the disk for 3 minutes then apply vacuum to remove the solvent completely. Apply 10 mL of methanol and allow it to soak into the disk for 3 minutes. Do not allow the disk to run dry until the end of the extraction step. Add 10 ml of DI water to keep the disk wet. Then add 1 liter of sample into the filtration reservoir, add the surrogate standard, Laboratory fortified blanks, matrix spikes and apply vacuum. Adjust the vacuum pressure to 50 ml/min flow rate.

After the sample is processed, pull air through disk for 10 minutes to remove residual water from the disk. Remove the filter base and place a small Zymark or a clear 40-ml vial into the

receiver. Add 5 ml of acetonitrile to the reservoir (rinsing the sides of the reservoir) and allow to soak for 3 minutes before applying vacuum. Repeat with a second and third with 5 ml acetonitrile. Repeat the eluting step once more with one 5 ml aliquot of methylene chloride. Transfer the combined extract into 250 ml Zymark tube. Rinse the clear vial with two 2ml portions of acetonitrile, adding the rinse to the Zymark tube. Evaporate the eluant with a Turbo-Vap concentration workstation to almost dryness. Add Methanol to bring the volume to 1 ml, then transfer to 2 brown autosampler vials and store in the refrigerator at 4 ℃

3.4.2 ANALYSIS

Initial Calibration: A five point calibration curve is required for this method; using standards STD-A (5ppt), B (10ppt), C (20ppt), D (50ppt) and E (100ppt)). For each analyte and surrogate, calculate the mean RF and the relative standard deviation (RSD). The %RSD of any analyte or surrogate within the calibration range must be less than 30%. If not – inject other standards or perform system maintenance and begin the system analysis and performance check over.

3.4.2.1 Continuous Calibration check:

Reagent blank must be monitored for every extraction run - monitoring of reagent blank is essential to the success of this method. Verify the calibration by measurement of two mid-point calibration check standards, one at the beginning and one at the end of the run. The check standard area counts must be within +/- 30% of the standard curve. LFBs (laboratory fortified blanks) should also be analyzed with each extraction run. Standards used for these QA/QC samples must be ordered from a second source whenever possible. For extended runs, check standards should be interspersed with samples at regular intervals. If the response of any analyte varies from the predicted response by more than +/- 20%, test must be repeated using fresh calibration standards. Data is collected and processed by Millennium 4.0 software. Any results above the highest calibration standard must be confirmed with a standard that is within +/- 20% of the actual result or the sample must be diluted to within the calibration range.

3.4.2.2 LC Conditions:

Column Temperature: 35 ℃

Gradient elution:

		Solvent A	Solvent B	Solvent C	
Time	Flow	H_2O	ACN	0.1%NH_4OH	Curve
0	0.25 ml/min	0	25	75	--
23	0.25	0	95	5	6
25	0.25	0	25	75	1
30	0.25	0	25	75	4
45	0.25	0	25	75	6
70	0.25	20	80	0	6
90	0	10	90	0	6

3.4.2.3 MS Conditions:

Scan: SIR (Single Ion Monitoring)

Acquire time: 0 to 16 Min. for ES-

6 to 17 Min. for ES +

	Mass (m/z)	Dwell (secs)	Cone Volts
ES-	267.29	0.40	60.0
	269.30	0.40	56.0
	271.31	0.40	65.0
	287.39	0.40	65.0
	295.33	0.40	55.0
	331.10	0.40	55.0
ES+	289.32	0.40	35.0
	315.32	0.50	35.0

4.0 RESULTS AND DISCUSSION

There are three different types of extraction techniques such as solid phase extraction using extraction disks or cartridges, liquid-liquid extraction method, and purge-trap technique for volatile compounds. OCWD Laboratory has examined three different extraction procedures to find the most suitable technique for OCWD laboratory to examine trace level of estrogens/hormones from water samples.

Solid Phase Extraction (SPE) of trace organic compounds in reverse phase sorbent is an attractive alternative to the more traditional methods that utilize liquid-liquid extraction for the removal of these pollutants from aqueous samples. The universal and C18 disk worked well for drinking water samples, but it had plugging problem with wastewater type samples. Adding glass beads above the extraction disk could help the extraction of wastewater samples without serious plugging problem. Both precision and accuracy of the analytical process must be demonstrated for each batch of samples by the analysis of matrix spike and matrix spike duplicates at the reporting level. The developed simultaneous method for estrogens/hormones should meet the detection limit of 5 ppt with less than 30% of relative standard deviation (RSD) for mean response factor (RF).

To find suitable analytical procedure of estrogens/hormones detection, the first step involves for setting sensitive and selective analytical method for the determination of the compounds in the drinking water, ground water and wastewater. OCWD Laboratory has examined extraction efficiency with different extraction disks such as a styrene divinylbenzene, C8, C18 and universal resin disk. As reported in the Table 11-2, most of target compounds had been well recovered with C18 and universal resin disk. The recovery of tested compounds ranged from 70 to 110 % except Diethylstilbestrol due to the poor resolution and low response with 4.0 micron particle size column. The resolution and response was improved with Hybrid C18, 3.5 micron particle size column (Xtera, waters).

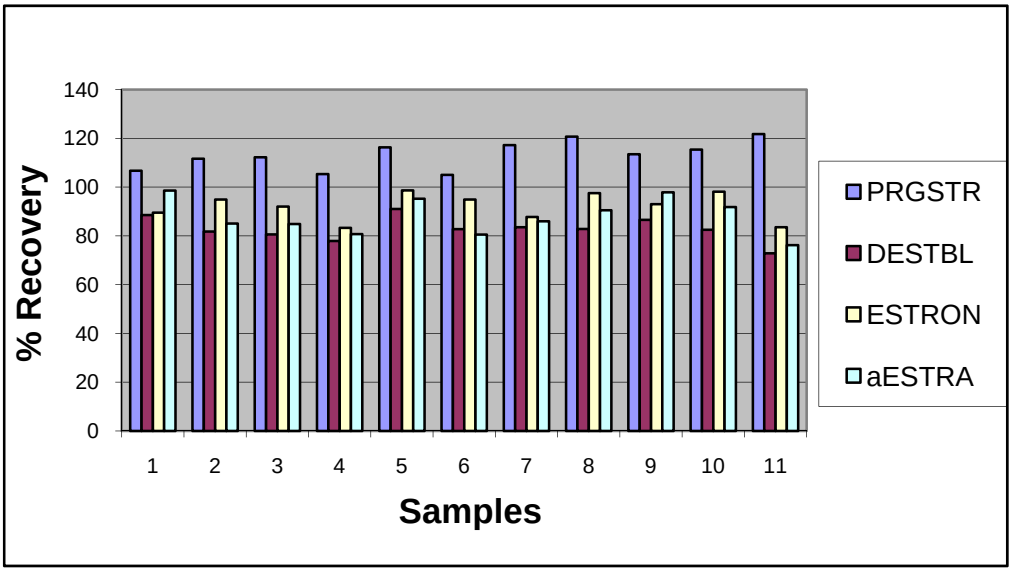
After OCWD Laboratory has experienced unfavorable reproducibility of extraction efficiency due to the high sample volume of 1-2 liter and some polar nature of analytes, the flow rate of sample was reduced to 50 ml per minutes and soaked the disk for 3 minutes with 5 ml of acetonitrile followed by 2-5 ml methylene chloride, which also was soaked for 3 minutes each time. Those reduced sample filtration time and soaking of disk with organic solvents could improve the extraction efficiency and generated acceptable reproducibility, which presented in Table 11-3.
The injection volume was limited less than 25 micro liter due to the capacity of 2.1 mm LC/MS

column. Regular LC column didn't have a problem up to 100 –200 micro liter of sample size, but the MS column didn't allow the large volume of sample size over 25-30 micro liter. When the large volume of sample was injected through LC column, the 9 target compounds eluted together as one peak. Also the LC column switching valve caused poor peak shape because of too much void volume. Then sample was introduced directly to the column by-passing the column switching valve.

Though acetonitrile was used for the sample elution from the extraction disk, estriol was not separated well with HPLC column when the extract is in 100% acetonitrile. As the methanol ratio in the extract was increased, the resolution and peak shape of the estriol was improved. The other target compounds were not affected the ratio of methanol in the final extract. The different particle size columns; 3.0, 3.5 and 4.0 micron columns eluted and separated the estrogens/hormones successfully, among the tested columns, 3.5 particle size column was the most reliable reproducibility and separation with 2,000 –3,000 psi of column pressure at 0.25 ml of column flow rate.

It was noticed that each compounds had a similar molecular weight and several of them had exact same molecular weight. Each compound was injected individually to determine the exact ion mass to optimize the single ion monitoring of mass spectrometry analysis. The molecular weight and exact mass of each compounds for single ion mode analysis were reported in the table 11-1. The detection limit of the ESI/LC/MS system with 1 liter sample volume and 1 ml final extract, was close to 5 part per trillion for 9 tested compounds. Though the triple quadruple mass spectrometry could generate a lot higher sensitivity compared to the single stage mass spectrometry, the detection limit of single stage could be improved with the increased sample volume and reduced the final extract volume without investing the three times of expense.

This study was initiated to ensure quality of the reclaimed water processed by micro filtration membrane, reverse osmosis membrane, and ultra violet treatment. The series of spiking tests and different particle size columns, optimized gradient elution of the mobile phase with the different matrix samples demonstrated that the developed method using ESI/LC/MS coupled solid phase extraction technique could be applied for the routine analysis of estrogens/hormones from different types of water samples, such as ground water, surface water and reclaimed water.



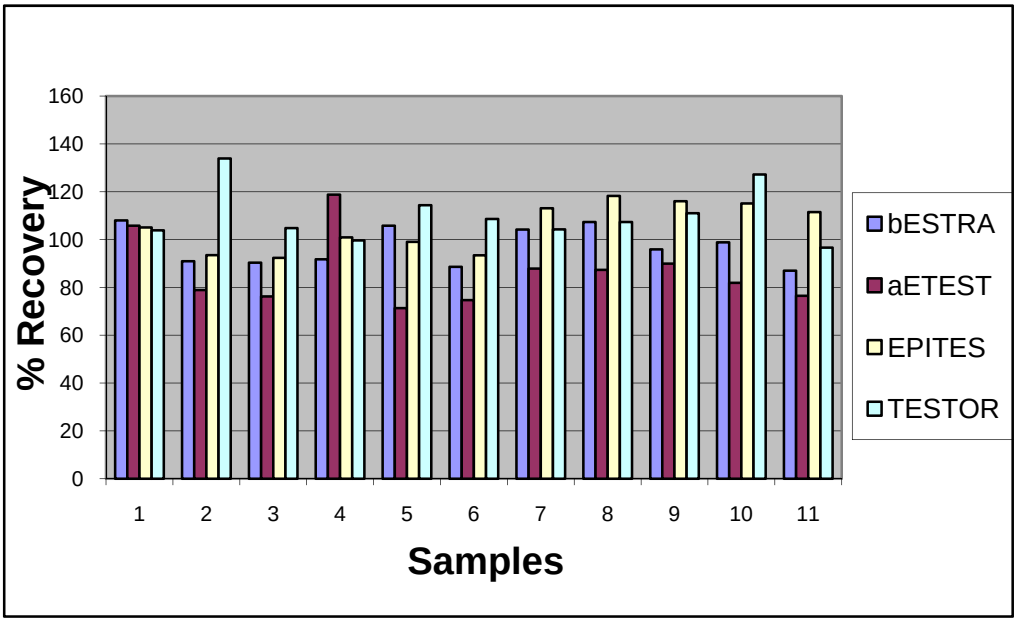


Fig. 11-1. % Recovery of 10 ppt spike

Table 11-1. % Recovery of estrogens/hormones with different particle size columns

	PRGSTR % Rec.			DESTBL % Rec.			ESTRON % Rec.			aESTRA % Rec.		
Column Used	Synergi	Varian	Varian	Synergi	Varian	Varian	Synergi	Varian	Varian	Synergi	Varian	Varian
	2/27/04	2/25/04	2/26/04	2/27/04	2/25/04	2/26/04	2/27/04	2/25/04	2/26/04	2/27/04	2/25/04	2/26/04
10ppt Low 1 (UR)	77	64	132	64	58	53	67	81	106	76	49	79
10ppt Low 2 (UR)	77	101	108	52	51	43	74	85	102	83	55	81
10ppt Low 3 (UR)	67	100	141	70	64	64	84	94	127	87	69	90
10ppt Low 1 (C-18)	83	112	112	27	37	35	49	79	75	60	51	60
10ppt Low 2 (C-18)	86	108	218	14	22	15	43	63	67	58	43	52
50ppt LFB 1 (UR)	93	93	103	96	70	76	81	79	97	92	69	81
50ppt LFB 2 (UR)	93	91	107	57	54	60	77	70	88	81	64	71
50ppt LFB 3 (UR)	104	89	84	60	58	65	80	72	91	87	69	77
50ppt LFB 4 (UR)	102	97	125	66	61	73	91	79	93	88	78	86
50ppt LFB 1 (C-18)	102	95	120	54	54	60	82	77	92	93	69	86
50ppt LFB 2 (C-18)	99	93	123	55	52	60	78	73	81	81	64	71

Table 11-2. % Recovery of estrogens/hormones with different particle size columns

	aETEST % Rec			EPITES % Rec.			TESTOR % Rec.			bESTRA % Rec.'		
Column Used	Synergi	Varian	Varian	Synergi	Varian	Varian	Synergi	Varian	Varian	Synergi	Varian	Varian
	2/27/04	2/25/04	2/26/04	2/27/04	2/25/04	2/26/04	2/27/04	2/25/04	2/26/04	2/27/04	2/25/04	2/26/04
10ppt Low 1 (UR)	70	77	149	71	91	100	82	109	98	82	77	79
10ppt Low 2 (UR)	67	76	111	80	101	99	81	115	102	70	79	81
10ppt Low 3 (UR)	88	88	148	77	119	104	83	130	111	82	90	92
10ppt Low 1 (C-18)	71	77	83	89	109	79	87	121	103	65	76	66
10ppt Low 2 (C-18)	61	58	63	83	123	104	89	144	129	47	58	52
50ppt LFB 1 (UR)	95	93	96	91	98	89	97	109	97	104	83	82
50ppt LFB 2 (UR)	84	82	92	100	101	92	93	100	95	87	74	71
50ppt LFB 3 (UR)	88	82	86	99	104	90	97	102	97	90	70	73
50ppt LFB 4 (UR))	101	93	102	105	108	97	109	107	105	94	80	80
50ppt LFB 1 (C-18)	98	87	86	102	110	94	107	112	96	96	83	80
50ppt LFB 2 (C-18)	87	87	78	99	110	91	96	112	98	86	75	72

Table 11-3. Verification of Reportable Detection Limit and Method Detection Limit

	LC/MS HORMONE				Analysis Date: 4/6/04 - 4/7/04			
	Varian Column				Instrument Method: Hormone_NoPDA			
	Sample extracted on 4/5/04							
	ESTRIO	% Rec	aETEST	% Rec	EPITES	% Rec	TESTOR	% Rec
	ppt		ppt		ppt		ppt	
5ppt Front Check Std	n/a	n/a	5.06	101	6.26	125	5.39	108
20ppt Front Check Std	n/a	n/a	20.41	102	20.58	103	20.92	105
5ppt RDL 1	n/a	n/a	5.17	103	3.07	61	5.64	113
5ppt RDL 2	n/a	n/a	5.81	116	3.14	63	5.43	109
5ppt RDL 3	n/a	n/a	5.41	108	3.20	64	6.16	123
5ppt RDL 4	n/a	n/a	7.17	143	3.82	76	5.94	119
10ppt RDL 1	n/a	n/a	10.58	106	10.50	105	10.39	104
10ppt RDL 2	n/a	n/a	7.89	79	9.35	94	13.39	134
10ppt RDL 3	n/a	n/a	7.62	76	9.24	92	10.48	105
10ppt RDL 4	n/a	n/a	11.88	119	10.09	101	9.96	100
10ppt RDL 5	n/a	n/a	7.13	71	9.90	99	11.43	114
10ppt RDL 6	n/a	n/a	7.47	75	9.34	93	10.86	109
10ppt RDL 7	n/a	n/a	8.78	88	11.31	113	10.42	104
10ppt RDL 8	n/a	n/a	8.74	87	11.82	118	10.74	107
10ppt RDL 9	n/a	n/a	8.99	90	11.60	116	11.10	111
10ppt RDL 10	n/a	n/a	8.19	82	11.51	115	12.72	127
10ppt RDL 11	n/a	n/a	7.65	77	11.15	111	9.66	97
20ppt MDL 1	n/a	n/a	22.45	112	22.45	112	20.44	102
20ppt MDL 2	n/a	n/a	19.12	96	22.72	114	19.91	100
20ppt MDL 3	n/a	n/a	25.55	128	28.67	143	25.04	125
20ppt MDL 4	n/a	n/a	22.46	112	24.46	122	21.67	108
20ppt MDL 5	n/a	n/a	22.86	114	23.43	117	22.12	111
20ppt MDL 6	n/a	n/a	22.40	112	22.83	114	21.14	106
20ppt MDL 7	n/a	n/a	21.40	107	23.29	116	20.97	105

20ppt MDL 8	n/a	n/a	22.70	114	25.72	129	22.14	111
5ppt Back Check Std	n/a	n/a	5.41	108	6.47	129	6.03	121
10ppt Back Check Std	n/a	n/a	10.61	106	10.85	108	10.00	100
20ppt Back Check Std	n/a	n/a	20.48	102	24.41	122	21.54	108

	PRGSTR	% Rec	DESTBL	% Rec	ESTRON	% Rec	aESTRA	% Rec	"bESTRA	% Rec
	ppt		ppt		ppt		ppt		ppt	
5ppt Std	6.55	131	6.03	121	5.45	27	4.88	98	5.55	111
20ppt Std	20	100	20.66	103	21.01	105	19.25	96	20.16	101
5ppt RDL 1	6.84	137	3.73	75	3.19	64	3.48	70	5.43	109
5ppt RDL 2	5.9	118	4.36	87	4.76	95	3.83	77	5.31	106
5ppt RDL 3	6.37	127	3.16	63	4.51	90	3.28	66	5.36	107
5ppt RDL 4	6.48	130	3.55	71	3.78	76	3.36	67	6.06	121
10ppt RDL 1	10.68	107	8.86	89	8.95	90	9.86	99	10.80	108
10ppt RDL 2	11.16	112	8.18	82	9.94	99	8.51	85	9.09	91
10ppt RDL 3	11.23	112	8.06	81	9.20	92	8.49	85	9.04	90
10ppt RDL 4	10.53	105	7.79	78	8.33	83	8.08	81	9.17	92
10ppt RDL 5	11.63	116	9.10	91	9.86	99	9.52	95	10.58	106
10ppt RDL 6	10.5	105	8.28	83	9.49	95	8.05	81	8.86	89
10ppt RDL 7	11.73	117	8.38	84	8.78	88	8.60	86	10.42	104
10ppt RDL 8	12.07	121	8.29	83	9.76	98	9.05	91	10.73	107
10ppt RDL 9	11.35	114	8.66	87	9.30	93	9.79	98	9.59	96
10ppt RDL 10	11.54	115	8.25	83	9.81	98	9.18	92	9.88	99
10ppt RDL 11	12.17	122	7.29	73	8.36	84	7.62	76	8.70	87
20ppt MDL 1	22.06	110	16.65	83	17.96	90	18.94	95	20.55	103
20ppt MDL 2	21.73	109	15.59	78	16.60	83	18.26	91	18.55	93
20ppt MDL 3	25.53	128	17.84	89	21.54	108	21.09	105	22.52	113
20ppt MDL 4	23.72	119	17.37	87	20.09	100	21.01	105	20.61	103
20ppt MDL 5	21.64	108	16.33	82	19.29	96	19.04	95	18.15	91

20ppt MDL 6	22.02	110	16.07	80	19.38	97	17.75	89	18.82	94
20ppt MDL 7	21.83	109	15.44	77	17.55	88	16.99	85	18.94	95
20ppt MDL 8	22.5	113	17.01	85	19.01	95	18.77	94	19.19	96
5ppt Back Std	6.02	120	5.23	105	6.57	33	4.65	93	5.53	111
10ppt Back Std	12.14	121	10.68	107	8.94	45	9.70	97	9.35	94
20ppt Back Std	21.28	106	19.00	95	17.56	88	17.19	86	17.87	89

5.0 CONCLUSION

5.1 Among the different particle size columns, 3.0, 3.5 and 4.0 micron, 3.5 micron hybrid particle column exhibited the most reproducible and reliable elution of 9 tested estrogens/hormones.

5.2 Since the target compounds had similar nature and molecular weight, the exact ion mass of each compound is necessary to determine part per trillion levels of these compounds using single ion monitoring mode of mass spectrometry.

5.3 The solvent of final extract was exchanged with at least 50% of methanol from 100% acetonitrile to improve the peak resolution and shape of estriol.

5.4 The universal resin, C-18 extraction disk generated acceptable recovery and precision of the target compounds with 90% solvent reduction and 80% less sample preparation time and labor for processing 1liter sample, which contributed reduction of the analytical cost over 80%.

5.5 Optimization steps for sample and instrument make it possible to use the ESI/LC/MS coupled with solid phase extraction for determination of sub part per trillion concentrations of estrogens/hormones form the different matrices of water samples such as ground water, surface water and reclaimed water.

6.0 FURTHER DEVELOPMENT

6.1 Initiate Performance Evaluation (PE) study to improve data reliability among the participating laboratories.

6.2 Automated solid phase extraction system will be examined to replace the manual extraction of estrogens/hormones.

6.3 Preservation study for different matrix samples will be performed to verify holding time, sample pH and amount of ascorbic acid or sodium thiosulfate.

6.4 Other possible contaminants from personal care products will be examined in wastewater, surface water, and groundwater using ESI/LC/MS system.

7.0 ACKNOWLEDGEMENTS

Funding for this project was provided by Orange County Water District. The authors wish to thank our organic chemists, Waters Incorporated, especially, Mr. Jay Kim, for their technical support and encouragement.

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SIMULTANEOUS ANALYTICAL METHOD FOR 1,4- DIOXANE AND N-NITROSOAMINES USING SOLID PHASE EXTRACTION AND GC/MS/MS/CI FROM AQUEOUS SAMPLES

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1.0 ABSTRACT

Among the emerging contaminants in the surface and treated wastewater, nitrosamines and 1,4-Dioxane are very prevalent contaminants in the water systems. The formation mechanism of N-nitrosdimethyl amine (NDMA) in the water treatment facility has been an urgent task to reduce NDMA in the final effluent. The mass consumption of 1,4-dioxane in many industrial applications for many years influenced level of this compound not only in surface water but also in groundwater with the significant concentrations between 2 part per billion and 3,000 part per billion.

Orange County Water District (OCWD) Laboratory has developed cost-effective and very sensitive detection method for NDMA and 1,4-dioxane using GC/MS/MS/CI and GC/MS/MS/Purge-Trap, respectively. Since the demand of these methods is very high to process over 2,000 samples each year. The lab initiated the multi-residual analytical method to incorporate 1,4-dioxane with the existing analytical method for nitrosamines. The solid phase extraction (SPE) with 80 to 100 meshes of granular carbon uses 10 ml of methylene chloride for extracting the interested analytes from the absorbent materials. The 10 ml extract was concentrated to final volume of 1ml to be analyzed by GC/MS/MS with positive chemical ionization with methanol reagent solution.

The developed analytical method generated the acceptable recovery and precision for both nitrosamines and 1,4-dioxane for different matrix of aqueous samples such as ground water, surface water and reclaimed water. The reportable detection limit of 1,4-dioxane was 0.05 part per billion while the purge-trap method was 1.0 part per billion.
Recently, 1,4-dioxane, which the US EPA classifies as a B2 probable human carcinogen, has been detected in the specific ground and surface waters. The findings of 1,4-dioxane in the water systems prompted the need of extensive monitoring of the compound in the drinking water. But the currently available methods have high detection limits of 10 to 50 μg/L. The high reportable detection limits are the results of poor extraction efficiency and volatile nature of the compound. Orange County Water District (OCWD) Laboratory has examined extraction and analysis techniques to establish more sensitive and reliable method to analyze for 1,4-dioxane to less than 1 μg/L in drinking water samples.

OCWD Laboratory has been developed a very reliable method to determine sub part per trillion levels of N-nitrosodimethylamine (NDMA) by GC/MS using positive chemical ionization with methanol or acetonitrile. Since 1,4-dioxane and nitrosamines have similar

properties; very water soluble, volatile and polar, 1,4-dioxane was successfully included in the existing method for nitrosamines. To accommodate 1,4-dioxane into the existing analytical method, 1,4-dioxane-d_8 and NDPA-d_{14} were both used as internal standards. The pH of 500 mL sample was adjusted between 4 and 11 and extracted three times with 60 ml of methylene chloride. The extract was evaporated to 1 ml using nitrogen gas at 35 celsius degrees in a water bath. The retention times of 1,4-dioxane and NDMA were 11.43 and 11.86 minutes with 60 m capillary column, respectively. The retention time differences of 0.43 minutes was far enough to change chemical ionization settings from 1,4-dioxane to NDMA, which multiple settings are necessary to perform simultaneous determination of these compounds without decreasing instrument sensitivity. The modified extraction and instrument techniques generated method detection limits, as the basis of a 1 L sample size, range from 0.1 to 0.2 µg/L for 1,4-dioxane and 0.2 to 0.3 ng/L for NDMA.

For the positive chemical ionization, methanol or acetonitrile have several advantages over ammonia gas for safety and system maintenance. Also the modified liquid-liquid extraction technique using separatory funnel could save 80% of extraction time compared to the continuous liquid-liquid extraction technique. The results of recent work on the analysis of 1,4-dioxane and NDMA from groundwater, surface water and reclaimed water samples are included. This study clearly demonstrated the GC/MS using chemical ionization with methanol was very sensitive, reliable and cost effective for the simultaneous analysis of 1,4-dioxane and NDMA from aqueous samples.

2.0 INTRODUCTION

Currently, N-nitrosodimethylamine and 1,4-dioxane are encountered in variety of industrial waste streams and ground water systems. Based on reproductive effects, both compounds are classified as B2 probable human carcinogens. Health authorities have established action levels or drinking water health advisories of 3 µg/L for 1,4-dioxane and 20 ng/L for N-nitrosodimethylamine.

Many studies have set up to measure environmental contamination with nitrosamines. Voaltile N-nitrosamines are a small part of the larger class of nitroso compounds, which are wide spread and often carcinogenic food contaminants.

Because of the health threat of nitrosamines, it is recommendable to limit their presence by

avoiding the addition of their precursors nitrate and nitrite. Human liver damage due exposure to N-nitrosodimethylamine and demonstration of the toxic and carcinogenic of this nitrosamine in animals were reported in 1956[1].

A survey of beers from the United States and Canada in 1990 by Scanlan et al. indicated that NDMA levels ranged from none detected to 580 ppt, approximately 1-5% of what the levels were in 1980. The reduction of NDMA in beer was done by introduction of sulfur dioxide into the drying air and indirect-fired type for drying malt[2].
NDMA was also reported in processed food such as cheese, milk, cured meats. Average concentrations of NDMA detected in food range from 90 to 100 ppt for whole milk, 2,600 to 2,700 ppt for bacon, and 300 to 800 ppt for cheese[4]. Groundwater contamination of NDMA was associated with liquid rocket fuel and detected in reclaimed water, and has recently detected in chlorinated surface water[3].

Despite reduced levels of N-nitrosodimethylamine (NDMA) found in the environment today compared to levels found over a decade ago, the detection of N-nitroso compounds are important because of their carcinogenicity and mutagenicity. The determination of NDMA at sub ppt levels in the aqueous samples is not easily accomplished due to it's small molecular size and polarity nature of amine compound.

Recently, the formation of NDMA in conventional drinking water treatment process has been reported and this was not eliminated by alteration of the types of treatment chemicals or their points of application. The exact formation mechanism was not yet determined. However, there is strong evidence that hydrogen ion, produced on alum addition, and either chlorine or chloramines both react with precursor compound to increase NDMA in conventional water treatment plant[6].

1,4-dioxane is a common solvent found in industrial waste and by-products. 1,4-dioxane is also a constituent of landfill leachates and a contaminant of ground waters. [8]1,4-dioxane is degraded slowly and is highly mobile in aquifer materials.

So far, NDMA and 1,4-dioxane are not regulated in drinking water. There is no official method available for the measurement of NDMA and 1,4-dioxane in drinking water in the part per trillion (ppt) range. US EPA method 625 includes NDMA as one of its' target analyte in the waste water, but the detection limit of the method is 50 ppb, which is way over interim regulation limit of 20 ppt, set by the California health department.

Only few research projects have dealt with the analytical method of nitrosamine in aqueous samples for measurement in the part per trillion range. The main subject of this study is establishing instrument and extraction method which generate high production, low detection limit of part per trillion range with rugged performance.

3.0 EXPERIMENTAL SECTION

3.1 Chemicals, Reagents, and Components

All solvents used were distilled in glass (Burdick and Jackson). Reagent grade water was obtained from the milli-Q purifier system manufactured by Millipore Corporation (Bedford,MA). All other chemicals were reagent grade. The N-nitrosoamines stock solutions were obtained from the Ultra Scientific ; 250 Smith Street, Notrth Kingstown, RI and Accu Standard , 125 Market Street, New Haven, CT.

N-nitrosodimethylamine-d_6 and 1,4-dioxane-d_8 were acquired from Cambridge Isotope Laboratories; 50 Frontage Road, Andover, MA. Empore^{tm} extraction disks, Carbon and C18, 47 mm, were obtained from Varian sample preparation products, Harbor City, CA. The stock solutions from Accu Standard were diluted with dichloromethane to serve as reference for recovery studies.

3.2 Apparatus and operating conditions

Glass filtration apparatus, 2 L separatory funnel, 47 mm, 300 mL funnel, 1000 mL flask, all glass assembly, Kontes, Cat.#953825 (Vineland, NJ). Six station extraction manifold, Varian sample preparation products, Cat.#1214-6001. TurboVap II concentration workstation, Zymark, Cat.#ZW8003.

The analysis of nitrosamines and 1,4-dioxane were carried out using Varian Gas chromatography 3800 / Ion trap detector using chemical ionization with methanol or acetonitrile liquid. The system was equipped with 60 m x 0.32 mm ID, 1.8 micron film thickness, Varian VRX column.

Analyze a 5-point calibration at the beginning of each analytical run. Verify the calibration by

measurement of two calibration check standards, one at the beginning and one at the end of the run. The check standards should be at two different concentration levels to verify the calibration curve. LFBs (laboratory fortified blanks –low: 2 ppt and high; 25 ppt for NDMA; 1ppb and 5 ppb for 1,4-dioxane) should also be analyzed with each extraction run. Standards used for these QA/QC samples must be ordered from a second source whenever possible. For extended runs, check standards should be interspersed with samples at regular intervals. If the response of any anlayte varies from the predicted response by more than +/- 30%, test must be repeated using fresh calibration standards. The concentration of nine point calibration is; 2, 5, 7, 10, 25, 50, 100, 150, and 200 ppt for NDMA and 0.1, 0.2, 0.5, 1.0 ,2.0, 5.0, 7.0,10, and 20 ppb for 1,4-dioxane .

Instrument Conditions:

1. Initial column temperature: 35 ºC
2. Hold time: 4 min
3. Final temperature: 200 ºC
4. Temperature rate: 20 ºC /minute to 140 ºC
   140 ºC to 200 ºC @ 50 ºC /min
5. Hold time: 4.55 min @ 200 ºC
6. Helium linear velocity: 1 mL/min
7. Splitless injection: 8 µl with 48 second delay
8. injector temperature: 37 ºC initial and programmed to 200 ºC
9. Detector: ion trap

10. Transfer line 215 °C.
11. Mass Range 40 to 100 m/z
12. Trap temperature 135 °C.
13. Background Mass 40 m/z
2. Ionization mode CI Auto
15. CI max ioniz. time 2000 (µsec)

16. CI max reaction time 120 (ms)
17. CI ioniz. storage time 5.0 m/z
18. Reagent ion eject 20 V
19. CI reaction storage 35.0 m/z
20. CI background mass 40 m/z

3.3 Procedure

3.3.1 LIQUID / LIQUID EXTRACTION:

3.3.1.1 Transfer one liter of the sample to the two-liter separatory funnel. Spike the sample with 5 µL of the surrogate standard spiking solution (5 µg/ml, NDMA-d_6, 250 µg/ml, 1,4-dioxane-d_8 and 5 µg/ml, NDPA-d_{14}), which gives 25 ppt concentrations of NDMA-d_6 and 25 ppt of NDPA-d14. Add 100 g of NaCl to the sample, seal, and shake to dissolve the salt. Add 60 ml of methylene chloride to the separatory funnel and extract the sample by vigorously shaking the funnel for 2 minutes with periodic venting to release excess pressure. Allow the organic layer to separate from the water phase for a minimum of 10 minutes. If the emulsion interface between layers is more than one-third the volume of the solvent layer, the analyst must employ mechanical techniques to complete the phase separation. The optimum technique depends upon the sample, but may include stirring, filtration through glass wool, centrifugation, or other physical methods. Collect the methylene chloride extract in a 500-mL Erlenmeyer containing approximately 7 grams of anhydrous sodium sulfate.

3.3.1.2 Add a second 60-mL volume of methylene chloride to the separatory funnel and repeat the extraction procedure a second time, combing the extracts in the Erlenmeyer flask. Perform a third extraction in the same manner. Swirl flask to dry extract; allow flask to sit for 15 minutes.

3.3.1.3 Transfer combined extract into concentrate tube and rinse the flask with 2-25 mL of methylene chloride. Concentrate the extract to 1mL final volume with Zymark turbo-Vap using nitrogen gas, less than 10 psi pressure at 35 °C of water bath temperature.

3.3.1.4 Transfer the extract into an appropriate sized TFE-fluorocarbon-sealed, screw-cap vial and store. Refrigerate at 4 °C until analysis by GC/MS.

3.3.2 SOLID PHASE EXTRACTION:

3.3.2.1 Transfer 500 mL of sample to 1-liter amber bottles. All QA/QC samples must be added sodium sulfite. Add 5 µL of the surrogate (SS) and 5 µL of internal standard.

3.3.2.2 Cartridge Clean Up and Conditioning: Rinse each cartridge with three, 3mL

methylene chloride. Let the cartridge drain dry after each flush. Then rinse the cartridge with three, 3mL methanol, DO NOT allow the methanol to elute below the top of the cartridge packing. From this point, do not allow the cartridge packing to go dry. Adjust the vacuum so that the approximate flow rate is 20 mL/min (50 min for a 1L sample) After all of the sample has passed through the SPE cartridge, draw air or nitrogen through the cartridge for 15-30 min at high vacuum (10-15 in Hg). The cartridge should appear dry (light tan color) before continuing with the elution steps.

3.3.2.3 Rinse the inside of each sample bottle with 8-10 mL methylene chloride and use vacuum to pull the solvent through the transfer tube and through the cartridge, colleting the solvent in a collection tube. Remove the transfer tubing from the top of the cartridge. Add 2-3mL methylene chloride to the top of the cartridge with a disposable pipette. Pull this solvent through the cartridge at low vacuum, such that the solvent exits the cartridge in a dropwise fashion. Pass the eluate through the drying column, which is packed with approximately 5-7 g of anhydrous sodium sulfate, and collect in a clean collection tube. Wash the sodium sulfate with at least 2 mL methylene chloride and collect in the same tube.

3.3.2.4 Concentrate the sample to approximately 0.9 mL – do not concentrate the extract to less than 0.5 mL. Make any volume adjustments with methylene chloride – to the 1.0 mL marked level. Transfer the 1.0 mL extract into a autosampler vial and store the sample extract in the refrigerator at 0 °C or below.

4.0 RESULTS AND DISCUSSION

The sample preparation method had to be applicable to raw and treated drinking water samples as well as industrial process and effluent storm samples. The extraction method should be simple and reproducible to accommodate a large number of samples. Extracts are analyzed either by low-resolution GC/MS with chemical ionization (using methanol, acetonitrile etc) and high-resolution electron impact mass spectrometry or other mass spectrometric techniques with equivalent sensitivity are acceptable. Both precision and accuracy of the analytical process must be demonstrated for each batch of samples by the anlaysis of matrix spike and matrix spike duplicates at the report level. The developed method for nitrosamines and 1,4-dioxane should meet the detection limit of 2 ppt and 1ppb, respectively, with less than 20% of relative standard deviation (RSD) for mean response factor (RF).

Since, the formation of NDMA during the methylene chloride extraction of chlorinated water containing precursor amines were reported[6], a sample of the reaction mixture was de-chlorinated with ascorbic acid or sodium thiosulphate prior to extraction to stop possible increase of NDMA.

To find suitable analytical procedure of nitrosamines and 1,4-dioxane detection, the first step involves for setting sensitive and selective analytical method for the determination of the compounds in the drinking water, ground water and waste water.

There are some existing analytical methods available for nitrosamines determination. But the precision and efficiency of the method is not well recognized. There are three different types of extraction procedures such as solid phase extraction using carbon disk, granular adsorbent, ambersorb method and liquid-liquid extraction method. We have examined the three different extraction procedures to find most suitable technique for our laboratory.

Solid Phase Extraction (SPE) of trace organic compounds in reverse phase sorbent is an attractive alternative to the more traditional methods that utilize liquid-liquid extraction for the removal of these pollutants from aqueous samples[9]. The carbon disk worked well for drinking water samples, but it had plugging problem with waste water type samples. Also the supplier of carbon disk stopped manufacturing of the product.

The recovery of granular ambersorb extraction only yielded 15-20 % of nitrosamines recovery. To use ambersorb method, we need high resolution mass spectrometer (HRMS) to detect around 2 ppt concentration of nitrosamines.

The previous work of NDMA extraction using liquid- liquid extraction with separatory funnel generated 20-30% of recovery for NDMA. After we modified the current liquid-liquid extraction method, the recovery of extraction increased to 55-65%. We are adding 25 ppt of NDMA-d_6 as a internal standard to monitor the extraction efficiency and also to ensure the uniform injection of autosampler. The recovery of NDMA using internal standard calibration, ranged between 80 to 120% regardless of sample matrix. The ion mass 75 was used for NDMA and 81 for NDMA-d_6 for sample analysis by GC/MS using chemical ionization with ammonia gas or methanol liquid.

The recovery of 1,4-dioxane is 30-40% and has some problems with the matrix of sample due to volatility and poor extraction efficiency of the analyte. After we have experienced unfavorable reproducibility of extraction efficiency, the purge-trap technique was applied instead of liquid-

liquid extraction technique. 20 minutes of purging time for 25 ml samples using helium and 15 minutes of baking at 260 °C , could improve the extraction efficiency and generated acceptable reproducibility.

When we compared the efficiency each extraction techniques for processing 10 samples, there are not much difference of labor and time consumption for extracting 10 samples between each extraction techniques. Each method takes about 4 to 5 hours to extract 10 samples for nitrosamines analysis. Our lab could process 20 samples for NDMA every day without additional investment of new glassware and equipment.

We modified the US EPA method 507, which use 2 L separatory funnel and processing 1 L sample. Due to the carcinogenic nature of NDMA even at very low levels, large volumes of water have to be extracted to achieve the required detection limits[5].

Since, the formation of NDMA during the methylene chloride extraction of chlorinated water containing precursor amines were reported[3], a sample of the reaction mixture was de-chlorinated with ascorbic acid or sodium thiosulphate prior to extraction to avoid possible formation of NDMA during extraction step.

First, the pH of sample was not changed by phosphate buffer solution as directed in the US EPA method 507. We examined extraction efficiency at different pH 2,4,7,9 and 11. NDMA has been recovered most at pH 7 and least at pH 2. The recoveries of NDMA are 87% and 81%, respectively. Since NDMA is neutral compound, it had same extraction efficiency at different pH conditions and generated acceptable results at the tested pH conditions. Actually, we have applied this result to remove some polar compounds from NDMA extraction in order to improve chromatographic analysis. Before extraction with methylene chloride, add enough 6-NaOH to increase pH of sample above 11 and extract sample to avoid acid extractable compound. After extract sample 3 times with 60 ml methylene chloride, rinse the extract with 10 ml 1:1 sulfuric acid to remove base extractable compounds. NDMA was well carried over to the final stage of extraction without reducing recovery of NDMA from base-acid extraction procedures.

The nature of NDMA is very water soluble and a little bit volatile during concentration step using Kudena Danish (K-D) concentrator. Instead of K-D concentrator, apply 9 psi of nitrogen gas and 35ºC of water bath to concentrate the extract to final volume of 1 ml, which data are shown in the Table 12-2. We also added 100 gm of sodium chloride to increase salting out effect from aqueous phase to organic layer during extraction, which improved recovery of NDMA

from water samples significantly.

After we have developed very sensitive and economical analytical techniques for the determination of nitrosamines and 1,4-dioxane, we applied our techniques to determine NDMA in different matrix samples such as ground water, surface water and wastewater. The results from different matrix samples were very reliable and reproducible.

The inclusion of 1,4-dioxane into the developed nitrosamines method could reduce the labor and analysis time without additional instrumentation. Orange County Water District is intensively monitoring nitrosamines and 1,4-dioxane in the waste water and ground water to protect source water.

5.0 CONCLUSION

5.1 Optimization steps for sample and instrument make it possible to use the existing GC/MS/MS/CI to analyze trace concentrations of nitrosamines and 1,4-dioxane from different matrix samples with high production.

5.2 Since nitrosamines and 1,4-dioxane are neutral compound, NDMA and 1,4-dioxane were well extracted at basic and acidic conditions, which was applied to remove background compounds to improve chromatographic analysis.

5.3 This study also revealed the optimum temperature and nitrogen gas pressure to concentrate the extracts

5.4 Chemical ionization using methanol or acetonitrile liquid both generated very acceptable data of nitrosamines and 1,4-dioxane.

5.5 Selection of column is important to avoid the matrix problem from chlorinated samples.

5.6 Sunlight could reduce NDMA levels in the water and showed no reformation of NDMA until 3days of sample storage. The UV treatment will be effective way to destruct NDMA from water matrix.

6.0 FURTHER DEVELOPMENT

6.1 The necessity of establishing suitable solid phase extraction (SPE) methods for the analysis of nitrosamines and 1,4-dioxane that requires very low detection limits of 2 ppt, to reduce the use of methylene chloride by 90 % and to reduce the labor by 70 %.

6.2 Initiate Performance Evaluation (PE) study to improve data reliability among the participating laboratories.

6.3 Study the precursors for NDMA formation in the wastewater to prevent formation of NDMA during wastewater treatment process.

6.4 Need to develop more reliable analytical method to determine sub ppt levels of nitrosamines, since the NDMA formation in the wastewater, could lead to form other nitrosamines.

7.0 ACKNOWLEDGEMENTS

Funding for this project was provided by Orange County Water District. The authors wish to thank our organic chemists for their technical support and encouragement.

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Table 12-1 MS/MS instrument parameters

Segment	Time Window (min)	Analyte	Parent Ion	CID Amplitude (Volts)	Product Ion For Quantitation (m/z)
1	7.00~10.00	NDMA	75	0.56(0.41)	43(47)
		NDMA-d^6	81	0.50(0.40)	46(50)
2	10.00~13.00	NMEA	89	0.56(0.36)	61
3	13.00~15.50	NDEA	103	0.46(0.39)	75
4	15.50~21.40	NDPA-d^{14}	145	0.46(0.37)	97
5	21.40~23.08	NPYR	101	0.44(0.39)	55
		NDPA	117	0.44(0.33)	89
		NMOR	131	0.44(0.35)	86
6	23.08~25.64	NPIP	115	0.44(0.39)	69
7	25.64~30.64	NDBA	159	0.44(0.40)	57

BIBLIOGRAPHIC DATA SHEET

(1) Report ID	(2) Project Classification	(3) Report Date
KIWE-WARC-08-01	International Collaborative Research	July 31, 2008

(4) Title/Subtitle

Development of Multi-residual Analytical Methods for Pharmaceuticals, Perfluorinated Compounds, Nitrosamines, Hormones, Persistent Organic Pollutants in Water

(5) Research Period

Apr 18, 2007 -- Apr 17, 2008

(6) Performing Organization

Water Analysis & Research Center (Kwater)

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Technology Center for Water(TZW), Germany
National Institute for Agro-Environmental Sciences (NIAES), Japan
Orange County Water Dsicict (OCWD), USA

(11) Contract or Authorization

Water Analysis & Research Center (Kwater)

(12) Abstracts

To increase the efficiency of monitoring, risk assessment and policy making for emerging contaminants such as pharmaceuticals, hormone-like chemicals, disinfection byproducts, perfluorinated compounds and persistent organic pollutants, the participated institutions developed the multiresidual analytical methods of these compounds at aquatic environment. Main analytical methods were optimized using high-tech instruments such as tandem mass spectrometry or high resolution technique for wide range of water samples with guidelines of quality assurance/quality control.

(13) Keyword

Multi-residual Analytical Methods, Pharmaceuticals, PerFluorinated Compounds, Nitrosamines, Hormones, Persistent Organic Pollutants, Water

(14) Project ID

KIWE-WARC-08-01

(15) Security Class	(16) No. of Pages	(17) Circulation	(18) Price
-	228	50	Not for sale

Publisher Kuen-Ho Kim
President of K-water

Date July 31, 2008

Publishing office Korea Institute of Water and Environment
462-1, Jeonmin-dong, Yoosung-gu,
Daejeon 305-811
TEL. 81-42-870-7310, 7320

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