ENVIRONMENT AGENCY

The determination of steroid oestrogens in waters using chromatography and mass spectrometry (2008)

Methods for the Examination of Waters and Associated Materials

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Methods for the Examination of Waters and Associated Materials

This booklet contains three methods for the determination of steroid oestrogens in a variety of waters including environmental waters, waste effluents and drinking waters.

Whilst this booklet may report details of the materials actually used, this does not constitute an endorsement of these products but serves only as an illustrative example. Equivalent products are available and it should be understood that the performance characteristics of the method might differ when other materials are used. It is left to users to evaluate methods in their own laboratories. Only limited performance data are presented.

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About this series

Introduction

This booklet is part of a series intended to provide authoritative guidance on recommended methods of sampling and analysis for determining the quality of drinking water, ground water, river water and sea water, waste water and effluents as well as sewage sludges, sediments, soil (including contaminated land) and biota. In addition, short reviews of the most important analytical techniques of interest to the water and sewage industries are included.

Performance of methods

Ideally, all methods should be fully evaluated with results from performance tests. These methods should be capable of establishing, within specified or pre-determined and acceptable limits of deviation and detection, whether or not any sample contains concentrations of parameters above those of interest.

For a method to be considered fully evaluated, individual results from at least three laboratories should be reported. The specifications of performance generally relate to maximum tolerable values for total error (random and systematic errors) systematic error (bias) total standard deviation and limit of detection. Often, full evaluation is not possible and only limited performance data may be available.

In addition, good laboratory practice and analytical quality control are essential if satisfactory results are to be achieved.

Standing Committee of Analysts

The preparation of booklets within the series "Methods for the Examination of Waters and Associated Materials" and their continuing revision is the responsibility of the Standing Committee of Analysts. This committee was established in 1972 by the Department of the Environment and is now managed by the Environment Agency. At present, there are nine working groups, each responsible for one section or aspect of water quality analysis. They are

1 General principles of sampling and accuracy of results
2 Microbiological methods
3 Empirical and physical methods
4 Metals and metalloids
5 General non-metallic substances
6 Organic impurities
7 Biological methods
8 Biodegradability and inhibition methods
9 Radiochemical methods

The actual methods and reviews are produced by smaller panels of experts in the appropriate field, in co-operation with the working group and main committee. The names of those members principally associated with these methods are listed at the back of the booklet.

Publication of new or revised methods will be notified to the technical press. If users wish to receive copies of advance notice of forthcoming publications, or obtain details of the index of methods then contact the Secretary on the Agency's internet web-page (**http://www.environment-agency.gov.uk/nls**) or by post.

Every effort is made to avoid errors appearing in the published text. If, however, any are found, please notify the Secretary.

Dr D Westwood
Secretary
October 2005

Warning to users

The analytical procedures described in this booklet should only be carried out under the proper supervision of competent, trained analysts in properly equipped laboratories.

All possible safety precautions should be followed and appropriate regulatory requirements complied with. This should include compliance with the Health and Safety at Work etc Act 1974 and regulations made under this Act, and the Control of Substances Hazardous to Health Regulations 2002 (SI 2002/2677). Where particular or exceptional hazards exist in carrying out the procedures described in this booklet, then specific attention is noted. Numerous publications are available giving practical details on first aid and laboratory safety. These should be consulted and be readily accessible to all analysts. Amongst such publications are; "Safe Practices in Chemical Laboratories" and "Hazards in the Chemical Laboratory", 1992, produced by the Royal Society of Chemistry; "Guidelines for Microbiological Safety", 1986, Portland Press, Colchester, produced by Member Societies of the Microbiological Consultative Committee; and "Safety Precautions, Notes for Guidance" produced by the Public Health Laboratory Service. Another useful publication is "Good Laboratory Practice" produced by the Department of Health.

The determination of steroid oestrogens in waters using chromatography and mass spectrometry

Introduction

There is concern that the oestrogenic effects of certain chemical substances can cause endocrine disruption and the presence of such substances in sewage effluent has been linked with the feminisation of male fish. This effect produces a condition, known as the 'intersex' condition that may reduce the ability of the male fish to reproduce.

Steroid oestrogens are a group of such chemical substances with known endocrine disrupting effects. Oestrone and 17β-oestradiol are two naturally occurring oestrogen steroids and are present in effluents as a result of the excretion of urine from humans. The synthetic steroid, 17α-ethynyl oestradiol is also present in effluents and is contained in pharmaceutical products such as contraceptive pills, and is also excreted in urine.

These steroid oestrogens have been shown to cause effects in fish at very low concentrations, for example, at the ng l^{-1} level. Sensitive methods are therefore required to determine these compounds in rivers, effluents and drinking waters at these levels in order to monitor environmental impacts and assess the risks to human health.

The structures of some common steroids are shown in Appendix A; also shown are structures of the deuterated compounds used as internal standards in the methods described in this booklet and some steroid-like compounds.

Steroids oestrogens are not stable in water over prolonged periods of time. Hence, if a delay occurs between sampling and analysis, solutions containing steroids may need to be preserved, for example with 6 ml of hydrochloric acid and 0.5 g of copper(II) nitrate per 2 litres of sample. Alternatively, all glassware used in the sampling and analysis of steroid oestrogens may need to be silanised. Appendix B shows stability data for a selection of steroid oestrogens.

Typical chromatograms of selected steroids are shown in Appendix C.

A The determination of soluble oestrone, 17β-oestradiol and 17α-ethynyl oestradiol in waters using liquid chromatography with tandem mass spectrometric detection

This method describes a procedure for the determination of soluble oestrone, 17β-oestradiol and 17α-ethynyl oestradiol in sewage influents, sewage effluents and surface waters using solid phase extraction, clean-up and liquid chromatography with tandem mass spectrometry (LC-MS/MS) for detection and quantification. The method has been shown to be suitable for the determination of steroid oestrogens in waste water and surface water samples. The procedures described may be applicable (with suitable adaptation) for the analysis of other matrices, for example drinking waters. As the method has been validated in only one laboratory, details are included for information purposes only, as an example of procedures that are available. Information on the routine use of this method would be welcomed to assess its full capabilities.

A1 Performance characteristics of the method

A1.1	Substances determined	Oestrone, 17β-oestradiol and 17α-ethynyl oestradiol.
A1.2	Type of sample	Sewage influents, sewage effluents and surface waters.
A1.3	Basis of method	The aqueous sample is filtered and then spiked with deuterated steroid internal standards and extracted using a solid phase extraction cartridge. The extract is cleaned-up using gel permeation chromatography followed by further clean-up using an aminopropyl solid phase cartridge. The cleaned extract may be analysed without derivatisation using LC-MS/MS operated in negative ion electrospray ionisation (ESI) mode, Alternatively, the cleaned extract may be analysed after derivatisation (using dansyl chloride) using LC-MS/MS operated using positive ion atmospheric photo-ionisation mode.
A1.4	Range of application	Typically up to 20 ng l^{-1} for each derivatised and non-derivatised steroid in sewage effluents and surface waters and, up to 200 ng l^{-1} for sewage influents.
A1.5	Calibration curve	All calibrations are linear over the range of application of the method.
A1.6	Interferences	Any substance, which is co-extracted under the conditions used and is not removed by the clean-up procedure, exhibits similar chromatographic behaviour to any of the steroids being determined and has the same mass spectral transitions, will

		interfere. However, the method has high selectivity for the substances analysed.
A1.7	Standard deviation	See Appendices D and E.
A1.8	Limit of detection	Non-derivatised steroids: Oestrone: 0.7 ng l^{-1} 17β-oestradiol: 0.1 ng l^{-1} 17α-ethynyl oestradiol: 0.1 ng l^{-1} Derivatised dansyl steroids: Oestrone: 0.1 ng l^{-1} 17β-oestradiol: 0.1 ng l^{-1} 17α-ethynyl oestradiol: 0.1 ng l^{-1}
A1.9	Sensitivity	This is instrument dependent.
A1.10	Bias	See Appendices D and E.

A2 Principle

The aqueous sample is filtered to remove particulate matter. The filtered sample is then spiked with internal standards (deuterated oestrogens) and extracted using a styrene divinyl benzene solid phase extraction cartridge. The extract is cleaned-up using gel permeation chromatography followed by further clean-up using an aminopropyl solid phase cartridge. The cleaned extract is analysed either without derivatisation (using LC-MS/MS operated in negative ion electrospray ionisation mode) or after derivatisation using dansyl chloride (using LC-MS/MS operated using positive ion atmospheric photo-ionisation). (Steroids adsorbed onto the particulate matter are not analysed by this method and may need to be analysed separately).

A3 Hazards

All of the natural steroids determined are oestrogenically active. It should be assumed that the deuterated internal standards are similarly active. Appropriate precautions should be taken when handling these compounds and solutions of these compounds. In addition, methanol and acetonitrile are toxic and flammable. Acetone is flammable. Ethyl acetate and hexane are flammable. Dichloromethane is narcotic. Copper salts are toxic and concentrated hydrochloric acid is corrosive.

A4 Reagents

All reagents should be of sufficient purity that they do not give rise to significant interfering peaks in the analysis. Purity should be checked for each batch of materials by running procedural blanks with each batch of samples analysed. Solvents suitable for high performance liquid chromatography or pesticide analysis use and analytical grade materials are normally suitable unless otherwise stated and details of preparation are given where appropriate. To avoid potential evaporation of solvents, standard solutions should be stored in a refrigerator. However, prior to use, all solutions and solvents should

be allowed to reach ambient room temperature before volumetric measurements are made.

A4.1 Water. This should be deionised or of similar quality.

A4.2 Acetonitrile.

A4.3 Methanol.

A4.4 Acetone.

A4.5 Ethyl acetate.

A4.6 Dichloromethane.

A4.7 Hexane.

A4.8 Ethyl acetate in hexane (30 v/v %). Mix 30 volumes of ethyl acetate (A4.5) with 70 volumes of hexane (A4.7).

A4.9 Ethyl acetate in acetone (50 v/v %). Mix equal volumes of ethyl acetate (A4.5) and acetone (A4.4).

A4.10 Ammonia solution (0.1 v/v %). Add 1 ml of concentrated ammonia solution to approximately 950 ml of water (A4.1). Mix well. Make to 1000 ml with water. Mix well.

A4.11 Sodium hydroxide solution (2M). Add 8 g of sodium hydroxide to approximately 80 ml of water (A4.1). Cool and mix well. Make to 100 ml with water. Mix well.

A4.12 Hydrochloric acid solution (30 v/v %). Add 30 ml of concentrated hydrochloric acid to approximately 60 ml of water (A4.1). Mix well. Make to 100 ml with water.

A4.13 Copper(II) nitrate.

A4.14 Dansyl chloride (1 mg ml^{-1} in acetonitrile). Dissolve 25 mg of dansyl chloride (5-dimethylamino-1-naphthalenesulfonyl chloride) in 25 ml of acetonitrile (A4.2) to produce a saturated solution.

A4.15 Sodium hydrogen carbonate buffer solution (0.1M). Dissolve 840 mg of sodium hydrogen carbonate in approximately 90 ml of water (A4.1). Adjust the pH of this solution to 10.5 using sodium hydroxide solution (A.4.11). Make to 100 ml with water (A4.1). Mix well.

A4.16 Solid phase cartridges. The following extraction cartridges, namely, a styrene divinyl benzene cartridge (Isolute ENV+) solid phase extraction cartridge (200 mg/6 ml) and Isolute aminopropyl solid phase extraction cartridge (500 mg/6 ml) have been used in this method. Whilst equivalent cartridges may also be used, all cartridges would need to be evaluated.

A4.17 Standard solutions

A4.17.1 Deuterated internal standard stock solutions (nominally 1 mg ml^{-1}). The following deuterated internal standards have been used in this method:

2, 4, 16, 16-d_4-oestrone:
2, 4, 16, 16-d_4-17β-oestradiol: and
2, 4, 16, 16-d_4-17α-ethynyl oestradiol.

Into a 10-ml volumetric flask, dissolve an accurately weighed amount of approximately 10 mg of 2, 4, 16, 16-d_4-oestrone in approximately 9 ml of acetone (A4.4). Make to 10 ml with acetone and mix well. Into a separate 10-ml volumetric flask, dissolve an accurately weighed amount of approximately 10 mg of 2, 4, 16, 16-d_4-17β-oestradiol in approximately 9 ml of acetone (A4.4). Make to 10 ml with acetone and mix well. Into a further 10-ml volumetric flask, dissolve an accurately weighed amount of approximately 10 mg of 2, 4, 16, 16-d_4-17α-ethynyl oestradiol in approximately 9 ml of acetone (A4.4). Make to 10 ml with acetone and mix well. The concentration of each deuterated steroid in these solutions is nominally 1 mg ml^{-1}. The solutions may be stored at -18 °C for up to one year.

A4.17.2 Intermediate deuterated internal standard stock solution (nominally 1 µg ml^{-1}). Add 50 µl of each deuterated internal standard stock solution (A4.17.1) into a 50-ml volumetric flask containing approximately 45 ml of acetone (A4.4). Make to 50 ml with acetone and mix well. The concentration of each deuterated steroid in this solution is nominally 1 µg ml^{-1}. This solution may be stored at -18 °C for up to one year.

A4.17.3 Spiking deuterated internal standard stock solution (nominally 20 ng ml^{-1}). Add 400 µl of intermediate deuterated internal standard stock solution (A4.17.2) into a 20-ml volumetric flask containing approximately 18 ml of acetone (A4.4). Make to 20 ml with acetone and mix well. The concentration of each deuterated steroid in this solution is nominally 20 ng ml^{-1}. This solution may be stored at -18 °C for up to one year.

A4.17.4 Steroid standard stock solutions (nominally 1 mg ml^{-1}). Into a 10-ml volumetric flask, dissolve an accurately weighed amount of approximately 10 mg of oestrone in approximately 9 ml of acetone (A4.4). Make to 10 ml with acetone and mix well. Into a separate 10-ml volumetric flask, dissolve an accurately weighed amount of approximately 10 mg of 17β-oestradiol in approximately 9 ml of acetone (A4.4). Make to 10 ml with acetone and mix well. Into a further 10-ml volumetric flask, dissolve an accurately weighed amount of approximately 10 mg of 17α-ethynyl oestradiol in approximately 9 ml of acetone (A4.4). Make to 10 ml with acetone and mix well. The concentration of each steroid in these solutions is nominally 1 mg ml^{-1}. These solutions may be stored at -18 °C for up to one year.

A4.17.5 Intermediate steroid standard stock solution (nominally 1 µg ml^{-1}). Add 50 µl of each steroid standard stock solution (A4.17.4) into a 50-ml volumetric flask containing approximately 45 ml of acetone (A4.4). Make to 50 ml with acetone and mix well. The concentration of each steroid in this solution is nominally 1 µg ml^{-1}. This solution may be stored at -18 °C for up to one year.

A4.17.6 Spiking steroid standard stock solution (nominally 20 ng ml^{-1}). Add 400 µl of

intermediate steroid standard stock solution (A4.17.5) into a 20-ml volumetric flask containing approximately 18 ml of acetone (A4.4). Make to 20 ml with acetone and mix well. The concentration of each steroid in this solution is 20 ng ml^{-1}. This solution may be stored at -18 °C for up to one year.

A4.17.7 Calibration standard solutions. Calibration standard solutions should be prepared. Each calibration solution should contain oestrone, 17β-oestradiol, 17α-ethynyl oestradiol and the deuterated internal standards. For example, each of the steroid concentrations should be 100, 50, 20, 10, 5, 1 and 0.5 ng ml^{-1} and the concentration of the deuterated internal standards should each be 1000 ng ml^{-1}.

The following table shows the volumes of intermediate steroid standard stock solution (A4.17.5) required to prepare 10 ml quantities of calibration standard solutions each containing 10 µl of each of the deuterated internal standard stock solutions (A4.17.1). Each flask should be made to 10 ml with acetone.

Concentration of steroid (ng ml^{-1})	Volume of intermediate steroid standard stock solution A4.17.5 (µl)
100	1000
50	500
20	200
10	100
5	50
1	10
0.5	5

A blank solution containing reagents (i.e. no steroids) and 10 µl of each of the deuterated internal standards (at the same concentrations) should also be prepared in acetone and made to 10 ml.

A5 Apparatus

In addition to general laboratory glassware, the following are required.

A5.1 Analytical balance capable of weighing to 0.0001g.

A5.2 Solid phase extraction apparatus. The manifold should be capable of handling several solid phase extraction cartridges. The flow rate through each individual cartridge should be controlled by adjusting the vacuum applied to each one, or by the application of air or nitrogen.

A5.3 Extract concentration equipment. Any suitable proprietary concentrator with thermostatically controlled water bath or equivalent system.

A5.4 Nitrogen or air blow-down apparatus or equivalent system.

A5.5 LC-MS/MS equipment. Suitable LC-MS equipment consisting of a binary or quaternary pump and a tandem mass spectrometer should be used. The suitability of the equipment will need to be evaluated.

The following conditions have been used in the performance testing of non-derivatised steroids by this method:

LC equipment:	Agilent 1100.
MS:	API 5000.
Column:	Phenomenex LUNA C18(2), 150 x 4.6 mm, 5 µm.
Mode:	Electrospray ionisation (negative ion).
Injection volume:	20 µl.
Flow:	0.5 ml per minute.
Mobile phase:	Solvent A: water containing 0.1 % ammonia. (A4.10). Solvent B: acetonitrile.

Gradient programme:

Time (minutes)	0	10	18	24	28
A:B solvent ratio	90:10	50:50	10:90	10:90	90:10

Equivalent equipment may be used.

The following conditions have been used in the performance testing of derivatised steroids by this method:

LC equipment:	Agilent 1100.
MS:	API3000.
Column:	Phenomenex Columbus - C18 column, 250 x 2 mm, 5 µm.
Mode:	Atmospheric pressure photo-ionisation (positive ion).
Injection volume:	50 µl.
Flow:	0.3 ml per minute.
Mobile phase:	Solvent A: water. Solvent B: acetonitrile.

Gradient programme:

Time (minutes)	0	0.5	12	15	18	22	26	30
A:B solvent ratio	25:75	25:75	0:100	0:100	80:20	80:20	25:75	25:75

Equivalent equipment may be used.

A5.6 Gel permeation chromatography. The following GPC equipment has been used in the performance testing of this method:

Column:	Polymer labs PL gel, 50 A, 300 x 7.5 mm, 5 µm.
Guard Column:	Polymer labs PL gel, 50 x 7.5 mm, 5 µm.
Mobile phase:	Dichloromethane.
Flow rate:	1 ml per minute.
Column temperature:	25 °C.
UV detector:	210 nm.
Injection volume:	95 µl.
Injection mode:	Standard.

Draw speed: 500 µl per minute.
Eject speed: 500 µl per minute.
Draw position: 3 mm.
Fraction collected: 3 ml fraction (7 - 10 minutes) in 10 ml vials.

Equivalent equipment may be used.

A6 Sample collection and storage

Samples should be collected in glass bottles of 2-litre capacity. Plastic screw tops may be suitable provided they are fitted with polytetrafluoroethylene or polytetrafluoroethylene-faced liners. Alternatively, ground-glass stoppers in glass bottles may be used. Prior to use, the bottles should be cleaned with a suitable cleaning agent (for example Decon 90). After cleaning, the bottles should be rinsed with water and then allowed to drain, before being dried. Oestrone, 17β-oestradiol and 17α-ethynyl oestradiol are not stable unless preserved. After collection, samples should be preserved, for example with 6 ml of hydrochloric acid (A4.12) and 0.5 g of copper(II) nitrate (A4.13) per 2 litres of sample. Samples should be analysed as soon as possible following collection and preservation. If storage of samples is unavoidable, samples should be stored in a refrigerator and kept at below 10 °C.

Appendix B shows some stability data for certain effluent samples analysed over a period of time.

A7 Analytical procedure

Step	Procedure	Note
A7.1	**Sample pre-treatment**	
A7.1.1	Prior to extraction, all samples should be filtered, see note a.	(a) Whatman GFD/GFF filter papers with a nominal pore size of 1 µm have been found suitable. Equivalent glass fibre membrane filters may be used.
A7.2	**Extraction**	
A7.2.1	To 1000 ml of filtered surface water or sewage effluent, or to 100 ml of filtered sewage influent, add 100 µl of spiking deuterated internal standard stock solution (A4.17.3). See note b.	b) This is equivalent to a concentration of 2 ng l^{-1} for surface water or sewage effluent samples, and (20 ng l^{-1} for sewage influent samples, (i.e. 2 ng of each deuterated internal standard added).

A7.2.2 To the solid phase extraction apparatus, attach sample lines and apply vacuum. Condition a styrene divinyl benzene cartridge (A4.16) solid phase extraction cartridge (see note c) by passing through the cartridge (in sequence) 5 ml of ethyl acetate (A4.5) followed by 5 ml of methanol (A4.3) followed by 5 ml of water (A4.1). See note d.

(c) A suitable flow rate of less than 10 ml per minute should be used.

(d) The cartridge should not be allowed to dry out during each elution stage.

A7.2.3 Pass the spiked filtered sample (A7.2.1) through the conditioned cartridge. See notes c and d.

A7.2.4 After extracting the sample, remove the lines and dry the cartridge under vacuum or using air or nitrogen (A5.2). Ensure that the cartridge is thoroughly dry, see note e.

(e) This is usually indicated by the observation of a colour change as the cartridge dries.

A7.3 Elution

A7.3.1 Prior to sample elution, place a clean dry collection vessel inside the extraction manifold. Apply a vacuum and elute the cartridge with 8 ml of dichloromethane (A4.6) see notes c and d. When the elution is completed, remove the collection vessel and using a concentrator (A5.3) concentrate the extract to 1 ml. Finally, using the blow down equipment (A5.4) concentrate the extract into an auto-sampler vial to 100 µl.

A7.4 GPC clean-up

A7.4.1 Using the GPC conditions described in section A5.6 carry out a clean-up procedure on 95 µl of the eluted sample extract (A7.3.1).

A7.4.2 Concentrate the GPC extract using a concentrator (A5.3) and blow down apparatus (A5.4) to 200 µl. To this extract, add hexane (A4.7) and make to 2.0 ml with hexane.

A7.5 SPE clean-up

A7.5.1 Prior to loading the cartridge, place a clean dry collection vessel inside the extraction manifold. To the solid phase extraction apparatus, attach sample lines, apply vacuum and condition an aminopropyl cartridge (A4.16) see note c, by passing through the cartridge 2 ml of hexane (A4.7). See note d. Pass the sample extract from section A7.4.1 through the cartridge, see note c,

(f) Do not discard this collection vessel or its contents.

and collect the eluate in the collection vessel. Remove the collection vessel from the manifold, see note f.

A7.5.2 Prior to sample elution, place another collection vessel inside the extraction manifold. Wash the cartridge with 2 ml of ethyl acetate in hexane (A4.8) and discard the washings. Repeat this procedure with a further 2 ml of ethyl acetate in hexane (A4.8).

A7.5.3 Place the original collection vessel (see section A7.5.1 and note f) inside the extraction manifold. Oestrone, 17β-oestradiol and 17α-ethynyl oestradiol are eluted from the cartridge with 2 ml of ethyl acetate in acetone (A4.9) see note g. Collect the eluate in the collection vessel. Repeat this procedure with a further 2 ml of ethyl acetate in acetone (A4.9). Remove the collection vessel and using a concentrator (A5.3) concentrate the extract to 1 ml. Using the blow down apparatus (A5.4) evaporate the extract to incipient dryness.

(g) A suitable flow rate of less than 2 ml per minute should be used.

A7.5.4 If non-derivatised steroids are to be determined, then to the dry residue add 100 µl of methanol (A4.3) and mix well, see note h. Transfer the extract to an auto-sampler vial and cap the vial. The extract is now ready for LC-MS/MS determination, see section A7.7.

(h) This solution should contain 1.9 ng of each deuterated internal standard.

A7.5.5 If derivatised steroids are to be determined, then to the dry residue add 100 µl of sodium hydrogen carbonate solution (A4.15) and 100 µl of dansyl chloride solution (A4.14). Cap the vial, mix the contents well (see note h) and place it in a suitable heater at 60 °C for 10 minutes. Remove the vial and allow it and the contents to cool to room temperature. The extract is now ready for LC-MS/MS determination, see section A7.8.

A7.6 Blank

A7.6.1 A sample blank (see note i) should be treated exactly as a sample, and taken through the complete analytical procedure described in sections A7.1 - A7.5.

(i) Water (A4.1) or a suitable ground water should be used.

A7.7 Determination of non-derivatised steroids

A7.7.1 Using manufacturer's instructions, optimise the

(j) Appropriate AQC

operating conditions of the LC-MS/MS system. Analyse the calibration standard solutions (A4.17.7) blank (A7.6.1) and sample extracts (A7.5.4). See note j. Determine the concentration of steroids in the sample extracts, see note k. Monitor the ions shown in Table A1.

samples should also be analysed.

(k) For any sample that exceeds the calibration range consideration should be give to repeating the analysis by taking a smaller volume of sample used for extraction (A7.2.1) or diluting the sample extract (A7.5.4) or injecting a smaller volume.

A7.8 **Determination of dansyl derivatives**

A7.8.1 Using manufacturer's instructions, optimise the operating conditions of the LC-MS/MS system. Analyse the calibration standard solutions (A4.17.7) blank (A7.6.1) and sample extracts (A7.7.5). See note j. Determine the concentration of the dansyl derivatives in the sample extracts, see note k. Monitor the ions shown in Table A2.

A8 Calculation of results

A calibration graph of the ratio of the respective peak area or height of the steroid of interest to the peak area or height of the corresponding deuterated internal standard should be constructed against the mass of deuterated internal standard injected. The original sample concentration should be calculated from the graph taking into account the volume of sample that is extracted (A7.2.1) and any dilutions (A7.5.4) that may have been used.

The respective peak area or height of each specific steroid should be measured. For each steroid, the response ratio should then be calculated.

$$\text{Response ratio} = \frac{\text{peak area or height (SS)}}{\text{peak area or height (DIS)}}$$

where

peak area or height (SS) is the peak area or height of the specific steroid,
peak area or height (DIS) is the peak area or height of the corresponding deuterated internal standard.

Plot the response ratio for the specific steroid against the concentration of the specific steroid in the calibration standards. From the plotted calibration curve, calculate the slope and intercept using linear regression.

Determining the corresponding response ratios in the unknown sample (RRu) and blank

sample (RRb) enables the concentration of each steroid, C_s, to be calculated.

$$C_s = ((RRu - RRb) - \text{intercept}) / \text{Slope}$$

A9 Additional reading material

1. UKWIR (2004) Endocrine Disrupters in Sewage Influent: Analytical Method Development, UKWIR, Report TX/04/B.

2. UKWIR (2003) Endocrine Disrupters in Sewage Sludge: Analytical Method Development, UKWIR, Report 03/TX/04/7 (ISBN 1 84057 307 4).

3. Development and validation of a high sensitivity liquid chromatography-tandem mass spectrometry (LC/MS/MS) method with chemical derivatisation for the determination of ethinyl estradiol in human plasma, W Z Shou, X Jiang, W Naidong, *Biomedical Chromatography,* 2004, **18**, pp414 - 421.

4. Quantification of 17α-ethynyl oestradiol in aquatic samples using liquid-liquid phase extraction, dansy derivatisation and liquid chromatography/positive electrospray tandem mass spectrometry, F Zhang, M Bartels, J Brodeur, E McClymont, K Woodburn, *Rapid Communications in Mass Spectrometry,* 2004, **18**, pp2739 - 2742.

Table A1 Ions monitored for non-derivatised steroids

Compound	Ion transition (1)* (m/z)	Ion transition (2)** (m/z)
oestrone	269.2 / 143.0	269.2 / 145.0
2, 4, 16, 16-d_4-oestrone	273.2 / 147.0	273.2 / 149.0
17β-oestradiol	271.1 / 143.1	271.1 / 145.1
2, 4, 16, 16-d_4-17β-oestradiol	275.2 / 147.0	275.2 / 149.0
17α-ethynyl oestradiol	295.2 / 143.0	295.2 / 145.0
2, 4, 16, 16-d_4-17α-ethynyl oestradiol	299.2 / 147.0	299.2 / 149.0

* These ions used for quantification
** These ions used for confirmation

Table A2 Ions monitored for derivatised dansyl steroids

Compound	Ion transition* (m/z)
Oestrone	504 / 170
2, 4, 16, 16-d_4-oestrone	510 / 170
17β-oestradiol	506 / 170
2, 4, 16, 16-d_4-17β-oestradiol	511 / 170
17α-ethynyl oestradiol	530 / 170
2, 4, 16, 16-d_4-17α-ethynyl oestradiol	535 / 170

* Qualifying ions were not used in this procedure and the selected ions were chosen as representing the most selective ions produced with minimal interference from isotopic contribution from the internal standard.

B The determination of soluble oestrone, 17β-oestradiol and 17α-ethynyl oestradiol in environmental waters by liquid chromatography with mass spectrometric time of flight detection (LC-MS/TOF)

This method describes a procedure for the determination of soluble oestrone, 17β-oestradiol and 17α-ethynyl oestradiol in sewage influents, sewage effluents and surface waters using solid phase extraction, clean-up and liquid chromatography with mass spectrometric time of flight detection. The method has been shown to be suitable for the determination of steroid oestrogens in waste water and surface water samples. The procedures described may be applicable (with suitable adaptation) for the analysis of other matrices, for example drinking waters. As the method has been validated in only one laboratory, details are included for information purposes only, as an example of procedures that are available. Information on the routine use of this method would be welcomed to assess its capabilities.

B1 Performance characteristics of the method

B1.1	Substances determined	Oestrone, 17β-oestradiol and 17α-ethynyl oestradiol.
B1.2	Type of sample	Final effluents, crude sewages and river waters.
B1.3	Basis of method	The aqueous sample is filtered and then spiked with deuterated steroid internal standards and extracted using a solid phase extraction cartridge. Steroids are then desorbed with ethyl acetate. The extract is cleaned-up using normal phase chromatography followed by further clean-up using gel permeation chromatography (GPC). The cleaned extract is analysed by high performance liquid chromatography using negative ion atmospheric photo-ionisation mode with mass spectrometer time of flight (LC-MS/TOF) detection.
B1.4	Range of application	Typically, up to 25 ng l^{-1} for each steroid in final effluents and river waters, and up to 125 ng l^{-1} in crude sewages.
B1.5	Limits of detection	See Appendix F.
B1.6	Calibration curve	Non-linear over the calibration range, a quadratic fit is used.
B1.7	Standard deviation	See Appendix F.
B1.8	Recovery	See Appendix F.
B1.9	Interferences	Any substance, which is co-extracted under the conditions used, is not removed by the clean-up procedure and exhibits similar chromatographic

		behaviour to any of the steroids being determined, will interfere**.** However, the method has high selectivity for the substances analysed.
B1.10	Time required for analysis	24 samples including AQC and blank samples can be extracted within the working day. The total analysis time for a batch of 24 samples is about 72 hours. Data handling may require a further 2 hours, but may be undertaken within the same time period as the preparative work.

B2 Principle

The aqueous sample is filtered to remove particulate matter. The filtered sample is passed through a styrene divinyl benzene polymer solid phase extraction cartridge. The cartridge is washed, dried and the steroids desorbed with ethyl acetate. This extract is evaporated to incipient dryness, dissolved in a 95:5 v/v cyclohexane:propan-2-ol mixture and then cleaned-up using normal phase chromatography using a Cyano LC column. The resulting extract is evaporated to incipient dryness, dissolved in dichloromethane and cleaned-up again using gel permeation chromatography (GPC). The extract is then evaporated to incipient dryness and dissolved in aqueous methanol. This extract is then ready for analysis using high performance liquid chromatography with negative ion atmospheric photo-ionisation mode and mass spectrometer time of flight detection. (Steroids adsorbed onto the particulate matter are not analysed by this method and may need to be analysed separately).

B3 Hazards

All of the natural steroids determined are oestrogenically active. It should be assumed that the deuterated internal standards are similarly active. Appropriate precautions should be taken when handling these compounds and solutions of these compounds. In addition, methanol is toxic and flammable. Propanol-2-ol and acetone are highly flammable. Ethyl acetate and cyclohexane are flammable. Dichloromethane is narcotic. Acetonitrile is toxic and highly flammable. Copper salts are toxic and concentrated hydrochloric acid is corrosive.

B4 Reagents

All reagents should be of sufficient purity that they do not give rise to significant interfering peaks in the analysis. Purity should be checked for each batch of materials by running procedural blanks with each batch of samples analysed. Solvents suitable for high performance liquid chromatography or pesticide analysis use and analytical grade materials are normally suitable unless otherwise stated and details of preparation are given where appropriate. To avoid potential evaporation of solvents, standard solutions should be stored in a refrigerator. However, prior to use, all solutions and solvents should be allowed to reach ambient room temperature before volumetric measurements are made.

B4.1 Water. This should be of distilled or deionised quality.

B4.2 Ethyl acetate.

B4.3 Methanol.

B4.4 Propan-2-ol.

B4.5 Cyclohexane.

B4.6 Dichloromethane.

B4.7 Acetone.

B4.8 Acetonitrile.

B4.9 Hydrochloric acid (30 v/v %). Add 30 ml of concentrated hydrochloric acid (36.5 - 38.0 %) to 70 ml of water (B4.1). Mix well.

B4.10 Copper(II) nitrate.

B4.11 Aqueous methanol (90:10 v/v). To a 100-ml volumetric flask add 10 ml of methanol (B4.3). Make to just less than 100 ml with water (B4.1) and mix well. Make to 100 ml with water and mix. This solution may be stored at ambient temperature for up to one year.

B4.12 Methanol:acetone (95:5 v/v). To 2375 ml of methanol (B4.3) add 125 ml of acetone (B4.7). Mix well. This solution may be stored at ambient temperature for up to 2 years.

B4.13 Purine (100 mg l^{-1}). To a 40-ml glass vial, add 4 mg of purine. Add 40 ml of methanol (B4.3). Mix well to dissolve. This solution may be stored between 1 - 10 °C for up to 2 years.

B4.14 Reserpine (100 mg l^{-1}). To a 40-ml glass vial, add 4 mg of reserpine. Add 40 ml of methanol (B4.3). Mix well to dissolve. This solution may be stored between 1 - 10 °C for up to 2 years.

B4.15 Locking mass solution. To a 1-litre glass bottle, add 500 ml of water (B4.1). Add 500 ml of methanol:acetone mixture (B4.12) and mix well. Add 2 ml of purine solution (B4.13) and 1 ml of reserpine solution (B4.14). Mix well. This solution may be stored at ambient temperature for up to one year.

B4.16 Aqueous methanol (40:60 v/v). To a 1000-ml volumetric flask add 600 ml of methanol (B4.3). Make to just less than 1000 ml with water (B4.1) and mix well. Make to 1000 ml with water. This solution may be stored at ambient temperature for up to one year.

B4.17 Cyclohexane:propan-2-ol (95:5 v/v). To a 100-ml volumetric flask, add 5 ml of propan-2-ol (B4.4). Make to just less than 100 ml with cyclohexane (B4.5) and mix well. Make to 100 ml with cyclohexane and mix well. This solution may be stored at ambient temperature for up to 2 years.

B4.18 Standard solutions

B4.18.1 Steroid standard stock solutions (nominally 0.1 mg ml^{-1}). Into a 100-ml volumetric flask, dissolve an accurately weighed amount of approximately 10 mg of oestrone in approximately 9 ml of acetonitrile (B4.8). Make to 100 ml with acetonitrile and mix well. Into a separate 100-ml volumetric flask, dissolve an accurately weighed amount of approximately 10 mg of 17β-oestradiol in approximately 9 ml of acetonitrile (B4.8). Make to 100 ml with acetonitrile and mix well. Into a further 100-ml volumetric flask, dissolve an accurately weighed amount of approximately 10 mg of 17α-ethynyl oestradiol in approximately 9 ml of acetonitrile (B4.8). Make to 100 ml with acetonitrile and mix well. The concentration of each steroid in these solutions is nominally 0.1 mg ml^{-1}. These solutions may be stored in the dark between 1 - 10 °C for up to one year. Alternatively, these solutions are available commercially. The manufacturer's expiry date should be adhered to.

B4.18.2 AQC steroid stock solutions (nominally 0.1 mg ml^{-1}). Ensure that AQC standards are obtained from a different supplier to that of the steroid standard stock solutions (B4.18.1). Alternatively, if the same supplier is used, the AQC standards should be of a different batch number to the steroid standard stock solutions (B4.18.1). Into a 100-ml volumetric flask, dissolve an accurately weighed amount of approximately 10 mg of oestrone in approximately 9 ml of acetonitrile (B4.8). Make to 100 ml with acetonitrile and mix well. Into a separate 100-ml volumetric flask, dissolve an accurately weighed amount of approximately 10 mg of 17β-oestradiol in approximately 9 ml of acetonitrile (B4.8). Make to 100 ml with acetonitrile and mix well. Into a further 100-ml volumetric flask, dissolve an accurately weighed amount of approximately 10 mg of 17α-ethynyl oestradiol in approximately 9 ml of acetonitrile (B4.8). Make to 100 ml with acetonitrile and mix well. The concentration of each steroid in these solutions is nominally 0.1 mg ml^{-1}. These solutions may be stored in the dark between 1 - 10 °C for up to one year. Alternatively, these solutions are available commercially. The manufacturer's expiry date should be adhered to.

B4.18.3 Individual deuterated internal standard stock solutions (nominally 0.1 mg ml^{-1}). The following deuterated internal standards have been used in this method:

2, 4, 16, 16-d_4-oestrone;
2, 4, 16, 16, 17-d_5-17β-oestradiol: and
2, 4, 16, 16-d_4-17α-ethynyl oestradiol.

Prepare individual solutions of each deuterated internal standard by, for example accurately weighing and adding to separate 40-ml glass vials, between 3 - 4 mg of deuterated internal standard. Record the weight and add an appropriate volume of acetonitrile (B4.8) to give a concentration of 0.1 mg ml^{-1}. These solutions may be stored between 1 - 10 °C for up to one year.

B4.18.4 Mixed deuterated internal standard stock solution (nominally 0.1 µg ml^{-1}). To a 100-ml volumetric flask, add 10 ml of methanol (B4.3) and approximately 85 ml of water (B4.1). Mix well. Add 100 µl of each individual deuterated internal standard stock solution (B4.18.3). Make to the mark with water (B4.1) and mix well. This solution may be stored between 1 - 10 °C for up to 6 months.

B4.18.5 Working calibration standard solutions. Calibration standard solutions should be prepared, each solution containing oestrone, 17β-oestradiol and 17α-ethynyl oestradiol and the deuterated internal standards. For example, each of the steroid concentrations should be 100, 40, and 8 ng ml^{-1} and the concentration of the deuterated internal standards should each be 20 ng ml^{-1}. To three separate 100-ml volumetric flasks add 10 ml of methanol (B4.3) and approximately 85 ml of water (B4.1). Mix well. To each flask, add 100, 40 or 8 µl of each steroid standard stock solution (B4.18.1). To each flask, add 20 µl of each individual deuterated internal standard stock solution (B4.18.3). Mix well. Make to 100 ml with water (B4.1) and mix well. These solutions may be stored between 1 - 10 °C for up to 6 months.

A blank solution containing reagents (i.e. no steroids) and the deuterated internal standards (each at a concentration of 20 ng ml^{-1}) should also be prepared and made to 100 ml.

B4.18.6 Recovery spiking solution (nominally 0.1 µg ml^{-1}). This is a mixed solution containing oestrone, 17β-oestradiol and 17α-ethynyl oestradiol, each at a concentration of 0.10 µg ml^{-1}. To a 100-ml volumetric flask, add 10 ml of methanol (B4.3) and approximately 85 ml of water (B4.1). Mix well. Add 100 µl of each steroid standard stock solution (B4.18.1). Make to 100 ml with water (B4.1) and mix well. The concentration of each steroid in this solution is 0.1 µg ml^{-1}. This solution may be stored between 1 - 10 °C for up to 6 months.

B4.18.7 AQC spiking solution (nominally 0.1 µg ml^{-1}). This is a mixed solution containing oestrone, 17β-oestradiol and 17α-ethynyl oestradiol, each at a concentration of 0.1 µg ml^{-1}. To a 100-ml volumetric flask, add 10 ml of methanol (B4.3) and approximately 85 ml of water (B4.1). Mix well. Add 100 µl of each AQC steroid standard stock solution (B4.18.2). Make to 100 ml with water (B4.1) and mix well. This solution may be stored between 1 - 10 °C for up to 6 months.

B4.18.8 Normal phase standard solution (nominally 0.02 µg ml^{-1}). This is a mixed solution containing oestrone, 17β-oestradiol and 17α-ethynyl oestradiol in a cyclohexane-propan-2-ol mixture, each at a concentration of 0.02 µg ml^{-1}. To a 40-ml glass vial, add 2 ml of propan-2-ol (B4.4) and 38 ml of cyclohexane (B4.5). Mix well. Add 8 µl of each steroid standard stock solution (B4.18.1). Mix well. This solution may be stored between 1 - 10 °C for up to one year. This solution should not be used for quantification purposes.

B4.18.9 GPC standard solution (nominally 0.1 µg ml^{-1}). This is a mixed solution containing oestrone, 17β-oestradiol and 17α-ethynyl oestradiol in dichloromethane. To a 40-ml glass vial, add 40 ml of dichloromethane (B4.6). Mix well. Add 40 µl of each steroid standard stock solution (B4.18.1). Mix well. The concentration of each steroid in this solution is 0.1 µg ml^{-1}. This solution may be stored between 1 - 10 °C for up to one year. This solution should not be used for quantification purposes.

B5 Apparatus

In addition to normal laboratory glassware the following apparatus may be required.

B5.1 Sample bottles. 1000 ml glass bottles with screw cap.

B5.2 Solid phase extraction cartridges. For example, Baker SDB1 (200 mg/3 ml) styrene divinyl benzene cartridges, or equivalent.

B5.3 Blow-down apparatus. Any device capable of directing a gentle stream of air or nitrogen into a 4-ml vial. Available with a heating block capable of maintaining a temperature of 45 ± 3 °C.

B5.4 Automatic solid phase extraction workstation. Capable of conditioning, loading (i.e. pumping the sample through the cartridge) and drying and de-sorbing solid phase extraction cartridges.

B5.5 Vials. A variety of sizes with screw cap and polytetrafluoroethylene-faced seals.

B5.6 LC/MS-TOF system. Fitted with atmospheric photo-ionisation source, binary pump, auto-injector with chiller unit, column heater compartment, time of flight mass spectrometer capable of producing a mass ion resolution of 0.04 - 0.06 mass units at the mass range of interest, data station and an additional isocratic pump to deliver the locking mass solution. The suitability of the equipment will need to be evaluated.

The following conditions were used to determine performance data.

LC equipment: Agilent 1100.
MS: Agilent LCMS-TOF.
Column: Luna phenyl hexyl, 2 x 150 mm, 3 µm, thermostatted at 60 °C.
Guard column: Luna phenyl propyl, 4 x 2 mm.
Mobile phase: Solvent A: water.
Solvent B: methanol:acetone mixture (B4.11).
Gradient programme

Time (minutes)	0	0.5	1.0	12	14	14.5
A:B solvent ratio	95:5	80:20	60:40	20:80	20:80	95:5

Flow rate: 0.3 ml per minute.
Locking mass pump: Flow-rate of 0.2 ml per minute.
Injector volume: 100 µl.
Detector: Mass spectrometer TOF.

Equivalent equipment may be used.

B5.7 Fraction collection system. Fitted with binary pump, auto-injector with chiller unit, column heater compartment, fraction collector with chiller unit, UV detector and data system.

B5.8 HPLC clean-up system.

The following conditions were used to determine performance data.

Column: Zorbax Cyano 4.6 x 150 mm, 5 µm thermostated at 55 °C.
Guard column: Zorbax Cyano 4.6 x 12.5 mm, 5 µm.

Mobile phase: Solvent A: cyclohexane.
Solvent B: propan-2-ol.
Flow-rate: 1 ml per minute.
Gradient programme

Time (minutes)	0	8.5	8.55	10.4	10.5
A:B solvent ratio	95:5	95:5	75:25	75:25	95:5

Run time: 15 minutes.
Injection volume: 250 µl (Draw speed 200 µl per minute, eject speed 200 µl per minute).
UV detector: 280 nm.

Equivalent equipment may be used.

B5.9 GPC clean-up system.

The following conditions were used to determine performance data

Column: Polymer labs PL gel 50 A, 7.5 x 300 mm, thermostatted at 30 °C.
Mobile phase Isocratic elution with dichloromethane.
Flow rate: 1 ml per minute.
Run time: 13.5 minutes.
Injection volume: 250 µl (Draw speed 125 µl per minute, eject speed 250 µl per minute.
UV detector: 280 nm.

Equivalent equipment may be used.

B6 Sample collection and preservation

Samples should be collected in 2 litre amber glass bottles and should be preserved at the time of sampling with 6.0 ± 0.2 ml of hydrochloric acid solution (B4.9) and 0.5 ± 0.1 g of copper(II) nitrate (B4.10) per 2 litres of sample. Samples should be stored between 1 - 10 °C. Appendix B shows some stability data for certain effluent samples analysed over a period of time. Analysis should begin as soon as possible after receipt at the laboratory. Once extraction has taken place, the resulting extracts may be stored in a spark-proof refrigerator for up to 4 weeks prior to analysis.

B7 Analytical procedure

Step	Procedure	Note
B7.1	**Extraction of samples**	
B7.1.1	Prior to extraction, all samples should be filtered, see note a.	(a) Whatman GFD/GFF filter papers with a nominal pore size of 1 µm have been found suitable. Equivalent glass fibre membrane filters may be used.
B7.1.2	For river waters and final effluents, to a clean 1-litre glass bottle, add 1000 ml of filtered sample. Add 50 µl of mixed deuterated internal standard stock solution (B4.18.4) see note b. Mix well.	(b) The concentration of each of the deuterated internal standards is equivalent to 5 ng l^{-1} (i.e. 5 ng of each deuterated internal standard added).
B7.1.3	For crude sewages, to a clean 1-litre glass bottle, add 200 ml of filtered sample and 800 ml of water (B4.1). Add 50 µl of mixed deuterated internal standard stock solution (B4.18.4) see note c. Mix well.	(c) The concentration of each of the deuterated internal standards in the sample is equivalent to 25 ng l^{-1} (i.e. 5 ng of each deuterated internal standard added).
B7.1.4	Condition a solid phase extraction cartridge (B5.2) see note d, with 5 ml of ethyl acetate (B4.2) followed by 5 ml of methanol (B4.3) followed by 3 ml of water (B4.1) see note e. Pass 800 ml of the spiked filtered sample through the conditioned cartridge, see notes e and f. Rinse the cartridge with 3 ml of aqueous methanol (B4.16) followed by 3 ml of water (B4.1) see notes e and g.	(d) A flow rate of about 15 ml per minute should be used. (e) The cartridge should not be allowed to dry out during each elution stage. (f) A flow rate of about 10 ml per minute should be used. (g) A flow rate of about 20 ml per minute should be used.
B7.2	**Elution**	
B7.2.1	After extracting the sample, dry the cartridge by passing air through it. Elute the cartridge with two quantities of ethyl acetate (B4.2) each of 1.5 ml, and a further 1-ml quantity of ethyl acetate, see	(h) A flow rate of about 2 ml per minute should be used.

notes e and h. Collect the eluate. Using a gentle stream of air or nitrogen (B5.3) evaporate this extract at 45 °C to incipient dryness. Dissolve the residue in 0.25 ml of cyclohexane:propan-2-ol (B4.17). Quantitatively transfer this extract to a vial fitted with a limited-volume insert and cap.

(i) At this stage, extracts can be stored between 2 - 8 °C for up to 4 weeks.

B7.3 Clean-up

B7.3.1 Using the HPLC conditions described in section B5.8 and normal phase chromatography, carry out a clean-up procedure on the eluted sample extract, see note j. Collect the fraction containing the steroids, quantitatively transfer this fraction to a 4-ml vial, and cap, see note i. Using a gentle stream of air or nitrogen (B5.3) at 45 °C evaporate the fraction to incipient dryness. Dissolve the residue in 0.25 ml of dichloromethane (B4. 6). Quantitatively transfer this extract to a vial fitted with a limited-volume insert and cap.

(j) Prior to clean-up of the sample, using these conditions, inject 250 µl of the normal phase standard solution (B4.18.8) and determine the retention time (typically 6 - 8 minutes) of the steroids. Adjust accordingly for sample clean-up.

B7.3.2 Using the GPC conditions described in section B5.9 carry out a clean-up procedure on the dichloromethane extract, see note k. Collect the fraction containing the steroids. Quantitatively transfer the fraction to a 4-ml vial and cap, see note i. Using a gentle stream of air or nitrogen (B5.3) at 45 °C evaporate the fraction to incipient dryness. Dissolve the residue in 0.20 ml of aqueous methanol (B4.11) and transfer this extract to a vial fitted with a limited-volume insert, see note l. The extract is now ready for LC-MS/TOF analysis.

(k) Prior to clean-up of the sample, using these conditions, inject 250 µl of the GPC standard solution (B4.18.9) and determine the retention time (typically 9 - 12 minutes) of the steroids. Adjust accordingly for sample clean-up.

(l) This solution should contain 5 ng of each deuterated internal standard.

B7.4 Blank, AQC and Recovery

B7.4.1 A sample blank (see note m), AQC (B4.18.7) and recovery solution (B4.18.6) should be treated exactly as a sample and taken through the complete analytical procedure described in sections B7.1 - B7.3.

(m) Water (B4.1) or a suitable ground water should be used.

B7.5 LC-MS/TOF analysis

B7.5.1 Transfer the working calibration standard solutions (B4.21) to glass vials, and cap. Using manufacturer's instructions, optimise the operating

(n) For any sample that exceeds the calibration range consideration should

	conditions of the LC-MS/TOF system. Analyse the calibration standard solutions (B4.18.5) blank,AQC, recovery and sample extracts (B7.3.2) see note m. Determine the concentration of steroids in the sample and blank extracts, see note n.	be given to repeating the analysis by taking a smaller volume of sample used for extraction (B7.1.2 or B7.1.3) diluting the sample extract (B7.3.2) or injecting a smaller volume.

B8 Calibration

A calibration graph of the ratio of the peak area of the steroid to the corresponding deuterated internal standard against the concentration of standard injected should be constructed via the data handling system. The original sample concentration should be calculated from the graph taking into account the sample volume extracted, the sample volume injected, and any dilutions that may have been used.

The peak area of each specific steroid should be measured. For each steroid, the response ratio should then be then calculated.

$$\text{Response ratio} = \frac{\text{peak area (D)}}{\text{peak area (IS)}}$$

where:

peak area (D) is the peak area of the steroid,
peak area (IS) is the peak area of the corresponding deuterated internal standard.

Plot the response ratio against the concentration for the calibration standards. Calculate the response ratio of the unknown samples and controls and from the calibration curve calculate the concentration of steroids in the sample.

B9 Confirmation of results

When analysed using negative ionisation, the exact m/z mass units of the three steroids are:-

Oestrone:	269.1547
17β-oestradiol:	271.1703
17α-ethynyl oestradiol:	295.1703

B10 Quality control

The quality of the analysis can be assured via reproducible calibration and testing of the extraction, GPC clean-up and LC-MS/TOF systems. A series of analytical quality control (AQC) samples should be analysed with each batch of samples and monitored, using for example control charts and other quality review procedures.

C The determination of oestrone, 17β-oestradiol and 17α-ethynyl oestradiol in drinking waters using gas chromatography with mass spectrometric detection

This method describes a procedure for the determination of oestrone, 17β-oestradiol and 17α-ethynyl oestradiol in drinking waters using solid phase extraction and gas chromatography with mass spectrometry (GC-MS) for detection and quantification. Performance data have been generated using silanised glassware throughout all the procedures.

C1 Performance characteristics of the method

C1.1	Substances determined	Oestrone, 17β-oestradiol and 17α-ethynyl oestradiol.
C1.2	Type of sample	Drinking waters.
C1.3	Basis of method	Using silanised glassware throughout, the aqueous sample is spiked with deuterated oestrogenic internal standards and extracted using a solid phase extraction cartridge. The extract is cleaned-up using reversed-phase HPLC and then derivatised using N-methyl-N-t-butyldimethyl-silyltrifluoroacetamide (MTBDMSTFA). The derivatised extract is analysed using GC-MS.
C1.4	Range of application	Typically, up to about 20 ng l^{-1} for each steroid in drinking waters.
C1.5	Calibration curve	Calibrations are linear over the range of application of the method.
C1.6	Interferences	Any substance, which is co-extracted under the conditions used, is not removed by the clean-up procedure, exhibits similar chromatographic behaviour to any of the steroids being determined and has the same mass spectral transitions, will interfere.
C1.7	Standard deviation	See Appendix G.
C1.8	Limit of detection	Derivatised MTBDMSTFA steroids: Oestrone: 1.0 ng l^{-1} 17-β-oestradiol: 0.5 ng l^{-1} 17α-ethynyl oestradiol: 0.9 ng l^{-1} (calculated as 5-times the within-batch standard deviation of deionised water spiked at 3 ng l^{-1} for each steroid).

C1.9 Sensitivity	This is instrument dependent.
C1.10 Bias	See Appendix G.

C2 Principle

Using silanised glassware throughout, known amounts of deuterated oestrogenic internal standards are added to 1 litre of the water to be analysed. The spiked sample is then passed through a solid phase extraction cartridge and oestrone, 17β-oestradiol and 17α-ethynyl oestradiol eluted with acetone. After evaporating to dryness, the residue is dissolved in aqueous methanol and cleaned-up on a reversed-phase HPLC column. The appropriate fraction from the HPLC column is collected, evaporated to dryness, and derivatised using N-methyl-N-t-butyldimethyl-silyltrifluoroacetamide (MTBDMSTFA). The derivatised sample is extracted into toluene and injected into a single quadrupole GC-MS using a programmed temperature vaporisation (PTV) injector. The derivatised oestrogen compounds are detected using selected ion monitoring of 3 or 4 characteristic ions and quantification is based on the measurement of a single ion of each compound (as highlighted in Table C2).

C3 Hazards

All of the natural steroids determined are oestrogenically active. It should be assumed that the deuterated internal standards are similarly active. Appropriate precautions should be taken when handling these compounds and solutions of these compounds. In addition, methanol and acetonitrile are toxic and flammable. Toluene, hexane and acetone are flammable. Dichloromethane is narcotic, and dimethyldichlorosilane is flammable. Strong oxidising agents should be avoided with dimethylformamide and ethanethiol.

C4 Reagents

All reagents should be of sufficient purity that they do not give rise to significant interfering peaks in the analysis. Purity should be checked for each batch of materials by running procedural blanks with each batch of samples analysed. Solvents suitable for high performance liquid chromatography or pesticide analysis use and analytical grade materials are normally suitable unless otherwise stated and details of preparation are given where appropriate. To avoid excessive evaporation of solvents, standard solutions should be stored in a refrigerator. However, prior to use, all solutions and solvents should be allowed to reach ambient room temperature before volumetric measurements are made.

C4.1 Water. This should be deionised or of similar quality.

C4.2 Acetonitrile.

C4.3 Methanol.

C4.4 Acetone.

C4.5 Toluene.

C4.6 Hexane.

C4.7 Dichloromethane.

C4.8 Aqueous methanol (30:70 v/v). To a 100-ml volumetric flask, add 70 ± 1 ml of methanol (C4.3). Make to just less than 100 ml with water (C4.1) and mix well. Make to 100 ml with water and mix.

C4.9 Silanising reagent. A solution of 10:90 v/v of dimethyldichlorosilane in dichloromethane is used to deactivate the surfaces of all glassware used in this method. Excess silanising reagent should be washed off with 10:90 v/v methanol:dichloromethane mixture.

C4.11 Derivatising reagent. Add 2.0 ± 0.2 ml of N-methyl-N-t-butyldimethyl-silyltrifluroacetamide (MTBDMSTFA) to 1.0 ± 0.1 ml of dimethylformamide. To this solution, add 12 ± 1 mg of ammonium iodide and 20 ± 2 µl of ethanethiol. Mix well. The prepared reagent may be stored at 20 °C for up to 1 month. The reagent may turn a light-yellow colour on standing, but this does not affect its subsequent use. This reagent may be prepared in a test tube with a ground neck stopper.

C4.12 Ascorbic acid solution. To a 1000-ml volumetric flask, add 4.0 ± 0.2 g of ascorbic acid and dissolve in approximately 900 ml of water (C4.1). Mix well. Make to the mark with water and mix well.

C4.13 Equilin (5 µg ml^{-1} in 70:30 v/v water:methanol).

C4.14 Standard solutions

C4.14.1 Mixed deuterated internal standard stock solution (nominally 1 µg ml^{-1}). The following deuterated internal standards have been used in this method:

d_4-oestrone:
d_4-17β-oestradiol: and
d_4-17α-ethynyl oestradiol.

Prepare a mixed deuterated internal standard stock solution containing d_4-oestrone, d_4-17β-oestradiol and d_4-17α-ethynyl oestradiol, each a concentration of 1 µg ml^{-1} in methanol. This solution may be stored at -18 °C for up to 3 months.

C4.14.2 Mixed steroid standard stock solution (1 µg ml^{-1}). Prepare a mixed steroid standard stock solution containing oestrone, 17β-oestradiol and 17α-ethynyl oestradiol each at a concentration of 1 µg ml^{-1} in methanol. This solution may be stored at -18 °C for up to 3 months.

C4.14.3 Mixed steroid and deuterated internal standard stock solutions. Prepare three solutions, each containing oestrone, 17β-oestradiol, 17α-ethynyl oestradiol and 100 ng ml^{-1} of each of the deuterated internal standards in methanol. The concentration of each of the steroids should be, for example 500, 100 and 10 ng ml^{-1}.

C4.15 Solid phase cartridges. The following extraction cartridges have been used in

this method. Waters Oasis HLB (500 mg/12 ml). Equivalent cartridges may also be used.

C5 Apparatus

All glassware should be silanised before use by rinsing all glass surfaces (which come into contact with steroids and deuterated steroids) with silanising reagent (C4.9) and allowing the glassware to drain. Excess silanising reagent should be washed off with 10:90 v/v methanol:dichloromethane (C4.10) and the glassware allowed to drain.

In addition to general laboratory glassware, the following are required.

C5.1 Analytical balance capable of weighing to 0.0001g.

C5.2 Solid phase extraction apparatus. The manifold should be capable of handling several solid phase extraction cartridges. The flow rate through each individual cartridge should be controlled by a positive pressure peristatic pump or vacuum manifold system.

C5.3 Membrane filters, polytetrafluoroethylene type of nominal pore size of 0.2 µm.

C5.4 Nitrogen blow-down apparatus or equivalent system.

C5.5 Derivatisation system. For example, a glass test tube with a ground glass stopper and a tapered base has been found to be suitable for the derivatisation step.

C5.6 HPLC system for clean-up. The system should be fitted with gradient pump and UV detection. The suitability of the equipment would need to be evaluated.

The following conditions were used to generate performance data.

HPLC equipment:
Column: HP Hypersil ODS, 100 x 4.6 mm, 5 µm, C18 guard column.
Mobile phase: Solvent A: water.
Solvent B: methanol.
Flow rate: 1 ml per minute.
Gradient programme:

Time (minutes)	0	1.5	15	15.1	17.9	18
A:B solvent ratio	70:30	70:30	19:81	0:100	0:100	70:30

Total run time: 25 minutes.
Column temperature: 40 °C.
Injection: 100 µl.
UV detector: 282 and 270 nm.

Equivalent columns and conditions may be used.

C5.7 GC-MS system. The suitability of the equipment would need to be evaluated.

The following conditions were used to generate performance data.

GC equipment:

Column:	ZB 5, 30 m, 250 µm and 0.25 µm thickness.
Gas type:	Helium.
Mode:	Constant flow at 1.1 ml per minute.
Average velocity:	39 cm per second.
Temperature programme:	Initial temperature at 100 ° C for 2.5 minutes, then 30 °C per minute to 260 ° C, 5 °C per minute to 310 °C, hold time for 2 minutes.
Equilibration time:	0.5 minutes.
Maximum temperature:	325 °C.
Injection volume:	10 µl.
Injector:	Gerstel PTV type in solvent vent mode.
Liners:	Straight line packed with deactivated glass wool.
Injector programme:	Initial temperature at 100 °C, for 0.5 minute, then 720 °C per minute to 250 °C.
Vent time:	0.5 minute.
Vent flow:	125 ml per minute.
Vent pressure:	14 psi.
Purge flow:	50 ml per minute.
Purge time:	3.0 minutes.
Total flow:	53.7 ml per minute.
Detector:	Quadrupole MS, electron impact ionisation, 1.96 points per second, detector voltage, 500 v, dwell time, 0.01 second, span:1.00
MS temperature:	150 °C.
MS source:	230 °C.
Transfer line:	280 °C.

C6 Sample collection and storage

Samples should be collected in 1 litre (or 2 litre) silanised glass bottles, with screw cap plastic stoppers fitted with polytetrafluoroethylene liners.

C7 Analytical procedure

Step	Procedure	Note
C7.1	**Extraction**	
C7.1.1	To 1000 ml of drinking water, add 4.0 ± 0.2 ml of ascorbic acid solution (C4.12) and 10.0 ± 0.2 µl of the mixed deuterated internal standard stock solution (C4.14.1) (see note a) and 10 ± 1 ml of methanol (C4.3). Mix thoroughly.	(a) This is equivalent to 1 ng l^{-1} (i.e. 1 ng of each deuterated internal standard added).

C7.1.2 Condition a solid phase extraction cartridge (C4.15) by passing 10 ± 1 ml of acetone (C4.4) through the cartridge followed by 10 ± 1 ml of methanol (C4.3) followed by 10 ± 1 ml of water (C4.1) see notes b and c.

(b) A flow rate of 10 - 15 ml per minute should be used.

(c) The cartridge should not be allowed to dry out during each elution stage.

C7.1.3 Pass the spiked sample through the cartridge, see note b. Wash the cartridge with 20 ± 2 ml of aqueous methanol (C4.8), followed by 20 ± 2 ml of hexane (C4.6) see note c. Discard the washings.

C7.2 Elution

C7.2.1 Elute the cartridge with 20 ml of acetone (C4.4) see note d. Collect the eluate and filter the extract through a membrane filter (C5.3). Collect the filtrate in a suitable vessel and evaporate the filtrate at 60 °C using a stream of oxygen-free nitrogen (C5.4) to approximately 1 ml.

(d) A flow rate of 10 ml per minute should be used.

C7.2.2 Quantitatively transfer the evaporated filtrate to a small vial and rinse the vessel with 1 ml of acetone. Transfer the rinsings to the vial. Evaporate the extract to incipient dryness using a stream of oxygen-free nitrogen (C5.4). Dissolve the residue in 100 µl of aqueous methanol (C4.8).

C7.3 HPLC clean-up

C7.3.1 Using the HPLC system and conditions described in section C5.6, determine the retention time of equilin in the equilin solution (C4.11) see note e. Inject the sample extract from C7.2.2 onto the HPLC column and collect the fraction eluting between approximately ± 5 % of the retention time of equilin, see note f. Transfer the collected eluate to a suitable vessel for derivatisation (C5.5).

(e) Three injections should be made, and the mean retention time calculated. Typically, retention time should be approximately 13 minutes.

(f) Approximately 1.5 ml should be collected.

C7.4 Derivatisation of cleaned-up sample extract

C7.4.1 Using a stream of oxygen-free nitrogen (C5.4) evaporate the cleaned-up extract to incipient dryness at 60 °C, see note g. Add 100 ± 5 µl of derivatising reagent (C4.11) to the vessel, stopper and heat for 1 hour at 100 °C. Allow the vessel and its contents to cool to ambient room

(g) All traces of water should be removed at this stage.

temperature. Add 0.5 ± 0.1 ml of acetonitrile (C4.2) and 0.5 ± 0.1 ml of water (C4.1) and 2 ± 0.2 ml of hexane (C4.6). Mix thoroughly, see note h.

(h) Care should be taken as the pressure may build up and cause the glass stopper to be ejected.

C7.4.2 Allow the layers to separate and transfer the upper hexane layer to a clean tube. Repeat the extraction of the derivatised phase with a further 2-ml quantity of hexane. Combine the hexane fractions.

C7.4.3 Using a stream of oxygen-free nitrogen (C5.4) evaporate the combined hexane extract to dryness at 60 °C. Dissolve the residue in 100 µl of toluene (C4.5) see notes j and k.

(j) The toluene extract may be stored at room temperature for up to 1 month or at -18 °C for up to 3 months.

(k) This solution should contain 1 ng of each deuterated internal standard.

C7.5 Blank

C7.5.1 A sample blank should be treated exactly as a sample and taken through the complete analytical procedure described in sections C7.1 – C7.4.

C7.6 Derivitisation of standards

C7.6.1 Transfer 100 µl of the mixed steroid and deuterated internal stock solutions (C4.14.3) to appropriate derivatisation vessels (C5.5). Using a stream of oxygen-free nitrogen (C5.4) evaporate each portion to incipient dryness at 60 °C, see note g. Add 100 ± 5 µl of derivatising reagent (C4.11) to each vessel, stopper and heat for 1 hour at 100 °C. Allow each vessel and its contents to cool to ambient room temperature. Add 0.5 ± 0.1 ml of acetonitrile (C4.2) and 0.5 ± 0.1 ml of water (C4.1) and 2.0 ± 0.2 ml of hexane (C4.6). Mix thoroughly, see note h.

C7.6.2 Allow the layers to separate and transfer the upper hexane layer to a clean tube. Repeat the extraction of the derivatised phase with a further 2-ml quantity of hexane. Combine the hexane fractions.

C7.6.3 Using a stream of oxygen-free nitrogen (C5.4) evaporate the combined hexane extract to dryness at 60 °C. Dissolve the residue in 100 µl of toluene (C4.5) see note j.

C7.7 Determination of steroids

C7.7.1 Using manufacturer's instructions, optimise the operating conditions of the GC-MS system (C5.7). Analyse the derivatised sample extracts, mixed steroid and deuterated internal stock solutions and blank, and determine the concentration of steroids in the sample and blank extracts.

C8 Calculation of results

C8.1 Confirmation of the identity of the steroid

To confirm the identification of an oestrogenic compound in a sample extract the following criteria should be met.

- The retention time of a peak due to an oestrogenic compound in the sample extract should be within ± 0.5 % of the retention time of the peak of the corresponding deuterated internal standard in the same extract.

- The characteristic ions (see Table C2) should produce a maximum response within the appropriate retention time window. The relative intensities of the ions from an oestrogenic compound, obtained from integrated ion traces without background subtraction and expressed as a percentage of the base peak should correspond, within the tolerances shown in Table C1 with those found for the appropriate deuterated internal standard in the same extract.

- To ensure appropriate performance is achieved, it should be demonstrated that a signal:noise ratio of not less than 3:1 is obtained. If not, the performance of the method may suffer.

- The response of the deuterated internal standard relative to that of the steroids present in the sample (as shown in column one of Table C1) will affect the precision of the calculated concentration in the sample, see column two of Table C1.

Table C1 Tolerances

Relative intensity of an ion in the deuterated standard	Precision for the corresponding natural ion in a sample
more than 20%	± 15%
less than 20 % and more than 10 %	± 20%
less than or equal to 10%	± 50%

C8.2 Calculation of results

Following confirmation by the above criteria, the steroid should be quantified by measurement of a single ion for the compound concerned, see Table C2.

The response ratio of the mixed steroid and deuterated internal standard stock solutions (C4.14.3) closest in value to the corresponding response of the sample extract should be used to quantify the oestrogens present in the sample

Table C2 Ions monitored for MTBDMSTFA derivatised steroids

Compound (associated molecular mass)	Ion used for quantification (m/z)
Oestrone (270)	498
2, 4, 16, 16-d_4-oestrone	501
17β-oestradiol (272)	500
2, 4, 16, 16-d_4-17β-oestradiol	504
17α-ethynyl oestradiol (296)	524
2, 4, 16, 16-d_4-17α-ethynyl oestradiol	528

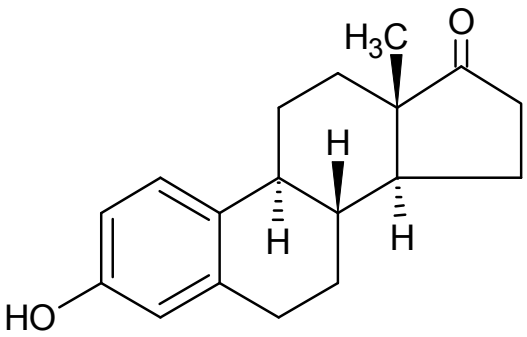
Confirmatory ions may need to be measured, as appropriate.

C9 References

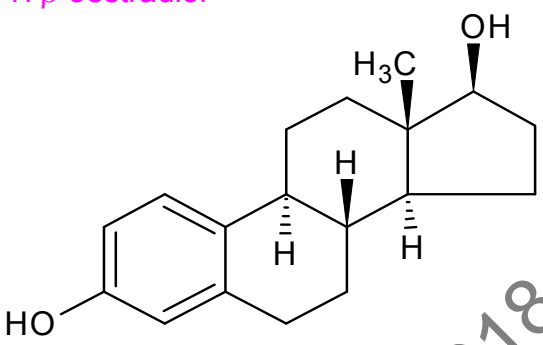
1 “Analysis of Oestrogens in Water: Supervision of Performance Tests” DWI 0836 February 2004.

Appendix A **Structures of common steroids, deuterated steroids and steroid-like compounds**

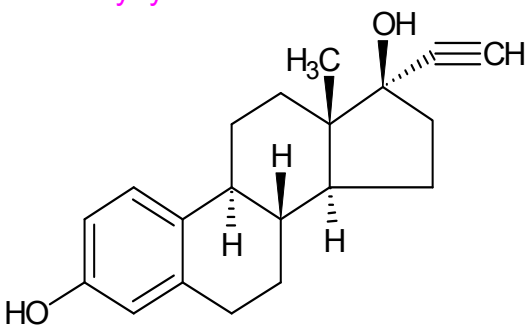
oestrone



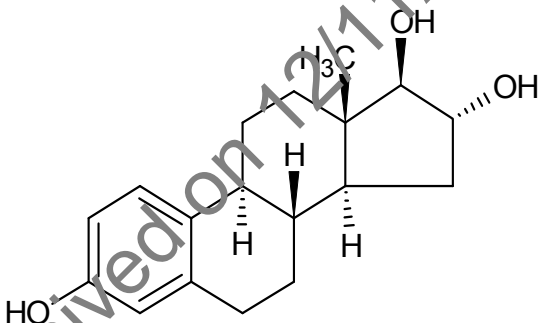
17β-oestradiol



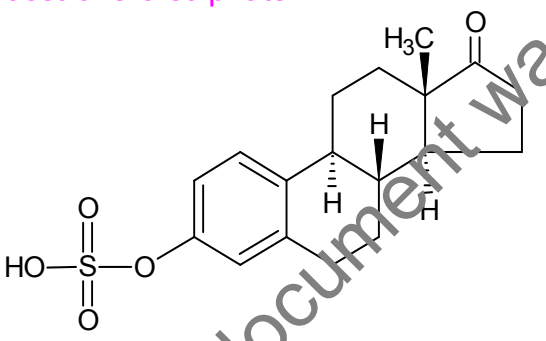
17α-ethynyl oestradiol



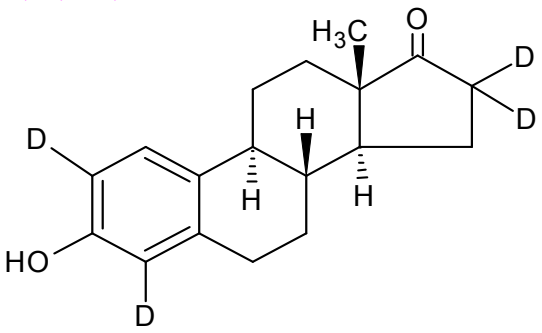
oestriol



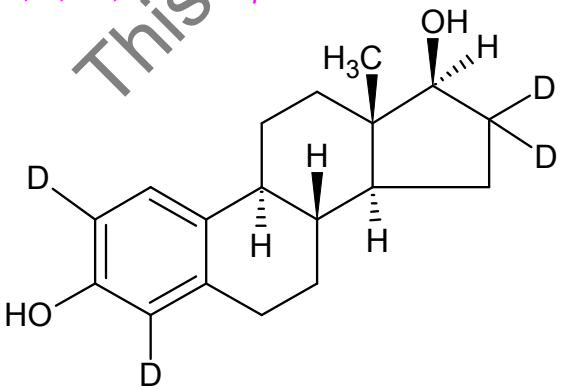
oestrone-3-sulphate



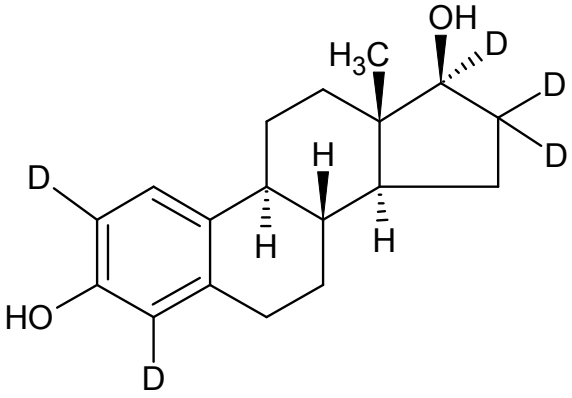
2, 4, 16, 16-d_4-oestrone



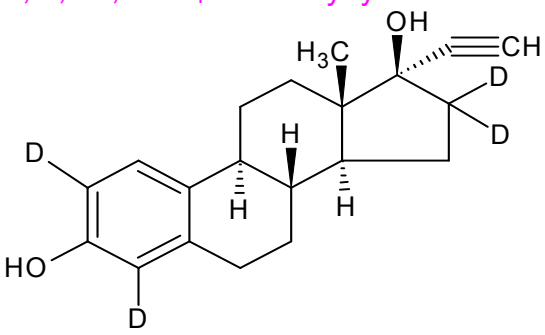
2, 4, 16, 16-d_4-17β-oestradiol



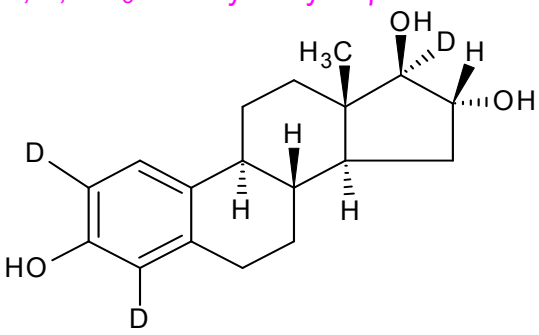
2, 4, 16, 16, 17-d_5-17β-oestradiol



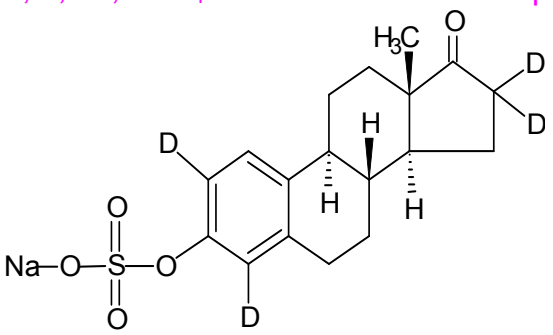
2, 4, 16, 16-d_4-17α-ethynyl oestradiol



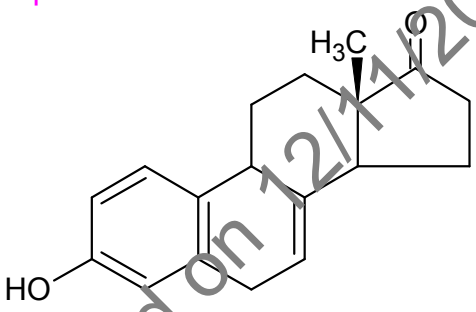
2, 4, 7-d_3-16α-hydroxy-17β-oestradiol



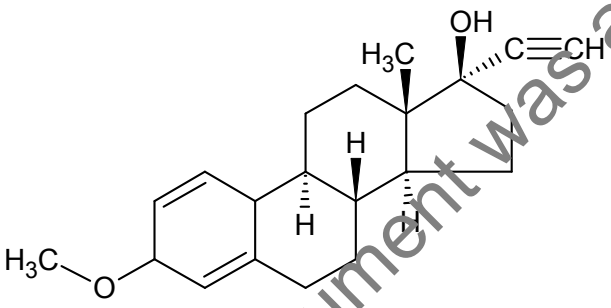
2, 4, 16, 16-d_4-sodium oestrone-3-sulphate



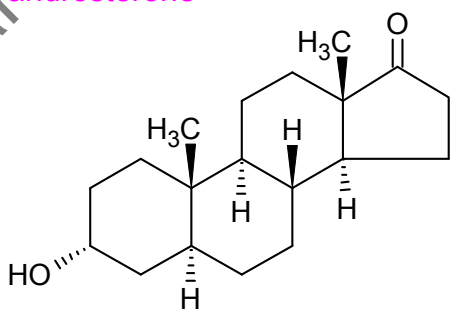
equilin



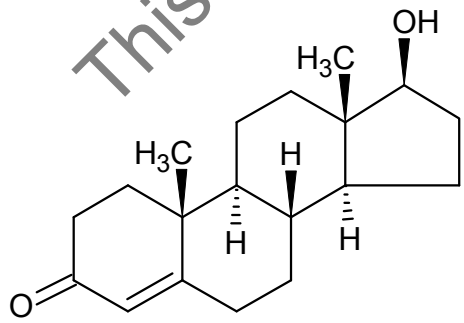
mestranol



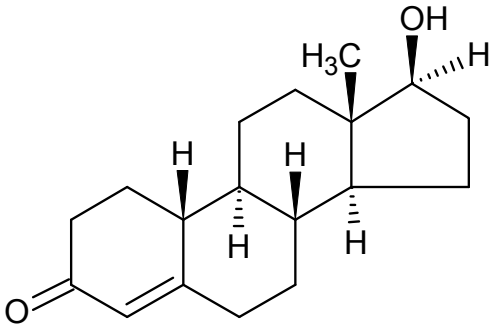
androsterone



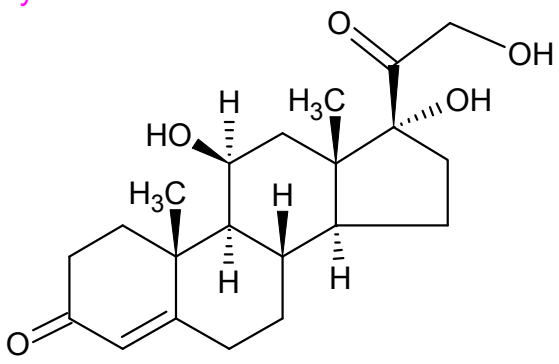
testosterone



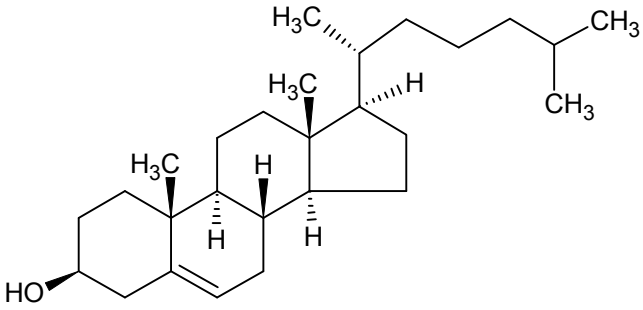
nandrolone



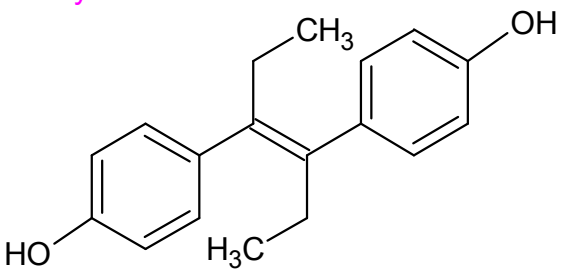
hydrocortisone



cholesterol



diethylstilbesterol

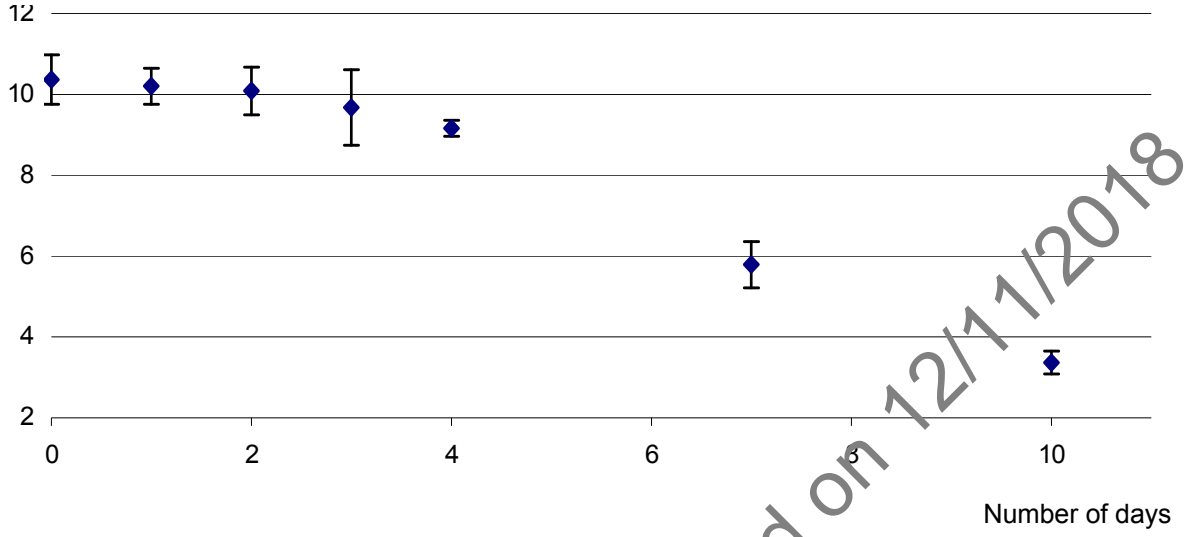


Appendix B Stability data for three oestrogen steroids in final effluent samples

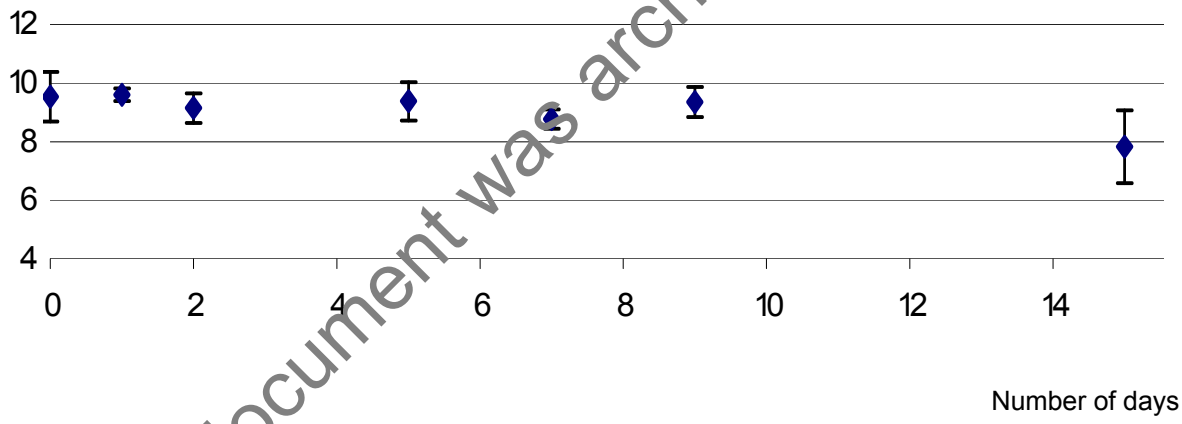
All graphs show the mean value (expressed as ng l^{-1}) and 95 % confidence intervals

B1 Oestrone

10 ng l^{-1} refrigerated final effluent without preservative



10 ng l^{-1} un-refrigerated final effluent with preservative added



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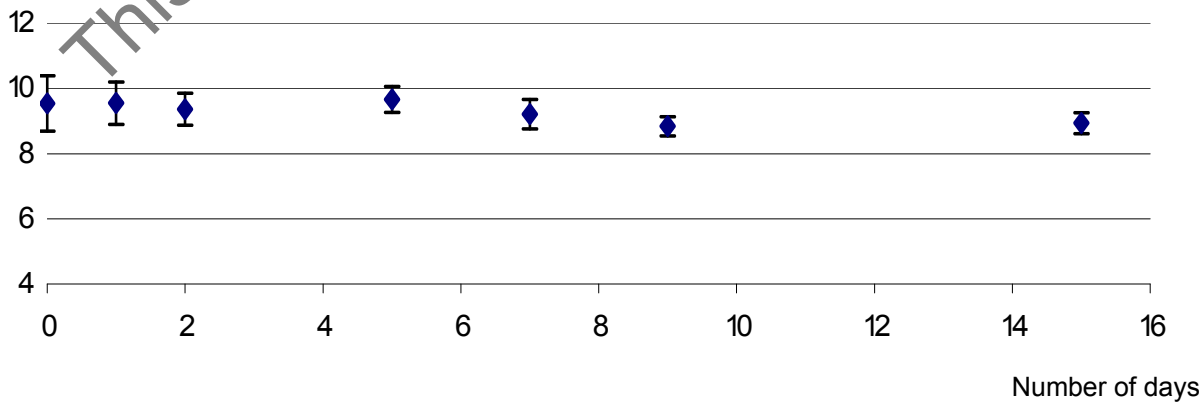
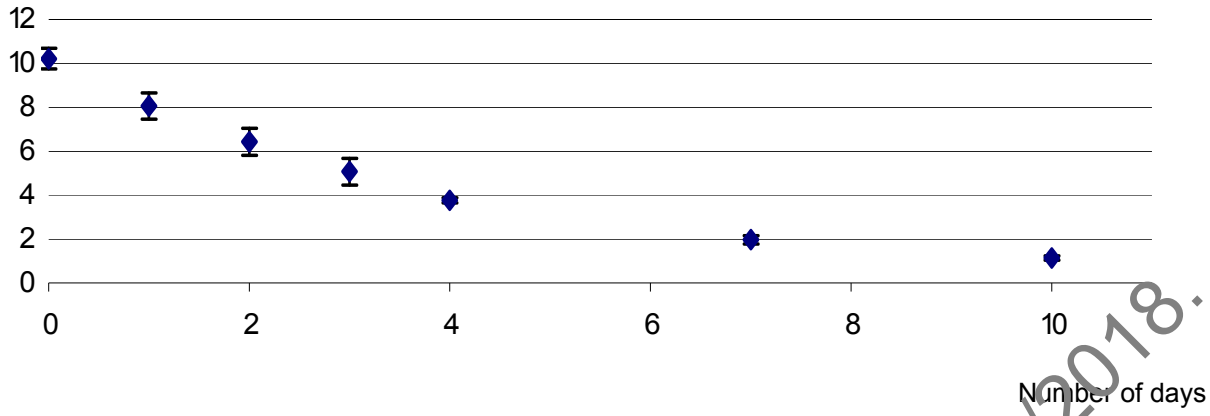
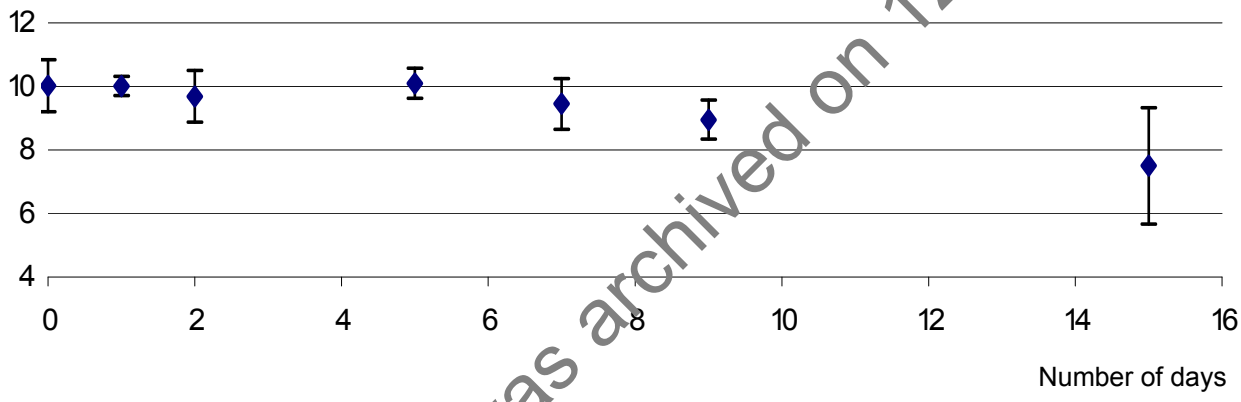


B2 17β-oestradiol

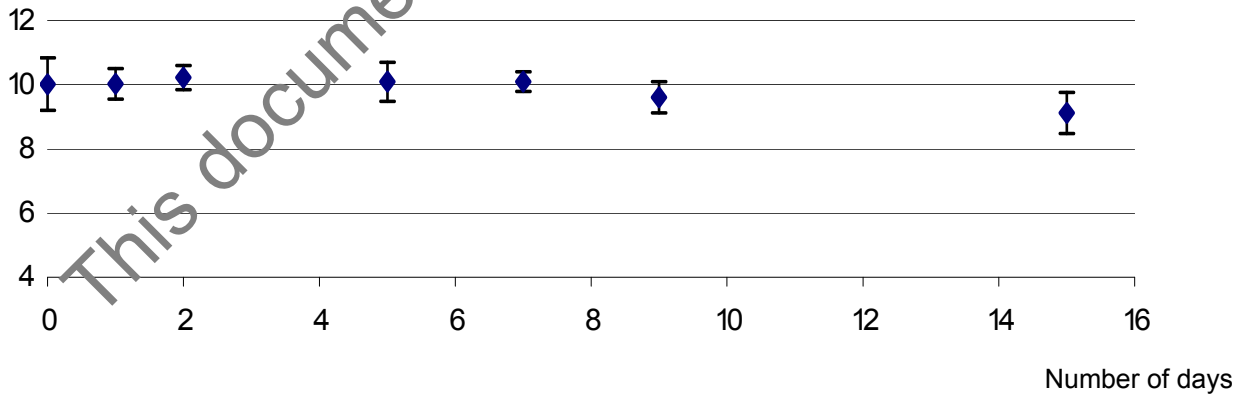
10 ng l^{-1} refrigerated final effluent without preservative



10 ng l^{-1} un-refrigerated final effluent with preservative added

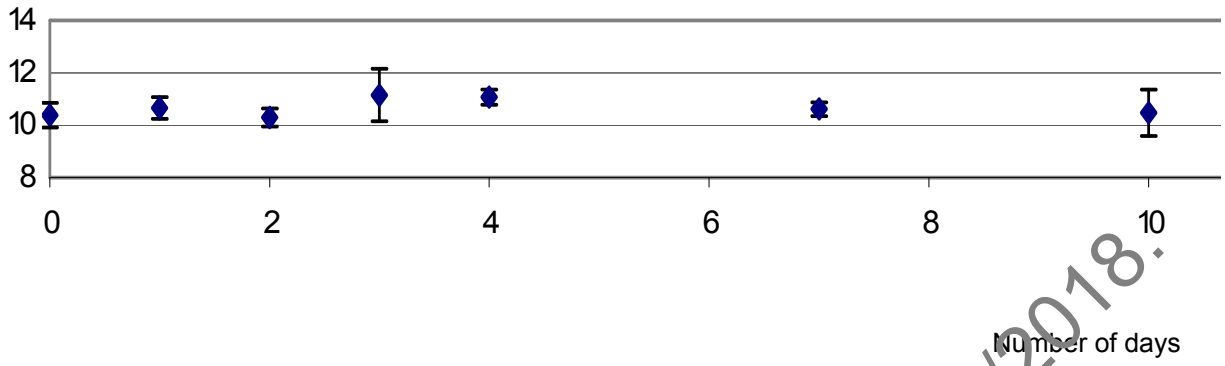


10 ng l^{-1} refrigerated final effluent with preservative added

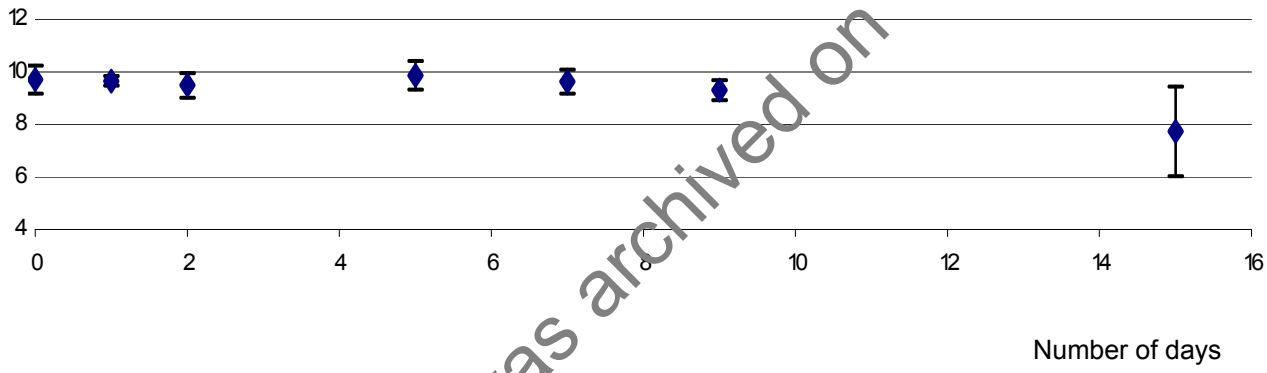


B3 17α-ethynyl oestradiol

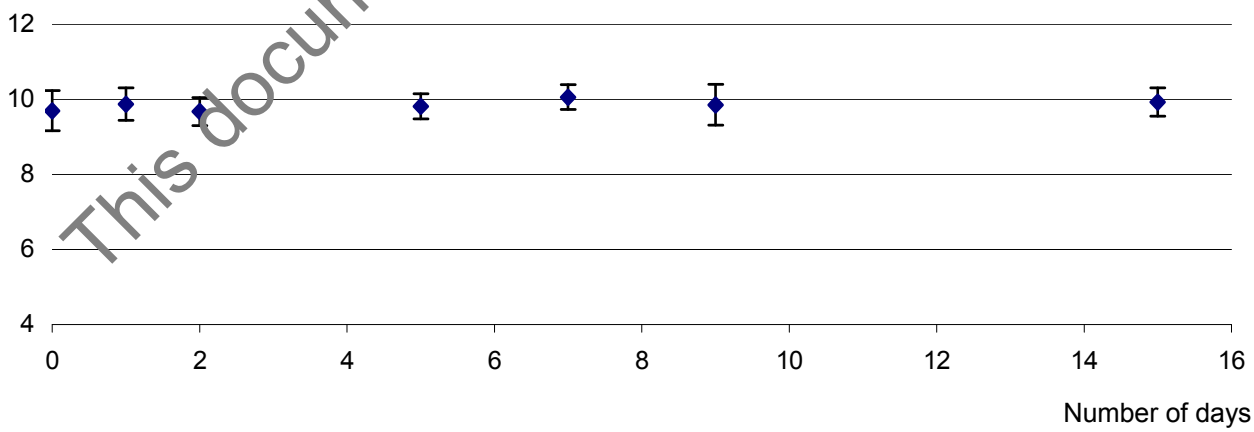
10 ng l^{-1} refrigerated final effluent, without preservative



10 ng l^{-1} un-refrigerated final effluent with preservative added

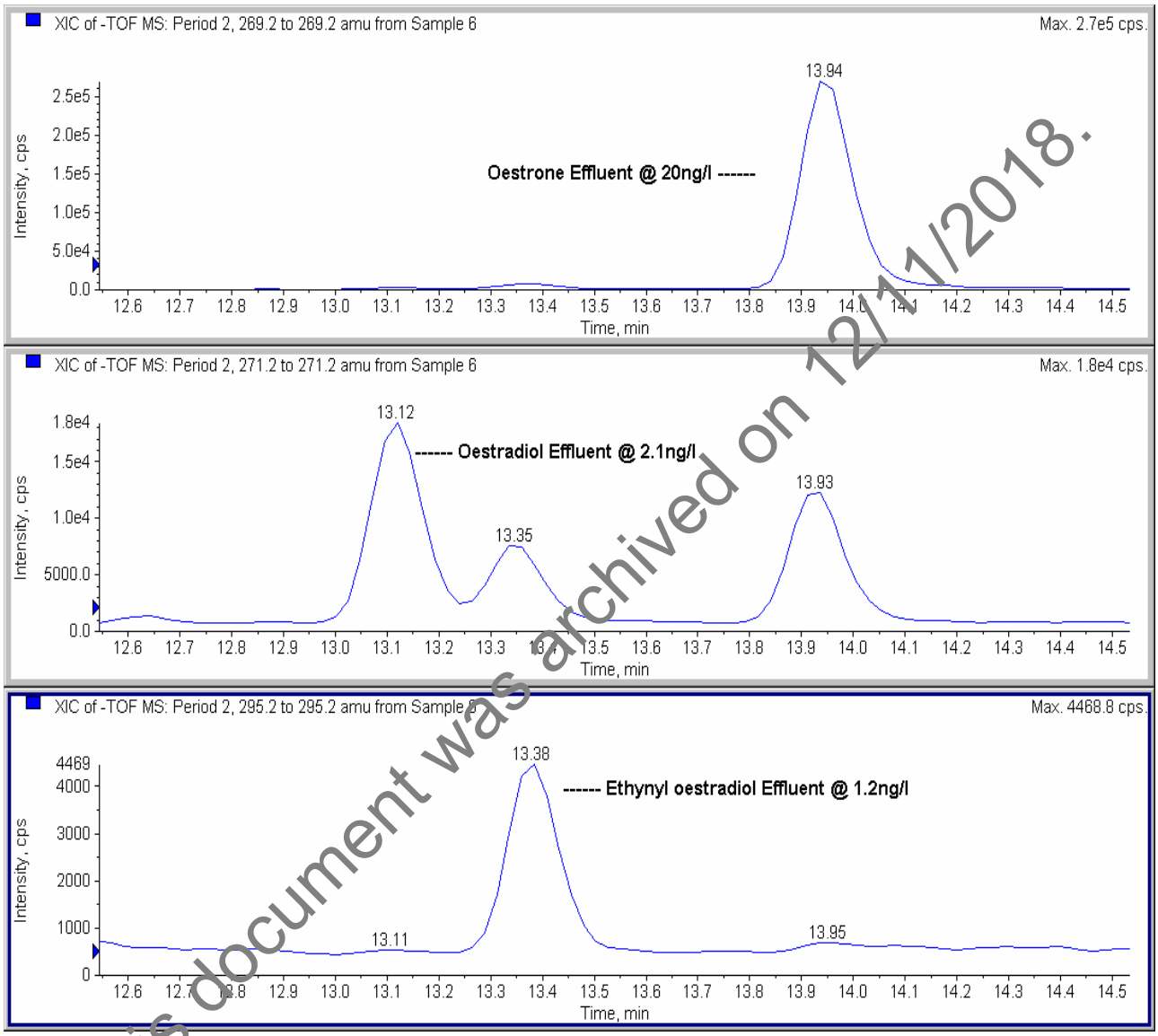


10 ng l^{-1} refrigerated final effluent with preservative added

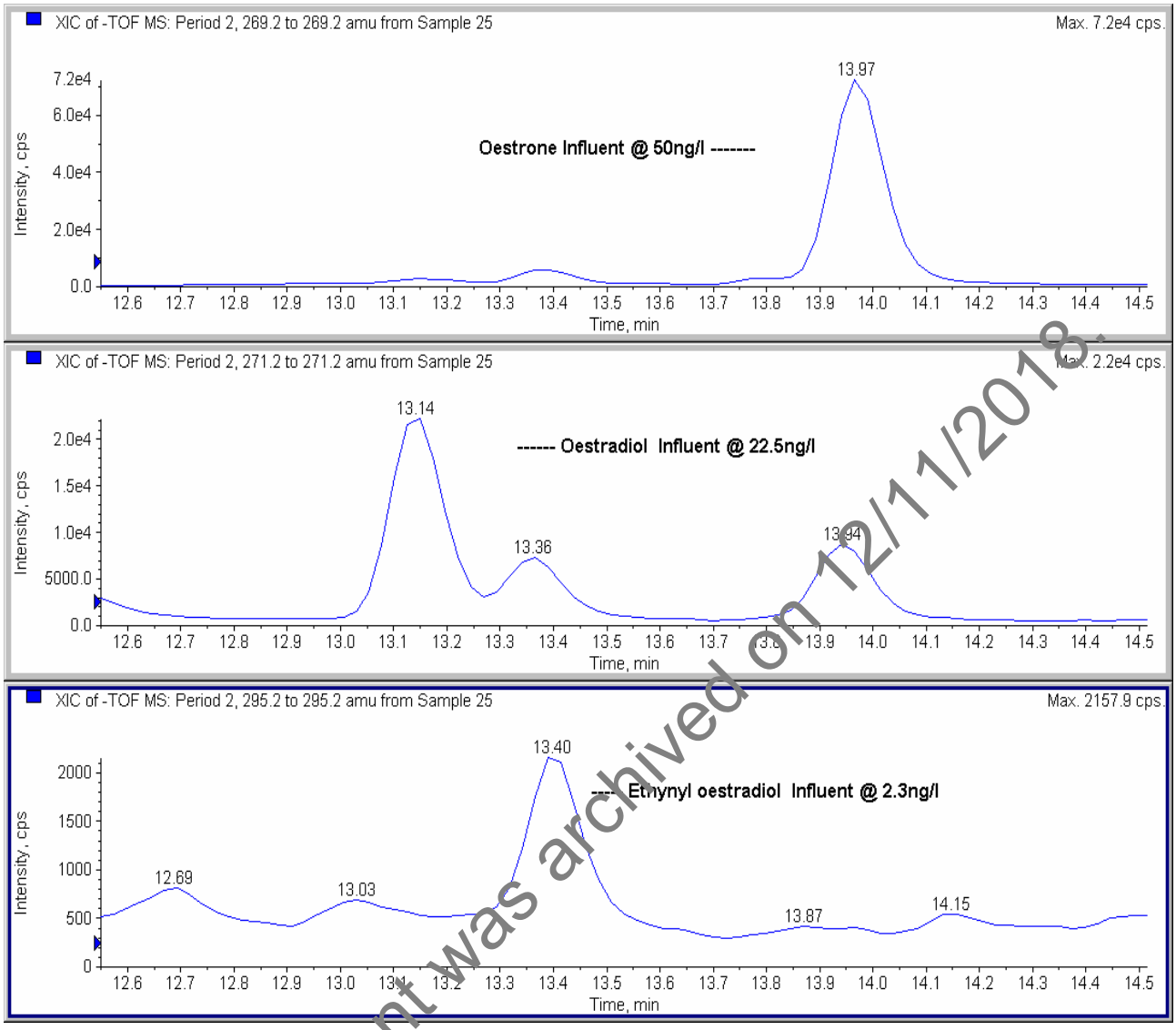


APPENDIX C Typical chromatograms by LC-MS/TOF

C1.1 An un-spiked final effluent from a sewage treatment works



C1.2 An un-spiked influent from a sewage treatment works



Data supplied by Anglian Water Services.

APPENDIX D Summary performance data for the non-derivatised steroids in method A

Sewage influent

	oestrone	17β-oestradiol	17α-ethynyl oestradiol
Crude sewage unspiked			
Number of batches analysed in duplicate	6	6	6
Range	59.2 - 72.5	11.2 - 14.9	1.6 - 3.9
Mean	67.4	13.1	2.4
Total standard deviation	4.3	1.1	0.6
%RSD	6.4	8.7	24.9
Crude sewage plus 10 ng l^{-1} steroids			
Number of batches analysed in duplicate	6	6	6
Range	71.3 - 82.3	20.4 - 23.8	9.2 - 12.8
Mean	77.7	22.0	11.6
Total standard deviation	4.5	1.1	1.5
%RSD	5.8	5.1	12.6
% Recovery	104	89	93
Crude sewage plus 40 ng l^{-1} steroids			
Number of batches analysed in duplicate	6	6	6
Range	102.0 - 112.0	44.7 - 55.8	36.4 - 47.6
Mean	107.8	50.4	40.6
Total standard deviation	3.6	3.6	3.9
%RSD	3.3	7.1	9.5
% Recovery	101	93	96

Sewage effluent

	oestrone	17β-oestradiol	17α-ethynyl oestradiol
STW effluent unspiked			
Number of batches analysed in duplicate	6	4	4
Range	1.27 – 3.36	0.40 – 0.62	0.82 – 1.17
Mean	1.6	0.5	1.1
Total standard deviation	0.6	0.1	0.1
%RSD	34.3	15.5	10.9
STW effluent plus 1 ng l^{-1} steroids			
Number of batches analysed in duplicate	6	6	6
Range	2.21 – 2.76	1.06 – 1.66	1.15 – 2.03
Mean	2.4	1.4	1.8
Total standard deviation	0.2	0.2	0.3
%RSD	6.9	12.3	17.0
% Recovery	81	82	70
STW effluent plus 4 ng l^{-1} steroids			
Number of batches analysed in duplicate	6	6	6
Range	4.45 - 5.46	3.51 - 4.96	4.13 – 5.48
Mean	5.0	4.2	4.8
Total standard deviation	0.4	0.4	0.4
%RSD	7.5	8.6	8.6
% Recovery	85	92	93

River water

	oestrone	17β-oestradiol	17α-ethynyl oestradiol
River water unspiked			
Number of batches analysed in duplicate	6	4	4
Range	1.76 – 4.13	0.51 – 0.74	0.67 – 1.99
Mean	2.4	0.6	1
Total standard deviation	0.7	0.1	0.4
%RSD	29.0	13.0	43.6
River water plus 1 ng l^{-1} steroids			
Number of batches analysed in duplicate	6	6	6
Range	2.83 – 3.56	1.23 – 2.59	0.93 – 2 05
Mean	3.1	1.6	1.6
Total standard deviation	0.2	0.4	0.4
%RSD	7.1	25.8	24.7
% Recovery	67	99	63
River water plus 4 ng l^{-1} steroids			
Number of batches analysed in duplicate	6	6	6
Range	5.67 - 6.40	3.86 – 4.53	3.75 – 4.91
Mean	6.1	4.2	4.6
Total standard deviation	0.3	0.2	0.3
%RSD	4.5	5.3	6.8
% Recovery	92	90	90

All concentrations expressed as ng l^{-1}.

Data provided by Severn Trent Laboratories.

APPENDIX E Summary performance data for the dansyl derivatised steroids in method A

Sewage influent

	oestrone	17β-oestradiol	17α-ethynyl oestradiol
Crude sewage unspiked			
Number of batches analysed in duplicate	6	6	6
Range	19.9 – 30.1	2.28 – 4.11	0.330 – 1.570
Mean	24.9	3.3*	0.7
Total standard deviation	3.6	0.6	0.4
%RSD	14.6	19.1	58.4
Crude sewage plus 20 or 10 ng l^{-1} steroids	(20)	(20)	(10)
Number of batches analysed in duplicate	6	6	6
Range	32.4 – 51.7	11.5 – 26.4	5.64 – 11.70
Mean	41.4	20.8	8.7
Total standard deviation	6.2	5	2
%RSD	14.9	23.9	22.8
% Recovery	83	88	81
Crude sewage plus 100 or 50 ng l^{-1} steroids	(100)	(100)	(50)
Number of batches analysed in duplicate	6	6	6
Range	100.0 – 143.0	53.0 – 122.0	30.1 – 59.4
Mean	119.8	94.8	44.0
Total standard deviation	15	22.3	9
%RSD	12.5	23.5	20.5
% Recovery	95	92	87

* one outlier rejected.

Sewage effluent

	oestrone	17β-oestradiol	17α-ethynyl oestradiol
STW effluent unspiked			
Number of batches analysed in duplicate	6	6	6
Range	4.50 – 6.99	0.279 – 1.020	0.171 – 0.299
Mean	5.83	0.49	0.24**
Total standard deviation	0.84	0.25	0.05
%RSD	14.5	50.8	19.0
STW effluent plus 2 or 1 ng l^{-1} steroids	(2)	(2)	(1)
Number of batches analysed in duplicate	6	6	6
Range	4.98 – 10.60	1.07 – 2.60	0.57 – 1.37
Mean	7	1.9	1.1
Total standard deviation	1.5	0.4	0.2
%RSD	21.4	22.2	20.1
% Recovery	58	72	88
STW effluent plus 8 or 4 ng l^{-1} steroids	(8)	(8)	(4)
Number of batches analysed in duplicate	6	6	6
Range	8.57 – 18.7	4.82 – 8.80	2.86 – 4.76
Mean	13.2	7.3	3.6
Total standard deviation	3.1	1.3	0.9
%RSD	23.4	17.9	23.4
% Recovery	92	85	85

** two outliers rejected.

River water

	oestrone	17β-oestradiol	17α-ethynyl oestradiol
River water unspiked			
Number of batches analysed in duplicate	6	6	6
Range	1.92 – 4.15	0.385 – 1.06	0.316 – 1.260
Mean	3.1	0.6**	0.8**
Total standard deviation	0.8	0.2	0.3
%RSD	26.6	43.8	35.3
River water plus 2 or 1 ng l^{-1} steroids	(2)	(2)	(1)
Number of batches analysed in duplicate	6	6	6
Range	3.05 – 7.11	1.27 – 3.79	1.11 – 2.00
Mean	4.8	2.1	1.5**
Total standard deviation	1.2	0.7	0.3
%RSD	25.8	31.2	16.9
% Recovery	87	77	65
River water plus 12 or 6 ng l^{-1} steroids	(12)	(12)	(6)
Number of batches analysed in duplicate	6	6	6
Range	7.78 – 18.70	3.23 – 14.8	3.65 – 9.31
Mean	13.2	10.2	6.1
Total standard deviation	3.6	2.9	1.7
%RSD	27.1	28.7	27.6
% Recovery	85	80	89

** two outliers rejected.

All concentrations expressed as ng l^{-1}.

Data provided by Environment Agency's National Laboratory Service.

Appendix F Performance data for method B using LC-MS/TOF

Standard solution

	oestrone	17β-oestradiol	17α-ethynyl oestradiol
Standard solution at 0.1 ng l^{-1} steroids			
Degrees of freedom	19	16	19
Mean	0.1	0.1	0.1
Total standard deviation	0.01	0.02	0.01
%RSD	11.1	14.9	11.9
% Bias	-2.4	+3.8	+0.1
Limit of detection	0.1	0.1	0.1
Standard solution at 2.0 ng l^{-1} steroids			
Degrees of freedom	12	16	15
Mean	1.9	1.9	2
Total standard deviation	0.2	0.2	0.2
%RSD	10.6	11.9	9.5
% Bias	-5.5	-2.8	0.0
Standard solution at 20.0 ng l^{-1} steroids			
Degrees of freedom	18	18	16
Mean	19.5	20.7	19.6
Total standard deviation	2	1.9	2.1
%RSD	10.2	9.0	10.6
% Bias	-2.9	+3.3	-1.9

River water

	oestrone	17β-oestradiol	17α-ethynyl oestradiol
River water spiked at 0.1 ng l^{-1} steroids			
Degrees of freedom	12	15	17
Mean	0.5	0.3	0.2
Total standard deviation	0.03	0.03	0.01
%RSD	6.2	9.5	8.2
Limit of detection	0.1	0.1	0.1
River water spiked at 2.0 ng l^{-1} steroids			
Degrees of freedom	14	12	13
Mean	2.3	2.4	2.1
Total standard deviation	0.2	0.2	0.1
%RSD	8.8	9.1	5.9
% Recovery	94	109	102
River water spiked at 20.0 ng l^{-1} steroids			
Degrees of freedom	13	17	11
Mean	18.4	20.1	19.9
Total standard deviation	1.98	1.45	1.58
%RSD	10.8	7.2	8.0
% Recovery	89	99	99

All concentrations expressed as ng l^{-1}.

Final effluent

	oestrone	17β-oestradiol	17α-ethynyl oestradiol
Final effluent unspiked			
Degrees of freedom	9	17	16
Mean	5.45	1.3	0.37
Total standard deviation	0.75	0.17	0.04
%RSD	13.7	13.1	10.2
Final effluent spiked at 2.5 ng l^{-1} steroids			
Degrees of freedom	10	13	12
Mean	8	4	2.8
Total standard deviation	0.85	0.46	0.24
%RSD	10.6	11.5	8.4
% Recovery	102	109	97
Final effluent spiked at 15.0 ng l^{-1} steroids			
Degrees of freedom	17	18	12
Mean	19.3	16.4	14.6
Total standard deviation	1.64	1.44	1.02
%RSD	8.5	8.8	7.0
% Recovery	92	100	95

Crude sewage

	oestrone	17β-oestradiol	17α-ethynyl oestradiol
Crude sewage unspiked			
Degrees of freedom	11	9	12
Mean	76.2	40.6	2.5
Total standard deviation	8.66	7.54	0.25
%RSD	11.4	18.6	10.1
Crude sewage spiked at 25 ng l^{-1} steroids			
Degrees of freedom	9	9	11
Mean	101.5	66.1	26.2
Total standard deviation	12.1	8.8	2.2
%RSD	11.9	13.3	8.5
% Recovery	101	102	95

All concentrations expressed as ng l^{-1}.
The limit of detection was calculated from the within batch standard deviations.
Data provided by Anglian Water.

Appendix G Performance data for derivatised MTBDMSTFA-steroids in method C

Drinking water Details of all raw data obtained in this inter-laboratory exercise can be found elsewhere[1], see section C9.

Laboratory 1

	oestrone	17β-oestradiol	17α-ethynyl oestradiol
Drinking water unspiked			
Number of batches analysed in duplicate	11	11	11
Range	0.04 – 0.79	0.01 – 0.48	0.07 – 1.14
Mean	0.2	0.1	0.4
Total standard deviation	0.2	0.1	0.3
%RSD	102	108	68
Drinking water plus 3 ng l^{-1} steroids			
Number of batches analysed in duplicate	11	11	11
Range	4.36 – 5.51	2.84 – 4.28	3.53 – 4.94
Mean	4.9	3.4	4.2
Total standard deviation	0.4	0.4	0.4
%RSD	8.0	12.0	10.4
% Recovery	158	110	127
Drinking water plus 10 ng l^{-1} steroids			
Number of batches analysed in duplicate	11	11	11
Range	12.63 – 17.42	7.8 – 13.75	8.2 – 15.08
Mean	14.4	10.1	11.2
Total standard deviation	1.4	1.5	1.6
%RSD	9.4	14.4	14.7
% Recovery	142	100	108

Laboratory 2

	oestrone	17β-oestradiol	17α-ethynyl oestradiol
Drinking water unspiked			
Number of batches analysed in duplicate	11	11	11
Range	0.48 – 2.57	0.06 – 1.47	0.04 – 1.17
Mean	1.3	0.34	0.21
Total standard deviation	0.67	0.34	0.24
%RSD	52	99	114
Drinking water plus 3 ng l^{-1} steroids			
Number of batches analysed in duplicate	11	11	11
Range	3.14 – 4.26	2.37 – 3.24	2.33 – 4.31
Mean	3.78	2.77	3.13
Total standard deviation	0.33	0.24	0.44
%RSD	8.8	8.8	14.1
% Recovery	83	81	97
Drinking water plus 10 ng l^{-1} steroids			
Number of batches analysed in duplicate	11	11	11
Range	8.19 – 10.89	7.4 – 10.08	6.98 – 9.82
Mean	9.38	8.58	8.81
Total standard deviation	0.72	0.76	0.89
%RSD	7.7	8.9	10.1
% Recovery	81	82	86

Laboratory 3

	oestrone	17β-oestradiol	17α-ethynyl oestradiol
Drinking water unspiked			
Number of batches analysed in duplicate	2	2	
Range	-0.05 – 1.85	0.13 – 3.09	
Mean	0.56	0.98	
Total standard deviation	0.9	1.41	
%RSD	162	144	
Drinking water plus 3 ng l^{-1} steroids			
Number of batches analysed in duplicate	7	10	7
Range	2.91 – 5.48	2.81 – 4.04	2.78 – 4.58
Mean	3.76	3.19	3.33
Total standard deviation	0.72	0.28	0.53
%RSD	19.3	8.8	15.8
% Recovery	106.7	73.7	
Drinking water plus 10 ng l^{-1} steroids			
Number of batches analysed in duplicate	10	11	10
Range	7.67 – 19.39	8.68 –14.17	8.67 – 14.19
Mean	13.61	10.99	10.79
Total standard deviation	4.17	1.58	1.59
%RSD	30.6	14.4	14.8
% Recovery	131	100	

All concentrations expressed as ng l^{-1}.

Data provided by Horse Forensic Laboratory, Severn Trent (Bridgend) and United Utilities

Combining results from the three laboratories gives:

	oestrone	17β-oestradiol	17α-ethynyl oestradiol
Drinking water *			
BLS or (BLS) as appropriate	0.678	(0.208)	0.186
BBS	0.381	-	0.145
WBS or (WLS) as appropriate	0.365	(0.409)	0.156
Mean	0.51	0.018	0.020
Expanded uncertainty (k=2)	1.72	0.92	0.57
Drinking water plus 3 ng l^{-1} steroids			
BLS or (BLS) as appropriate	0.648	0.316	0.645
BBS	0.197	0.224	0.147
WBS or (WLS) as appropriate	0.455	0.206	0.386
Mean	3.73	2.90	3.33
Expanded uncertainty (k=2)	1.63	0.88	1.53
Recovery (%)	107	90.7	104
Drinking water plus 10 ng l^{-1} steroids			
BLS or (BLS) as appropriate	(2.662)	1.171	1.232
BBS	-	0.742	0.748
WBS or (WLS) as appropriate	(2.549)	1.100	1.194
Mean	11.97	9.67	10.03
Expanded uncertainty (k=2)	7.37	3.54	3.74
Recovery (%)	115	94.9	98.3

* source - River Thames.

Where a between batch effect was present: (this would suggest the value is statistically significant)

BLS = Between laboratory standard deviation
BBS = Within laboratory between batch standard deviation
WBS = Within laboratory within batch standard deviation

Where a between batch effect was not present: (this would suggest the value is not statistically significant)

(BLS) = Between laboratory standard deviation
(WLS) = Within laboratory standard deviation

The expanded uncertainty is expressed as twice the square root of the sum of the between laboratory variance and within laboratory variance or variances.

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