Unifying Analytical Methods for the Determination of Oestrone, Oestradiol and Ethinyl Oestradiol in Water

Final Report to the Department of the Environment, Transport and the Regions

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UNIFYING ANALYTICAL METHODS FOR THE DETERMINATION OF OESTRONE, OESTRADIOL AND ETHINYL OESTRADIOL IN WATER

EXECUTIVE SUMMARY

Two methods to determine oestrone, 17β-oestradiol and 17α-ethinyl oestradiol (as free steroids) in waters were separately developed by the CEFAS laboratory at Burnham-on-Crouch and WRc in 1996 and 1997 respectively. The method produced by CEFAS, involving solid phase extraction, a clean-up step and GCMS (gas chromatography-mass spectrometry) analysis, was designed to determine these three compounds in sewage effluents and river waters, but its performance had not been assessed. WRc developed a method for drinking waters. This involved solid phase extraction, derivatisation, and analysis by GCMS/MS. Performance testing demonstrated that the detection limits for oestrone and 17β-oestradiol were 0.2 ng l^{-1}, and 0.4 ng l^{-1} for ethinyl oestradiol.

The Drinking Water Inspectorate invited tenders to unify the existing analytical methodology and to undertake limited performance testing using the chosen method. The proposal submitted by WRc, in which it was proposed that both WRc and CEFAS should be involved, was accepted and work commenced in August 1997.

During initial discussions it was decided to attempt to modify the method developed by WRc, by incorporating a clean-up step following the derivatisation of the steroids. This was designed to remove excess derivatising agent and by-products which were thought to be responsible for problems during the GC stage of the GCMS/MS analysis. Following experimental work both CEFAS and WRc concluded that there was no advantage to be gained from the clean-up steps that had been investigated and it was decided to proceed to performance test the WRc GCMS/MS method with river waters.

The results of the performance testing suggest that the limits of detection for the three steroids of interest in river waters are about 0.4 - 0.6 ng l^{-1}, with both laboratories reporting similar results. If these limits of detection are acceptable, more comprehensive performance testing (e.g. to the criteria currently accepted by the Standing Committee of Analysts) should be undertaken. The analytical method has been written in "Blue Book" format in the Appendix to this report.

A preliminary investigation of the stability of oestrone, 17β-oestradiol and 17α-ethinyl oestradiol in river waters, at a spiking level of about 10 ng l^{-1}, suggests that there is some interconversion of oestrone and 17β-oestradiol within less than ten days, and that within twenty days all of these compounds are significantly degraded.

CONTENTS Page

APPENDICES

LIST OF TABLES

1. INTRODUCTION

Under a contract jointly funded by the Department of the Environment, and UK Water Industry Research Ltd, and managed by the Drinking Water Inspectorate , an analytical method to determine oestrone, 17β-oestradiol and 17α-ethinyl oestradiol (as free steroids) in drinking water was developed by WRc in 1996-97. The method involved solid phase extraction, derivatisation to form t-butyldimethylsilyl (TBDMS) derivatives and analysis using gas chromatography-mass spectrometry/mass spectrometry (GCMS/MS). This latter technique involves monitoring the breakdown of a so-called parent ion to give a daughter ion and provides increased specificity compared to GCMS, as a response is only obtained when both the parent and daughter ions are simultaneously detected. Compounds giving rise to the parent ion, but not the daughter ion are not detected. Three isotopically-labelled internal standards (d_4-oestrone, d_4-17β-oestradiol and d_4-17α-ethinyl oestradiol) were used for quantification and to compensate for any variation in GC retention times. This latter effect had caused problems with certainty of detection when using an earlier GCMS method developed by WRc for these compounds.

Performance testing provided detection limits of 0.2 ng l^{-1} for oestrone and 17β-oestradiol and 0.4 ng l^{-1} for 17α-ethinyl oestradiol in drinking waters.

Work carried out by CEFAS (previously the MAFF fisheries laboratory) for the Environment Agency during 1994-96 to determine oestrogenically active compounds in treated sewage effluents led to the development of a method based on solid phase extraction, a clean-up step and GCMS (without derivatisation) for determining oestrone, 17β-oestradiol and 17α-ethinyl oestradiol again, as free steroids. The performance of this method was not established.

Because of general concern regarding the concentrations of these three steroids in environmental waters, a meeting involving interested parties was held at the Department of the Environment in June 1997, and it was agreed that further work was necessary to produce a single analytical method which could be applied to the various matrices of interest (drinking waters, river waters, estuarine waters and treated sewage effluents) and that the performance of any modified methodology needed to be assessed for these matrices.

In July 1997, the Department of the Environment, Transport and the Regions invited tenders to unify existing methodology for the determination of oestrone, 17β-oestradiol and 17α-ethinyl oestradiol in river waters. The proposal submitted by WRc, in which it was proposed that both WRc and CEFAS would be involved, was accepted by the Department and work commenced in August 1997.

2. OBJECTIVES AND WORK PROGRAMME

The objective of the work was to combine the analytical methodology developed by WRc for drinking and river waters with that developed by CEFAS for treated sewage effluents, to provide a method which could be applied to the various matrices of interest.

The work programme agreed was as follows:

- CEFAS analytical staff would familiarise themselves with the WRc methodology;
- both CEFAS and WRc would investigate clean-up steps following the derivatisation of extracts, prior to GCMS/MS;
- following agreement between CEFAS and WRc as to the optimal clean-up procedure, a large sample (about 150 litres) of R. Thames water would be collected by WRc. This would be filtered and split between CEFAS and WRc. A decision as to whether to spike these samples prior to distribution would be taken following some preliminary stability testing. Each laboratory would analyse six batches of duplicate samples, spiked at an appropriate level with each of the steroids of interest, and the results examined for (i) comparability and (ii) to establish whether the method was satisfactory for further performance testing;
- as different GCMS/MS instruments were to be used by CEFAS and WRc for the analysis, their performance would be assessed relative to the overall analytical requirements;
- if it appeared that a clean-up procedure provided considerable improvement with respect to removal of interferences, the utility of GCMS would be investigated, to assess likely detection limits;
- provided the fully developed method appeared to be satisfactory, it would be written up in "Blue Book" format;
- recommendations would be made as to the design of further performance tests.

3. INVESTIGATION OF CLEAN-UP STEPS AND PRELIMINARY STABILITY TESTING

Following a meeting between WRc and CEFAS it was agreed that WRc would investigate the use of silica cartridges for clean-up, while CEFAS would use Florisil® cartridges and, time permitting, other types of cartridges. WRc would carry out some preliminary stability testing prior to taking a decision on how best to spike the river water samples which were to be used for the limited performance testing, i.e. if the steroids of interest are stable in river water then a bulk river water sample could be spiked, however, if the steroids are not stable over a period of at least two weeks, then individual water samples would need to be spiked immediately prior to analysis.

3.1 Clean-up using silica cartridges

17β-Oestradiol (100 µg) was derivatised to form the TBDMS derivative, as previously described (James *et al.* 1997). As TBDMS-17β-oestradiol is the most polar of the three steroid TBDMS derivatives it was considered unnecessary to include the other steroid derivatives for this investigation. The derivative was dissolved in 14.2 ml of diethyl ether to give a solution containing nominally 10 µg ml^{-1}, and 100 µl of this solution diluted to 10 ml with diethyl ether to give a solution containing 100 ng ml^{-1}. 1 ml of this solution was loaded onto a silica cartridge (500 mg; Isolute SI) which had been conditioned using diethyl ether. The diethyl ether eluting from the cartridge during this loading step was retained as "Fraction 1". Five further 2 ml portions of diethyl ether were sequentially added to the cartridge, and on each occasion the diethyl ether eluting from the cartridge were retained (Fraction 2 through to Fraction 6). Finally, 2 ml of methanol was added to the cartridge and the eluent retained (Fraction 7). Fractions 1-6 were reduced in volume to 20 µl and each concentrated fraction examined by GCMS in full scan mode. Fraction 7 was taken to dryness and the residue dissolved in diethyl ether (20 µl) prior to examination by GCMS. Only Fraction 2 contained the TBDMS derivative of 17β-oestradiol, and although fewer additional peaks were detected than in the solution prior to the clean-up, it was estimated that the recovery of the derivative was only about 50%. It was considered that no advantage was gained using this clean-up procedure as no improvements were noted in the chromatographic performance of the GC column (i.e. it was still necessary to replace the retention gap and the remove the first portion of the GC column after 5 - 7 injections due to degradation of the chromatographic performance).

3.2 Clean-up using other cartridges

In addition to Florisil® cartridges (Isolute FL), CEFAS laboratory also investigated the utility of cyanopropyl (Isolute CN), aminopropyl (Isolute NH2), 2,3-dihydroxypropyl (Isolute DIOL) and silica (Isolute SI) cartridges. A mixture of the three steroids of interest and the corresponding deuterated internal standards was derivatised to form the

TBDMS derivatives, and this mixture of derivatives used for the investigation. Mixtures of pentane and diethyl ether as well as diethyl ether were used as elution solvents. The results of five separate experiments can be summarised as follows:

- Isolute CN cartridges - although the TBDMS derivatives of oestrone and ethinyl oestradiol (and the corresponding internal standards) were recovered with most eluents (e.g. 90:10 pentane:ether, 50:50 pentane:ether, 70:30 pentane:ether), TBDMS-17β-oestradiol and TBDMS-d_4-17β-oestradiol were generally only recovered when diethyl ether was used as the elution solvent, but the recoveries of these two compounds was <70%.

- Isolute NH2 cartridges - results were similar to those using Isolute CN cartridges, with TBDMS-17β-oestradiol and TBDMS-d_4-17β-oestradiol only recovered consistently when either 50:50 pentane:diethyl ether or diethyl ether were used as eluents. Recoveries of all compounds was poor (4-25%) even when using diethyl ether as eluent.

- Isolute DIOL cartridges - recoveries were extremely inconsistent, ranging from 0-200% for oestrone, 0-28% for 17β-oestradiol and 0-624% for 17α-ethinyl oestradiol, which suggests that interferences were being introduced.

- Isolute FL cartridges - recoveries were poor and variable.

- Isolute SI cartridges - when using 50:50 pentane:diethyl ether as eluent, recoveries of the steroid derivatives were in the range 20-46%, and when using diethyl ether as eluent recoveries were in the range 20-39%. Although all of the steroids and the d_4-internal standards were recovered it was considered that the losses were unacceptable, given the low limits of detection required.

Generally the results were inconsistent, and no improvement in GC performance was obtained. It was therefore considered that no advantage would be gained by using any of the cartridges tested.

3.3 Preliminary stability testing

A 20 litre sample of river water was collected (on 20.10.97) and spiked with approximately 10 ng l^{-1} of each of the three steroids of interest, and two 5 litre sub-samples analysed immediately to determine the actual concentrations present. The remaining 10 litres was stored in the laboratory at ambient temperature (c. 20 °C) and light conditions (10 hrs daylight, 14 hrs darkness per day). Another 5 litre sub-sample was analysed after 10 days, and the remaining 5 litres analysed after 20 days.

The concentrations found in the various sub-samples analysed are given in Table 3.1. As quantification is based on the responses obtained relative to isotopically-labelled internal standards added prior to the commencement of the analysis, the results are automatically compensated for recovery efficiencies.

Table 3.1 Results from stability testing of oestrone, 17β-oestradiol and 17α-ethinyl oestradiol in river water

Sample Description	Concentration found (ng l-1)		
	Oestrone	17β-Oestradiol	17α-Ethinyl oestradiol
Sample 1A (0 days)	15.9	9.1	13.8
Sample 1B (0 days)	14.9	9.9	13.3
Sample 2 (10 days)	21.6	2.2	14.2
Sample 3 (20 days)	3.9	5.1	4.1

These results suggest that for any extended performance testing, samples would need to be spiked immediately prior to extraction as the concentrations change significantly with time. It appears that there may be some conversion of 17β-oestradiol to oestrone during the first 10 day period, which may be due to oxidation, and although 17α-ethinyl oestradiol seems to be stable for this time, its concentration decreases markedly between 10 and 20 days.

Following this first stage of the work, a meeting was held between CEFAS and WRc and it was agreed that the methodology to be used for the limited performance testing exercise would be that developed by WRc for drinking waters, i.e. no clean-up step would be incorporated. The method has been written in "Blue Book" format in the Appendix to this report.

It was also agreed that the samples to be analysed for the performance testing would be spiked at a concentration of 1 ng l^{-1} with each of the three steroids of interest and the three deuterated internal standards immediately prior to the commencement of the analysis. WRc would provide CEFAS with standard solutions of the three internal standards.

4. PERFORMANCE TESTING OF STEROIDS METHODOLOGY FOR RIVER WATERS

A bulk sample (approximately 150 litres) of R. Thames water was collected by WRc staff in mid-October 1997. This was filtered through GF/D and GF/F filters using a stainless steel pressure filtration apparatus, and twenty-four 5 litre sub-samples stored in 5 litre glass bottles fitted with Teflon®-lined screw caps. Twelve of these samples were for WRc and twelve for CEFAS. These latter samples were collected by CEFAS staff on the day following the filtering operation.

As the primary purpose of the performance testing exercise was to establish the limit of detection achievable using the chosen method, it was decided that the samples should be spiked with 1 ng l^{-1} of each of the three steroids of interest and at the same level with the corresponding deuterated internal standards.

Although the method is given in full in the Appendix the main steps in the analysis are summarised below:

- the sample is spiked with the internal standards;
- the spiked sample is extracted using C_{18} solid phase extraction cartridges;
- the extract is concentrated and derivatised using N-methyl-N-t-butyldimethylsilyl trifluoroacetamide (MTBSTFA) containing 1% t-butyldimethylchlorosilane (TBDMCS) to form t-butyldimethylsilyl (TBDMS) derivatives;
- the derivatised extracts are analysed by GCMS/MS.

The river water samples analysed by WRc were spiked and extracted within a few days of the bulk sample being taken. The results obtained are given below in Table 4.1.

These results compare favourably with those found for drinking waters in that the limits of detection (LODs) are generally about a factor of two higher, which is to be expected given that better LODs are usually achieved for drinking waters.

The fact that the concentrations of oestrone found (mean c. 2 ng l^{-1})were higher than the intended spiking level (1 ng l^{-1}) is presumably due to the fact that oestrone is already present at a low level in the R. Thames at Medmenham. The samples analysed by CEFAS were stored for a few weeks prior to the analysis, and given the results of the stability testing (the concentrations of all of these steroids decreased significantly over a 20 day period) it is not surprising that increased concentrations of oestrone were not detected by CEFAS.

The results obtained by CEFAS are given in Table 4.2.

Table 4.1 WRc results from performance testing

Batch/sample	Concentration found (ng l^{-1})		
	Oestrone	17β-Oestradiol	17α-Ethinyl oestradiol
1a	1.738	0.961	1.061
1b	1.832	0.908	1.214
2a	1.863	0.996	1.184
2b	1.940	0.899	1.302
3a	2.247	1.151	1.001
3b	2.039	0.878	1.092
4a	2.067	1.030	1.275
4b	2.117	1.186	1.164
5a	1.994	1.348	1.450
5b	1.999	1.371	1.165
6a	1.973	1.081	1.193
6b	2.162	1.141	1.245
Mean	1.9976	1.0792	1.1955
M1	0.0357	0.0492	0.0166
M0	0.0080	0.0096	0.0118
Sw	0.0896	0.0980	0.1087
Sb	.1177	0.1407	0.0489
St	.1479	0.1714	0.1192
Rel SD (St%)	7.4	15.9	10.0
Est. LOD (ng l^{-1})	0.4	0.4	0.5
Est. Degs. F	7	7	10

Table 4.2 CEFAS results from performance testing

Batch/sample	Concentration found (ng l^{-1})		
	Oestrone	17β-Oestradiol	17α-Ethinyl oestradiol
1a	1.080	0.820	1.080
1b	1.100	1.260	1.040
1c	1.300	0.980	1.000
2a	1.120	1.300	1.040
2b	1.260	1.320	1.500
2c	1.480	1.240	1.140
3a	0.920	1.140	1.100
3b	1.120	1.200	0.820
3c	1.280	0.840	0.780
4a	0.820	0.800	1.180
4b	1.220	1.360	1.220
4c	1.240	1.060	1.040
Mean	1.1617	1.1100	1.0783
M1	0.0233	0.0432	0.0599
M0	0.0341	0.0418	0.0249
Sw	0.1847	0.2044	0.1577
Sb	0.0000	0.0215	0.1081
St	0.1847	0.2055	0.1912
Rel SD (St%)	15.9	18.5	17.7
Est. LOD (ng l^{-1})	0.6	0.6	0.6
Est. Degs. F	11	11	8

Although the performance testing undertaken by CEFAS was conducted on the basis of four batches of three replicates, rather than six batches of duplicates, the above data are not significantly different to those produced by WRc and demonstrate that the analytical method is robust.

On this basis, further inter-laboratory performance testing to "Blue Book" specifications is justified. This would involve the analysis of eleven duplicate batches of spiked samples at two concentrations ("low spike" and "high spike") within the range of interest (e.g. at 20 ng l^{-1} and 80 ng l^{-1} for river waters, assuming the range of interest to be 0 - 100 ng l^{-1}) for each matrix of interest. Additionally, eleven duplicate batches of a blank-spiked water would need to be analysed to establish detection limits.

Given the comparability of the two data sets, it does not appear that the performance of the methodology is dependent on the type of mass spectrometer used for the GCMS/MS analysis. The instrument used by WRc was a VG Trio-3 triple quadrupole mass spectrometer, while CEFAS used a Finnigan GCQ which has an ion trap design.

5. CONCLUSIONS

Modification of the method developed by WRc to determine oestrone, 17β-oestradiol and 17α-ethinyl oestradiol in drinking waters, to incorporate a clean-up step following derivatisation to form TBDMS derivatives, has been investigated by WRc and CEFAS.

The main purpose of doing so was to attempt to remove excess derivatising agent and by-products which cause problems with contamination of the GC column used for the GCMS/MS analysis, resulting in the need to replace the GC column retention gap and remove the first portion (10 - 20 cm) of the GC column after about six extracts have been analysed. It was concluded that no advantage was gained with any of the procedures applied, as the performance of the GC column was still adversely affected after 5-7 of the extracts subjected to clean-up had been analysed, and the recovery of the derivatised steroids in the clean-up step was generally no better than 50%. This poor recovery would inevitable lead to worse detection limits for all of the compounds of interest. It was therefore decided to proceed using the method as originally developed by WRc.

Both WRc and CEFAS analysed twelve river water samples spiked at 1 ng l^{-1} with oestrone, 17β-oestradiol and 17α-ethinyl oestradiol to determine the limits of detection of the method for river waters. The results obtained by the two laboratories were quite comparable with WRc finding detection limits of 0.4-0.5 ng l^{-1} and CEFAS finding detection limits of abut 0.6 ng l^{-1}. On the basis of these data it appears that further performance inter-laboratory testing to the standards required by the Standing Committee of Analysts is justified.

APPENDIX THE DETERMINATION OF OESTRONE, OESTRADIOL AND ETHINYL OESTRADIOL IN DRINKING AND RIVER WATERS

INTRODUCTION

Following the detection of oestrogenic activity in treated sewage effluents, fractionation of sewage effluents to isolate highly active fractions and investigative chemical analysis led to the identification of three steroids, *viz.* oestrone, 17β-oestradiol and 17α-ethinyl oestradiol, which were responsible for the majority of the detected activity. As treated sewage effluent is discharged into rivers in the UK, concern has arisen regarding potential deleterious environmental effects, as biochemical changes in fish can be triggered by extremely low concentrations of these compounds. Also, as water to be treated prior to use as drinking water is abstracted from rivers into which treated sewage effluents are discharged, there is also concern regarding the implications for drinking water consumers.

An analytical method to determine oestrone, 17β-oestradiol and 17α-ethinyl oestradiol at sub-ng l^{-1} levels in drinking waters and river waters has been developed (James *et al.* 1997). This forms the basis for the method as described in this publication.

Users should be aware that only free steroids are determined by this method. Steroid conjugates (e.g. sulphates and glucuronides) are not determined.

1. Performance characteristics of the method

1.1 Substances determined

Oestrone, 17β-oestradiol and 17α-ethinyl oestradiol.

1.2 Type of sample

Drinking waters and river waters.

1.3 Basis of method

Following the addition of internal standards, the water samples are extracted using C_{18} solid phase extraction cartridges. The extracts are derivatised to form the t-butyldimethylsilyl (TBDMS) ethers of the steroids of interest and the internal standards, and the derivatised extracts analysed using gas chromatography-mass spectrometry/mass spectrometry (GCMS/MS). Quantification is carried out by direct comparison of the response for each steroid and the response for the internal standards (a separate internal standard is used for each steroid).

1.4 Range of application

Up to 10 ng l^{-1}, when internal standards are added at a concentration of 1 ng l^{-1}; the range 1 - 100 ng l^{-1} when internal standards are added at a concentration of 10 ng l^{-1}.

1.5 Calibration coefficients

Not applicable.

1.6 Standard deviation

10-20% at 1 ng l^{-1}.

1.7 Limit of detection

TBA

1.8 Sensitivity

TBA

1.9 Bias

TBA

1.10 Interferences

Organic compounds which are co-extracted, derivatised, co-elute on the gas chromatographic column used, and which produce the same parent and daughter ions in their mass spectra as the steroids of interest could be interferences. Generally, however, GCMS/MS is extremely specific.

1.11 Time required for analysis

For one sample, results may be obtained within 10 hours of the commencement of the analysis; depending on the availability of equipment, results from six water samples (e.g. four samples, an AQC sample and a blank) could be available within 20 hours.

2. Principle

Water samples are filtered to remove particulate matter as its presence adversely affects the time needed for extraction. Drinking water samples should be dechlorinated using an aqueous solution of ascorbic acid, as free chlorine has been shown to react with the steroids to be determined and the internal standards.

A mixture of internal standards (d_4-oestradiol, d_4-17β-oestradiol and d_4-17α-ethinyl oestradiol) is added, and the sample extracted using C_{18} solid phase extraction cartridges. The cartridges are washed with 50% aqueous methanol prior to elution with aqueous methanol (85:15 methanol:water). The extracts are reduced to dryness, and derivatised with a solution in acetonitrile of a mixture of N-methyl-N-t-butyldimethylsilyl trifluoroacetamide (MTBSTFA) (99%) and t-butyldimethyl-chlorosilane (TBDMCS) (1%). Excess derivatising agent is removed by taking the derivatised extract to dryness and the residue is redissolved in diethyl ether prior to analysis by GCMS/MS. The losses monitored by MS/MS are the $M^{+\cdot} \rightarrow [M-57]^+$ losses for each of the steroids of interest and the internal standards, where $M^{+\cdot}$ is the molecular ion and the $[M-57]^+$ ion corresponds to the loss of the t-butyl group from the molecular ion.

3. Interferences

Other compounds present in samples, which are extracted using C_{18} solid phase cartridges and derivatised using MTBSTFA/TBDMCS, and which have identical GC retention times and produce MS/MS responses for the same ions which are monitored for the steroids of interest, could act as interferences.

4. Hazards

All of the steroids determined are oestrogenically active, and it must be assumed that the internal standards are also active. Appropriate precautions should be taken when handling the pure compounds and standard solutions of these compounds.

Several of the reagents used are potentially hazardous. Methanol, acetonitrile and diethyl ether are toxic and flammable, and the derivatising agent (99% MTBSTFA:1%TBDMCS) is toxic.

5. Reagents

5.1 Standards and internal standards

Oestrone, 17β-oestradiol and 17α-ethinyl oestradiol are available as crystalline solids from various sources. The internal standards used are 2,4,16,16-d_4-oestrone, 2,4,16,16-d_4-17β-oestradiol and 2,4,16,16-d_4-17α-ethinyl oestradiol. These are available from CDN isotopes (UK agents Univar plc, K&K-Greeff Ltd, Suffolk House, George Street, Croydon CR9 3QL).

Individual stock solutions of each of the steroids and internal standards are accurately prepared at about 1 mg ml^{-1} in acetone. For the steroids to be monitored, the stock solutions are diluted appropriately and mixed to give a spiking solution in acetone containing 0.1 ng μl^{-1} of each compound. For the internal standards, a spiking solution in acetone containing 0.1 ng μl^{-1} of each is prepared similarly.

5.2 Solvents and reagents

5.2.1 Silanising solution. A solution (10%; v/v) of a dimethyldichlorosilane in methylene chloride is used to deactivate the surface of all of the glassware which comes into contact with the samples and underivatised extracts. Sufficient of this solution (0.5 - 30 ml, depending on the surface area of the glassware involved) is poured into all of the sample bottles, separating funnels, round-bottomed flasks and Reacti-Vials® and swirled over the surface, ensuring complete coverage. Excess silanising solution is allowed to drain and disposed of. Silanising solution should preferable be made up as required, but excess solution can be stored for short periods (up to 1 week).

5.2.2 Solvents. Methanol (HPLC grade) is used for conditioning the solid phase extraction cartridges. Deionised water is used for conditioning the solid phase cartridges and for admixture with methanol for washing the cartridges prior to elution and for elution of the steroids of interest and the internal standards. Acetonitrile (silylation grade) is used as a solvent for the derivatisation step. Diethyl ether (HPLC grade or redistilled) is used as a solvent for the derivatised steroids and internal standards for the GCMS/MS analysis.

5.2.3 Ascorbic acid solution (0.4%). Dissolve 4.0 ± 0.2 g ascorbic acid in 1 l deionised water.

5.2.4 Solid phase cartridges. The cartridges used are 5 g Isolute C_{18} non-end-capped solid phase extraction cartridges. Similar cartridges from other manufacturers may be suitable but should be evaluated prior to use. At no time during the conditioning, extraction or washing stages should the cartridges be allowed to dry out.

5.2.5 Derivatising agent. N-methyl-N-t-butyldimethylsilyl trifluoroacetamide (MTBSTFA) mixed with t-butyldimethychlorolsilane (1%;v/v) is used to form t-butyl-dimethylsilyl (TBDMS) derivatives of the steroids of interest and the internal standards.

6. Apparatus

6.1 Sample bottles

Sampling bottles should be made of glass. Plastic screw tops are suitable provided they are fitted with PTFE® or PTFE®-faced liners. Alternatively, ground-glass stoppered glass bottles may be used. Bottles with a capacity of at least 5 l required for sampling and prior to use the bottles should be cleaned with a suitable proprietary cleaning agent (e.g. Decon 90) and, following thorough rinsing with deionised water and drying, should be deactivated with silanising solution (5.2.1) (20 - 30 ml).

6.2 Syringes and volumetric flasks

A range of glass syringes (e.g. between 10 µl and 1 ml) and low volume volumetric flasks (e.g. between 5 ml and 50 ml) should be available to prepare standard solutions and to spike samples.

6.3 Filtering equipment

Suitable pressure-filtering apparatus fitted with GF/F and GF/D filters (GF/D in top) should be available to filter samples.

6.4 Extraction apparatus

Various manually operated or automated solid phase extraction (SPE) systems are available. Typically a vacuum manifold to which several solid phase extraction cartridges can be attached is used, and the flow rate through each individual cartridge controlled by adjusting the vacuum applied to each.

6.5 Extract concentration equipment

6.5.1 Round-bottomed flasks (100 ml) for collection of eluate from the solid phase extraction.

6.5.2 Rotary evaporator fitted with a thermostatically controlled water bath.

6.5.3 Reacti-Vials® (3 ml) for concentration of the extracts.

6.5.4 Nitrogen blow-down equipment.

6.6 Derivatised extract concentration equipment

Nitrogen blow-down equipment.

6.7 GCMS/MS system

6.7.1 A capillary gas chromatograph with a temperature-programmable oven is required. Either a split/splitless heated injector (operated in the splitless mode) or an on-column injector is suitable, and injection can be manual or via a suitable autosampler.

6.7.2 The GC column used for the preliminary performance testing was a 30 m, 0.32 mm ID, 0.25 µm film thickness, DB5.625 manufactured by J&W. Other capillary columns of a similar polarity may be suitable. The GC column is linked to an on-column injector via a retention gap (20 - 60 cm) of deactivated uncoated 0.53 mm ID fused silica using a "press-fit" connector.

6.7.3 The GC temperature programme used is as follows:

- initial temperature 30 °C, held for 4 minutes following injection;
- the column temperature is then linearly programme at 10 °C min^{-1}, to a final temperature of 300 °C, which is maintained for a further 9 minutes.

6.7.4 A mass spectrometer fitted with an ion source suitable for electron impact ionisation and capable of operating in the MS/MS mode is required. This may be a triple quadrupole mass spectrometer, an ion trap type mass spectrometer controlled by software allowing MS/MS operation, or a magnetic sector double-focusing mass spectrometer.

6.7.5 A mass spectrometry data system is used to record the responses (as peak areas) for the various losses in MS/MS mode for the steroids of interest and the internal standards.

7. Sample collection and preservation

Samples (5 l) should be collected in suitable glass bottles (see 6.1). If drinking water samples are being collected, ascorbic acid solution (20 ml; see 5.2.3) should be added to dechlorinate the sample as soon as it is taken, as the steroids of interest react with free chlorine. As the steroids to be monitored are not stable in river waters for more than a few days, such samples should be analysed as soon as possible following collection. If storage is unavoidable, samples should be kept in a refrigerator at 4 °C.

8. Analytical procedure

8.1 Sample pre-treatment

The sample (5 l) is filtered, using a pressure filter, through a GF/D filter placed on top of a GF/F filter. A solution (50 µl; 0.1 ng μl^{-1}) of the internal standards in acetone is added to the sample.

8.2 Extraction

An Isolute C_{18} cartridge (5 g; non-end-capped) is conditioned by passing methanol (20 ml), then deionised water (20 ml) through it, using an applied vacuum to maintain a flow rate of 20 ± 5 ml min^{-1}, taking care to ensure that the cartridge does not dry out during this process or prior to passage of a sample through the cartridge. The sample is then passed through the cartridge at a flow rate of 20 ± 5 ml min^{-1}, the flow rate again being controlled by application of vacuum. After all of the sample has passed through the cartridge (3-4 fours), the cartridge is left under vacuum for a further 5 minutes prior to washing with a 50:50 mixture of methanol/water (40 ml). The wash mixture is discarded and the steroids eluted from the cartridge with a 85:15 mixture of methanol/water (20 ml) at a flow rate of 4 ± 1 ml min^{-1}. The extract is collected in a silanised round-bottomed flask (100 ml) and concentrated almost to dryness on a rotary evaporator on a water bath maintained at 40 ± 2 °C. The residue is dissolved in methanol (500 ± 50 µl) and the methanol solution transferred (using a micro-syringe) to a silanised Reacti-Vial® (3 ml). The flask is rinsed with more methanol (2 x 200 ± 20 µl) and the rinsings added to the solution in the Reacti-Vial®. The extract is then reduced to dryness using Nitrogen blow-down.

8.3 Derivatisation procedure

To the residue from the concentration extract (see 8.2) in the Reacti-Vial®, add silylation grade acetonitrile (100 µl) and the derivatising agent (MTBSTFA + 1% TBDMCS) (100 µl). Cap the Reacti-Vial® firmly, shake and then ultrasonicate for about 10 seconds. Leave for 15 ± 1 minutes and shake the Reacti-Vial®. Leave for a further 15 ± 1 minutes, remove the vial cap and concentrate to dryness using Nitrogen blow-down. Diethyl ether (50 µl) is then added to the residue and the mixture sonicated for about 10 seconds. The Reacti-Vial® is recapped, and left for a few minutes so that any undissolved material settles. The solution is then carefully transferred (using a micro-syringe, and avoiding the transfer of particulate matter) to a GC autosampler vial (100 µl) for GCMS/MS analysis. If the derivatised extract is not immediately analysed it may be stored in a freezer (-18 ± 4 °C). Should any particulate material precipitate, the clear solution should be transferred to a fresh GC vial prior to analysis.

8.4 GCMS/MS analysis

Suitable GCMS/MS systems are described in Section 7 above. The system performance should be optimised, with respect to collision energy and collision gas pressure, using a derivatised steroid standard, e.g. TBDMS-17β-oestradiol. If the mass spectrometer is fitted with a solid probe, the derivatised standard can be introduced in this way. Alternatively, the standard is introduced via the GC.

The mass spectrometer is set up in MS/MS mode to monitor the following dissociations:

- 384.25 → 327.18 (TBDMS-oestrone)
- 388.27 → 331.20 (TBDMS-d_4-oestrone)
- 386.26 → 329.20 (TBDMS-17β-oestradiol)
- 390.29 → 333.20 (TBDMS-d_4-17β-oestradiol)
- 410.26 → 353.20 (TBDMS-17α-ethinyl oestradiol)
- 414.29 → 357.22 (TBDMS-d_4-17α-ethinyl oestradiol)

The instrumental conditions for the MRM operation of the MS/MS system are set as recommended by the manufacturer, or based on experience. However, it is desirable that each dissociation is monitored no less frequently than once per second, so that responses are adequately defined.

8.5 Quantification

This is based on direct comparison of the peak areas obtained for each of the derivatised steroids with the peak areas of the corresponding deuterium-labelled internal standard. Provided that the peak areas being compared do not differ by more than an order of magnitude (i.e. an internal standard concentration of 2 ng l^{-1} covers the range 0.2 - 20 ng l^{-1}; an internal standard concentration of 10 ng l^{-1} covers the range 1-100 ng l^{-1}), it is generally accepted (Baldwin *et al.* 1997) that this means of quantification is satisfactory. For drinking waters it is recommended that the internal standards should be used at a concentration of 1 ng l^{-1}, whereas for river waters the internal standards should be used at 10 ng l^{-1}.

9. Sources of error

9.1 Contamination

Normal precautions should be taken to ensure that there is no cross-contamination between samples or extracts.

Since oestrone and 17β-oestradiol are human (both male and female) excretion products, and 17α-ethinyl oestradiol is used in the contraceptive "pill", there is a possibility of sample or extract contamination by laboratory workers during the extraction and derivatisation procedure. However, provided normal laboratory precautions are used (e.g. disposable gloves are worn), and given that these steroids are excreted mainly as conjugates, the risk of contamination in this way appears to be minimal.

9.2 Purity of standards

Suppliers of standard and internal standards should be contacted for this information.

9.3 Interfering substances

Any compound which is extracted and recovered using the procedures described in 8.2, and is derivatised using the procedures described in 8.3, and co-elutes and gives the same ions as either the three steroids of interest or the internal standards under the GCMS/MS conditions used will act as an interference. Given the specificity of the method, the most likely interferences will be other steroids. However, during preliminary performance testing no such interferences were observed.

10. Checking the validity of results

Once the method has been put into routine operation, various factors may affect the accuracy of the analytical results. Some of these have been noted in 9 above. It is recommended that at least one AQC sample is analysed in duplicate with each batch of samples.

11. References

Baldwin, R., Bethem, R.A., Boyd, R.K., Budde, W.L., Cairns, T., Gibbons, R.D., Henion, J.D., Kaiser, M.A., Lewis, D.L., Matusik, J.E., Sphon, J.A., Stephany, R.W. and Trubey, R.K. (1997) 1996 ASMS Fall Workshop: Limits to Confirmation, Quantitation and Detection. *Journal of the American Society for Mass Spectrometry*, **8**, 1180-1190.

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