



BSI Standards Publication

Water quality — Determination of selected estrogens in whole water samples — Method using solid phase extraction (SPE) followed by liquid chromatography (LC) or gas chromatography (GC) coupled to mass spectrometry (MS) detection

National foreword

This British Standard is the UK implementation of EN ISO 13646:2025. It is identical to ISO 13646:2025.

The UK participation in its preparation was entrusted to Technical Committee EH/3/2, Physical chemical and biochemical methods.

A list of organizations represented on this committee can be obtained on request to its committee manager.

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Date	Text affected

Water quality - Determination of selected estrogens in
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or gas chromatography (GC) coupled to mass
spectrometry (MS) detection (ISO 13646:2025)

Qualité de l'eau - Dosage d'œstrogènes sélectionnés
dans des échantillons d'eau totale - Méthode par
extraction en phase solide (SPE) suivie d'une détection
par chromatographie en phase liquide (CL) ou en
phase gazeuse (CG) couplée à la spectrométrie de
masse (SM) (ISO 13646:2025)

Wasserbeschaffenheit - Bestimmung ausgewählter
Estrogene in Gesamtwasserproben - Verfahren mittels
Festphasenextraktion (SPE) gefolgt von
Flüssigkeitschromatographie (LC) oder
Gaschromatographie (GC) gekoppelt mit
massenspektrometrischer Detektion (MS) (ISO
13646:2025)

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CEN-CENELEC Management Centre: Rue de la Science 23, B-1040 Brussels

European foreword

This document (EN ISO 13646:2025) has been prepared by Technical Committee ISO/TC 147 "Water quality" in collaboration with Technical Committee CEN/TC 230 "Water analysis" the secretariat of which is held by DIN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by April 2026, and conflicting national standards shall be withdrawn at the latest by April 2026.

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Endorsement notice

The text of ISO 13646:2025 has been approved by CEN as EN ISO 13646:2025 without any modification.

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO document should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 2, *Physical, chemical and biochemical methods*, in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 230, *Water analysis*, in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

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Introduction

Natural and synthetic estrogens are widely used worldwide, e.g. for contraception. Through application or improper disposal, these estrogens can enter the water cycle unchanged or transformed. They can therefore be detected in surface and groundwater, as well as in treated wastewater. It is known that estrogens can end up in surface waters via wastewater, and due to their physicochemical properties, they can partition in the different compartments [water and suspended particulate matter (SPM)] of water systems. They are of rising concern, due to their high estrogenic activity even at the measured ultra-trace levels (far below ng/l). Besides feminised fish and other endocrine disruptive effects in water ecosystems, they can also be a factor in biodiversity loss.^[16] Therefore, appropriate measurement methods are required to monitor estrogen levels below their ecotoxicological level [e.g. predicted no effect concentration (PNEC) or environmental quality standard (EQS)] and accordingly demonstrate if a water body is at risk.

This document specifies validated methods for analysing water samples in monitoring programs aiming at qualifying the quality of the water environment with respects to the selected estrogens.

Water quality — Determination of selected estrogens in whole water samples — Method using solid phase extraction (SPE) followed by liquid chromatography (LC) or gas chromatography (GC) coupled to mass spectrometry (MS) detection

WARNING — Persons using this document should be familiar with normal laboratory practice. This document does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices.

IMPORTANT — It is absolutely essential that tests conducted in accordance with this document be carried out by suitably qualified staff.

1 Scope

This document specifies methods for the determination of five selected estrogens in whole water samples listed in [Table 1](#) (see [Clause 4](#)). The methods are based on solid-phase extraction (SPE; disk or cartridge) followed by liquid or gas chromatography-mass spectrometry detection (tandem mass spectrometry or high resolution mass spectrometry). Depending on the sample preparation chosen, the sample preparation can be applicable to the analysis of selected estrogens in drinking water, groundwater and surface water containing suspended particulate matter (SPM) up to 500 mg/l, dissolved organic carbon (DOC) content up to 14 mg/l (whole water samples).

The lower application range defined as verified limit of quantification can vary depending on the methods, the sensitivity of the equipment used and the matrix of the sample. The range is 0,006 ng/l to 1 ng/l for 17alpha-ethinylestradiol (EE2) and 0,038 ng/l to 1 ng/l for the other estrogens in drinking water, ground water and surface water. The upper limit of the working range is approximately tens of nanograms per litre.

For application that targets the measurements of very low level concentrations (between the lowest LOQ and 0,1 ng/l), every single step of the procedure becomes critical.

The methods can be used to determine further estrogens or hormones in other types of water, for example treated wastewater, if accuracy has been tested and verified for each case as well as storage conditions of both samples and reference solutions have been validated.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 8466-1:2021, *Water quality — Calibration and evaluation of analytical methods — Part 1: Linear calibration function*

ISO 21253-1:2019, *Water quality — Multi-compound class methods — Part 1: Criteria for the identification of target compounds by gas and liquid chromatography and mass spectrometry*

ISO 11352:2025, *Water quality — Estimation of measurement uncertainty based on validation and quality control data*

3 Terms, definitions and subscripts

3.1 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

- ISO Online browsing platform: available at <https://www.iso.org/obp>
- IEC Electropedia: available at <https://www.electropedia.org/>

3.1.1

accuracy

closeness of agreement between a measured quantity value and a true quantity value of a measurand

[SOURCE: ISO/IEC Guide 99:2007, 2.13, modified — the terms “measurement accuracy” and “accuracy of measurement” and Notes 1, 2 and 3 to entry have been deleted.]

3.1.2

analyte

substance to be analyzed

[SOURCE: ISO 6107:2021, 3.31, modified — the domain and Notes 1 and 2 to entry have been deleted.]

3.1.3

blank

aliquot of reagent water (reagent blank) or of a matrix in which the *analyte* (3.1.2) is absent (matrix blank) that is treated exactly as a sample through the complete analytical procedure including extraction, clean-up, identification and quantification including all the relevant reagents and materials

Note 1 to entry: It is crucial that the laboratory specifies which blank is considered.

[SOURCE: ISO 21253-2:2019, 3.2]

3.1.4

calibration

operation that, under specified conditions, in a first step, establishes a relation between the quantity values with measurement uncertainties provided by measurement standards and corresponding indications with associated measurement uncertainties and, in a second step, uses this information to establish a relation for obtaining a measurement result from an indication

Note 1 to entry: A calibration may be expressed by a statement, calibration function, calibration diagram, calibration curve, or calibration table. In some cases, it may consist of an additive or multiplicative correction of the indication with associated measurement uncertainty.

Note 2 to entry: Calibration should not be confused with adjustment of a measuring system, often mistakenly called “self-calibration”, nor with verification of calibration.

[SOURCE: ISO/IEC Guide 99:2007, 2.39, modified — Note 1 to entry has been deleted.]

3.1.5

certified reference material

CRM

reference material, accompanied by documentation issued by an authoritative body and providing one or more specified property values with associated uncertainties and traceabilities, using valid procedures

[SOURCE: ISO/IEC Guide 99:2007, 5.14, modified — Notes 1, 2, 3, 4 and 5 to entry have been deleted.]

3.1.6**integrity**

property of the parameter(s) of interest, information or content of a sample stored in a container that has not been altered or lost in an unauthorized manner or that has been subject to loss of representativeness

[SOURCE: ISO 5667-3:2024, 3.2]

3.1.7**isotope dilution quantification**

process where isotopically labelled standards (e.g. deuterium- or carbon 13-labeled), which are chemically similar isotopic analogs of the target *analytes* (3.1.2), are added to all environmental and quality control and quality assurance samples before extraction and that follow the analytical procedure

Note 1 to entry: Isotope dilution quantification improves quantitative *accuracy* (3.1.1) by accounting for sample-specific procedural losses in the determined analyte concentration

3.1.8**limit of quantification****LOQ**

lowest value of a determinand that can be determined with an acceptable level of *accuracy* (3.1.1), which could be estimated by different means and verified in the intended matrix

[SOURCE: ISO 21253-2:2019, 3.4]

3.1.9**recovery****relative recovery**

extent to which a known, added quantity of determinant in a sample can be measured by an analytical system

Note 1 to entry: Recovery is calculated from the difference between results obtained from a spiked and an unspiked aliquot of sample and is usually expressed as a percentage.

[SOURCE: ISO 5667-14:2014, 3.8]

3.1.10**traceability**

property of a measurement result whereby the result can be related to a reference through a documented unbroken chain of *calibrations* (3.1.4), each contributing to the measurement uncertainty

[SOURCE: ISO/IEC Guide 99:2007, 2.41, modified — Notes 1, 2, 3, 4, 5, 6, 7 and 8 to entry have been deleted.]

3.1.11**yield****absolute recovery**

amount of *analyte* (3.1.2) added in the test sample corrected by the relative recovery of the internal standard (analyte-to-internal standard ratio)

Note 1 to entry: Yield is a value that accounts for both sample matrix effect and compound recovery.

[SOURCE: ISO 21253-2:2019, 3.11]

3.2 Subscripts

<i>a</i>	measuring variables for addition
<i>A</i>	addition
<i>e</i>	measuring variables for the calibration
<i>i</i>	identity of the substance
<i>I</i>	internal standard
<i>j</i>	consecutive number for value pairs
<i>g</i>	found value
<i>v</i>	default value
<i>M</i>	measuring solution
<i>P</i>	sample
<i>R</i>	reference

4 Principle

A water sample is spiked with an appropriate amount of isotopic labelled standard analogous of each targeted estrogen, before the sample is extracted by solid phase extraction (SPE) cartridge or disk and then cleaned up by SPE. The separation of the substances is achieved by liquid chromatography (LC) or gas chromatography (GC) with an identification and quantification based on mass spectrometry [tandem mass spectrometry (MS/MS) or high-resolution mass spectrometry (HRMS)]. The result is calculated by implementing isotope dilution calibration.

Table 1 — Characteristics of the five selected estrogens

Name	Structure	CAS RN ^a	Formula	Molecular weight g/mol	log <i>K</i> _{ow}
17alpha-ethinylestradiol (EE2) IUPAC name: (13R,17S)-17-ethynyl-13-methyl-7,8,9,11,12,14,15,16-octahydro-6H-cyclopenta[a]phenanthrene-3,17-diol		57-63-6	C ₂₀ H ₂₄ O ₂	296,40	4,52
17alpha-estradiol (17αE2) IUPAC name: (8R,9S,13S,14S,17R)-13-methyl-6,7,8,9,11,12,14,15,16,17-decahydrocyclopenta[a]phenanthrene-3,17-diol		57-91-0	C ₁₈ H ₂₄ O ₂	272,38	4,13
17beta-estradiol (17βE2) IUPAC name: (8R,9S,13S,14S,17S)-13-methyl-6,7,8,9,11,12,14,15,16,17-decahydrocyclopenta[a]phenanthrene-3,17-diol		50-28-2	C ₁₈ H ₂₄ O ₂	272,38	4,13
Estriol (E3) IUPAC name: (8R,9S,13S,14S,16R,17R)-13-methyl-6,7,8,9,11,12,14,15,16,17-decahydrocyclopenta[a]phenanthrene-3,16,17-triol		50-27-1	C ₁₈ H ₂₄ O ₃	288,38	2,94
Estrone (E1) IUPAC name: (8R,9S,13S,14S)-3-hydroxy-13-methyl-7,8,9,11,12,14,15,16-octahydro-6H-cyclopenta[a]phenanthrene-17-one		53-16-7	C ₁₈ H ₂₂ O ₂	270,37	3,69

^a CAS Registry Number® is a trademark of the American Chemical Society (ACS). This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

5 Interferences

5.1 General

Solvents, reagents, glassware and other sample processing hardware can yield artefacts, elevated baselines or lock-mass suppression causing misinterpretation of chromatograms.

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

Glassware should be rinsed with solvent and washed with a detergent solution as soon after use as is practical. Sonication of glassware containing a detergent solution for 30 s can aid in cleaning. After detergent washing, glassware should be rinsed first with solvent (e.g. methanol) and then with ultrapure water (6.1). Baking of glassware in an oven, programmable and capable of heating to at least 450 °C for 2 h can be warranted.

Sample contamination is a concern because estrogenic substances are biogenic and can be present on human skin or can be used as pharmaceuticals or personal care products. It is important that field and laboratory personnel exercise care to avoid contamination of the samples by avoiding consumption or contact with such materials immediately before and during sample collection and processing procedures. Exercising care is important for both the acquisition and subsequent handling of samples and sample extracts to avoid contamination.

5.2 Interferences with sampling, extraction and concentration

Use sampling containers of materials (7.1) that do not affect the analyte content during the contact time, preferably glass. Avoid plastics and organic materials during sampling, sample storage at (5 ± 3) °C or extraction especially if very low level of concentrations are targeted. High-density polyethylene (HDPE) or

polytetrafluoroethylene (PTFE) may be used but are not recommended if very low concentration levels ($< 0,1$ ng/l) are to be measured.

If automatic samplers are used, avoid the use of silicone or rubber material for the tubes. If these materials are present, ensure that the contact time is minimized. Rinse the sampling line with the water to be sampled before taking the test sample. ISO 5667-1 and ISO 5667-3 provide guidance.

The storage temperature is (5 ± 3) °C. For sampling and sample preservation, see [Clause 8](#).

During storage of the test samples, losses of components can occur due to adsorption on the walls of the containers. The extent of the losses depends on the storage time.

Commercially available solid-phase extraction (SPE cartridge or disk) can differ in quality. Variations in the selectivity of the materials also frequently occur from batch to batch, thus possibly causing significant deviations in extraction yield. This does not basically impair their suitability, apart from a resulting higher quantification limit for individual substances.

Avoid major fluctuations in the extraction times and elution procedures within one sample sequence when analyzing the samples.

Water samples containing high content of SPM or DOC can lead to clogging in case of SPE cartridge use (see [9.2.2](#)) that can be prejudicial to extraction recovery. To overcome this, a reduction of sample volume or switching to SPE disk (see [9.2.3](#)) extraction can be implemented. Another solution is the application of glass wool or sand to the cartridge (filling height 1 cm to 2 cm) to achieve the extraction of higher sample volumes.

Repeated uses and cleaning procedures of glassware can cause active sites on the glass surface that can irreversibly adsorb the selected estrogens and also be responsible of cross contamination.

To prevent cross contamination between series, SPE apparatus, tubing and items in contact with samples shall be cleaned with successively, for example, (hot) water with detergents, solvents (e.g. methanol, acetone, ethanol) then rinsed with water and dried.

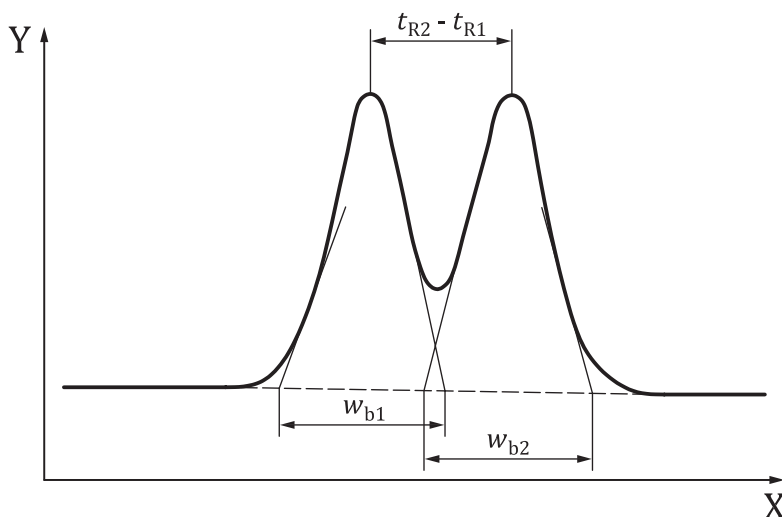
Interferences co-extracted from samples will vary considerably from source to source, depending on the diversity of the site being sampled. Interfering substances can be present at concentrations several orders of magnitude higher than the selected estrogens. The most frequently encountered interferences are humic and other acids. Given that very low levels of estrogens can be measured by this method, elimination of interferences is essential (implementation of clean-up, see [9.3](#)).

5.3 Interferences during high performance liquid chromatography and mass spectrometry

Substances with similar retention times and masses as the target estrogens can lead to interferences and overlapping or incompletely resolved peaks in the chromatogram. Depending on their intensity, those co-elutents can affect the trueness of the analysis.

17 α E2 and 17 β E2 are epi-isomers and as consequence have the same transitions in MS/MS. It is critical to separate both substances. A minimal chromatographic resolution of $R \geq 1,2$ must be achieved. If the criterion cannot be achieved, a suitable column shall be chosen to meet the required resolution (see [Annex E](#) and [Annex F](#) for examples).

The chromatographic resolution is calculated according to [Figure 1](#) and [Formula \(1\)](#).

**Key**

X	time
Y	intensity
t_{R1}, t_{R2}	retention time of eluting substances 1 and 2 in seconds (s)
w_{b1}, w_{b2}	peak width at the base of each peak in seconds (s)

Figure 1 — Resolution of chromatographic peaks

$$R = 2 \frac{(t_{R2} - t_{R1})}{w_{b1} + w_{b2}} \quad (1)$$

where

R	is the resolution;
t_{R1}, t_{R2}	is the retention time of eluting substances 1 and 2 in seconds (s);
w_{b1}, w_{b2}	is the peak width at the base of each peak in seconds (s).

NOTE Alternative calculation methods for R are also possible.

For some targeted estrogens, some sensitive transitions have shown a lack of selectivity or specificity leading to possibly false positive and overestimation of results.

Accompanying substances (matrix) can affect the ionization of the target substances (e.g. ion suppression or signal enhancement). This can result in underestimation or overestimation of concentration during quantification and significantly affect the sensitivity of the method. These interferences can be detected and corrected by implementing a clean-up step (see 9.3) and isotope dilution quantification.

5.4 Interferences during gas phase chromatography and mass spectrometry

Substances with similar retention times and masses as the target estrogens can lead to interferences and overlapping or incompletely resolved peaks in the chromatogram. Depending on their intensity, those co-elutents can affect the trueness of the analysis.

17 α E2 and 17 β E2 are epi-isomers and as consequence have the same transitions in MS. It is critical to separate both substances. A minimal chromatographic resolution of $R \geq 1,2$ is suitable. If the criterion cannot be reached, a suitable column shall be chosen to meet the required resolution (see Annex G and Annex H for an example).

Interferences from accompanying substances (matrix) can occur depending on methods applied.

5.5 Interferences from internal standards

For mass spectrometric detection, it is recommended to use a stable isotopically labelled analogue as an internal standard; ^{13}C or ^{15}N -labelled compounds are preferred rather than deuterated compounds. There is generally an observable drift in retention time compared to the natural compound when working with isotopically labelled standards (isotope effect).

Only a stable part of the molecule shall be labelled, and degree of labelling shall not change over the course of analysis. Exchanges can take place, via tautomerization processes for example, and so this type of mechanism shall be experimentally verified in the event that the abundance of internal standard is shown to vary.

Spectral overlapping between labelled analogue and native substances shall be avoided. Consequently, for the majority of applications, the labelled analogue should preferably have a mass difference of at least 3 Da to the native target substance.

The isotopic purity of the internal standards shall be checked. It is not a critical factor provided that its impurities, including unlabelled analogue, have preferably zero contribution on target compounds of the analytical method. In the event that this is not possible, the contribution of the labelled analogue to the unlabelled analogue should be negligible at LOQ level.

If internal standards with less than 3 Da are implemented, the above criteria shall be checked and documented to prevent from false positive and misquantification.

6 Reagents

As far as available, analytical grade or residue-analytical grade reagents shall be used. The content of impurities contributing to blank values or causing interfering signals shall be negligible (below 1/3 LOQ). This shall be checked in regular intervals.

Reagents, solvents and water used as eluents shall be suitable for LC or respectively GC and mass spectrometry.

When very low level of estrogens are to be measured (below 0,1 ng/l) and the lowest LOQ shall be reached the quality of reagents and materials become highly critical and shall be routinely monitored to prevent cross contamination and false positive results.

6.1 Water, H_2O .

Ultrapure water without any interfering blank values. The quality of water is checked by the same procedure as the sample to be measured.

Commercially available mineral water in glass bottles can also be used to determine method blank when ultrapure water has significant background interference.

6.2 Sodium thiosulfate pentahydrate, $\text{w}(\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O})$ of at least 99 %.

6.3 Disodium salt of ethylenediaminetetraacetic acid(EDTA), ($\rho \approx 0,025 \text{ g/ml}$), ($\text{C}_{10}\text{H}_{14}\text{N}_2\text{Na}_2\text{O}_8 \cdot 2\text{H}_2\text{O}$), at least 99 %.

Dissolve 25 g EDTA in 1 000 ml of water.

6.4 Methanol(MeOH), (CH_3OH), for extraction, purification, mobile phase and preparation of reference solutions.

6.5 Acetonitrile (ACN), (CH_3CN), for extraction, purification, mobile phase and preparation of reference solutions.

6.6 Ethyl acetate(EA), ($\text{C}_4\text{H}_8\text{O}_2$), for extraction and purification.

- 6.7 Acetone**, (C_3H_6O), for extraction and purification and derivatization (GC).
- 6.8 *n*-hexane**, (C_6H_{14}), for extraction and purification.
- 6.9 Methyl tert-butyl ether (MTBE)**, ($C_5H_{12}O$), for extraction and purification.
- 6.10 Acetone extra dry**, (C_3H_6O), for derivatization.
- 6.11 Acetic acid**, $w(CH_3COOH)$ of at least 99 %, for mobile phase (LC).
- 6.12 Formic acid**, $w(HCOOH)$ of at least 98 %, for mobile phase (LC).
- 6.13 Hydrochloric acid**, $w(HCl)$ of at least 36 %, for pH adjustment of water sample.
- 6.14 Sulphuric acid**, $w(H_2SO_4)$ of at least 98 %, for pH adjustment of water sample.
- 6.15 Sodium chloride**, $w(NaCl)$ of at least 98 %, for preservation of analytes in water sample.
- 6.16 Ascorbic acid**, $w(C_6H_8O_6)$ of at least 98 %, for preservation of analytes in water sample.
- 6.17 Ammonium fluoride**, (NH_4F), at least 98 %, for mobile phase (LC).
- 6.18 Pyridine**, (C_5H_5N), for derivatization (GC).
- 6.19 N-trimethylsilylimidazole (TMSI)**, ($C_6H_{12}N_2Si$), for derivatization (GC).
- 6.20 5-(dimethylamino)naphthalene-1-sulfonyl chloride, dansyl chloride(DNSCI)**, ($C_{12}H_{12}ClNO_2S$), LC grade for dansylation (LC).
- 6.21 Pyridine-3-sulfonyl chloride(PS-Cl)**, ($C_5H_4ClNO_2S$), for derivatization (LC).
- 6.22 Sodium carbonate concentrate (0,1 mol/l, pH = 10,5)**, (Na_2CO_3), in water, for derivatization (LC).
- 6.23 Sodium bicarbonate concentrate**, 0,1 mol/l, for derivatization (LC).
- 6.24 Sodium hydroxide solution**, 10 mol/l, for derivatization (LC) and pH adjustment of water sample.
- 6.25 Dansyl reagent solution, for dansylation (LC).**

The volume of dansyl reagent to be prepared shall be calculated beforehand because it depends on the number of samples to be dansyld. To do this, weigh 0,783 mg of dansyl chloride ([6.20](#)) for 1 ml of extra dry acetone ([6.10](#)) in an amber glass bottle of suitable volume, then mix well. The dansyl reagent thus prepared cannot be stored for more than 24 h. The vial of dansyl chloride, once opened, can be stored for one week at a controlled temperature $5\text{ }^{\circ}\text{C} \pm 3\text{ }^{\circ}\text{C}$.

6.26 Pyridine-3-sulfonyl chloride solution, for derivatization (LC).

Weigh 10 mg to 40 mg of pyridine-3-sulfonyl chloride (PS-Cl) ([6.21](#)) and add 10 ml to 40 ml of acetone ([6.7](#)) into a glass vial so that a solution of 1 mg/ml is formed. Transfer PS-Cl with a glass pipette. Mix well. PS-Cl is a reactive, moisture-sensitive and turbid liquid. It is recommended to add some nitrogen into the PS-Cl reagent vial after usage and into the PS-Cl solution vial after preparation and usage. If this is done, the opened PS-Cl reagent vial can be used at least for four months. Discard the PS-Cl reagent if it turns clearly more solid. Store both at $(5 \pm 3)\text{ }^{\circ}\text{C}$.

6.27 Bicarbonate buffer 100 mmol/ml, pH = 10,5, for dansylation.

Weigh 1,68 g of sodium bicarbonate into a 200 ml volumetric flask, then adjust the volume with laboratory grade water (6.1). Transfer the solution obtained into a 250 ml glass bottle. Using a pH meter, adjust the pH of the solution to $10,5 \pm 0,1$ with a 10 mol/l of sodium hydroxide solution (6.24) (e.g. about 1 ml) and homogenize. The buffer thus prepared can then be stored for one month, at a controlled temperature $(5 \pm 3) ^\circ\text{C}$.

6.28 Bicarbonate buffer 100 mmol/l, pH = 10,5, for derivatization.

Mix 80 ml of sodium carbonate concentrate (6.22) and 20 ml of sodium bicarbonate concentrate (6.23). Using a pH meter, adjust the pH of the solution to $10,5 \pm 0,1$, if needed, by adding either sodium carbonate concentrate (if pH is below 10,5) or sodium bicarbonate concentrate (if the pH is above 10,5) and homogenize. The buffer thus prepared can be stored for one month, at a controlled temperature of $(5 \pm 3) ^\circ\text{C}$.

6.29 Operating gases for the gas chromatography or liquid chromatography mass spectrometry, of high purity and in accordance with the manufacturer's specifications.**6.30 Standards.****6.30.1 Reference substances**, see [Table 1](#) for list of substances.

High purity standards shall be implemented.

Ensure and check that the single reference substance does not contain detectable concentrations of the other substances (see [Table 1](#)) to be determined.

NOTE 1 Solutions of single reference substances or mixed solutions of various combinations are commercially available.

NOTE 2 Certified reference materials to establish traceability to SI measurements are available.

6.30.2 Internal standards, as given in [Table 2](#).

High purity standards shall be implemented (see [5.5](#)).

NOTE Solutions are commercially available.

The degree of labelling shall not change over the course of analysis. Exchanges can take place, via tautomerization processes for example, and so this type of mechanism shall be experimentally verified in the event that the abundance of internal standard is shown to vary.

Table 2 — Names, acronyms and CAS numbers of isotopically labelled estrogens

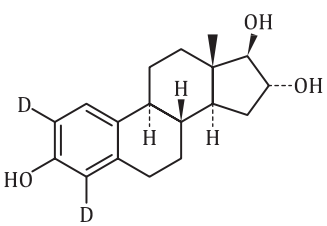
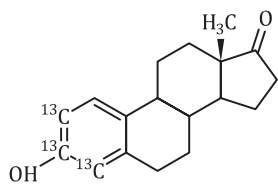
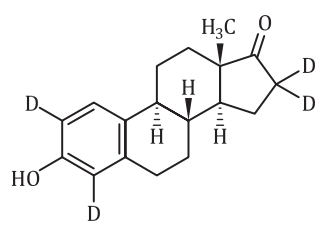
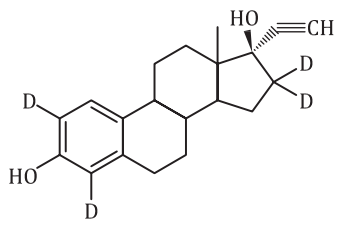
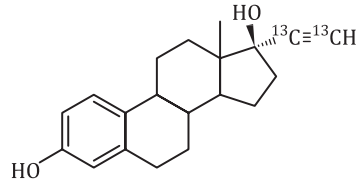
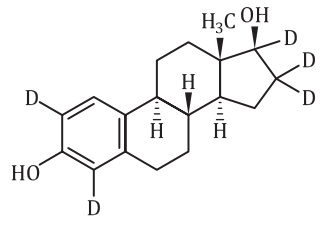
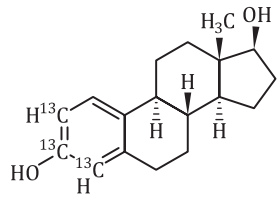
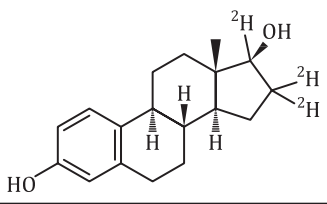
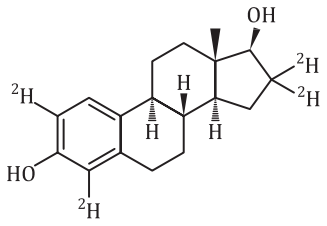
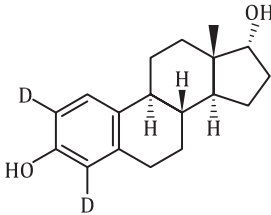
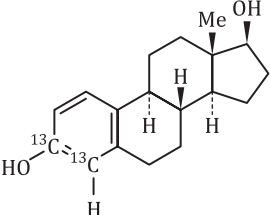
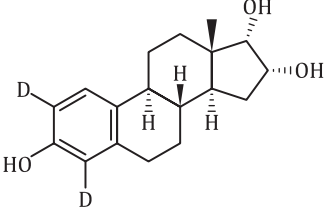
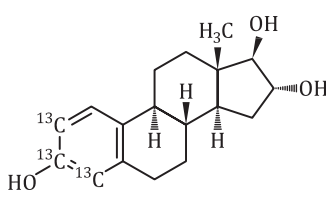
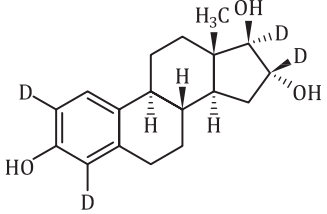
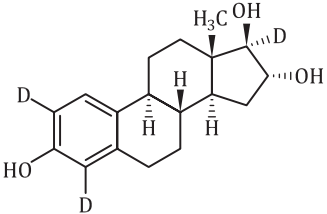
Substance	CAS RN	Substance	CAS RN
Estrone 2,4 D2 (E1-D2) 	56588-58-0	Estrone 2,3,4- ¹³ C ₃ (E1- ¹³ C ₃) 	1241684-29-6
Estrone 2,4,16,16-D4 (E1-D4) 	53866-34-5	/	/
17α-ethinylestradiol 2,4,16,16-D4 (EE2- D4) 	350820-06-3	17α-ethinylestradiol 20,21- ¹³ C ₂ (EE2- ¹³ C ₂) 	2483735-63-1
17β-estradiol 2,4,16,16,17-D5 (17βE2- D5) 	221093-45-4	17β-estradiol 2,3,4- ¹³ C ₃ (17βE2- ¹³ C ₃) 	1261254-48-1
17β-estradiol-16,16,17-D3 (17βE2- D3) 	79037-37-9	/	/
17β-estradiol-2,4,16,16-D4 (17βE2- D4) 	66789-03-5	/	/

Table 2 (continued)

Substance	CAS RN	Substance	CAS RN
17 α -estradiol 2,4-D2 (17 α E2- D2) 	81586-94-9	17 α -estradiol 3,4- ¹³ C ₂ (17 α E2- ¹³ C ₂) 	82938-05-4
Estriol 2,4-D2 (E3-D2) 	53866-32-3	Estriol- 2,3,4- ¹³ C ₃ (E3- ¹³ C ₃) 	1255639-56-5
Estriol 2,4,16,17-D4 (E3-D4) 	2111805-38-8	/	/
Estriol 2,4,17-D3 (E3-D3) 	79037-36-8	/	/

Some of the labelled standards can be used as an injection standard.

6.31 Preparation of solutions.

6.31.1 General.

Solutions of the reference substances ([6.30.1](#)) and of the internal standards ([6.30.2](#)) are required for the calibration according to [Clause 10](#) and for checking the procedure according to [Clause 11](#).

The solutions are commercially available and can be custom-made with a wide variety of mass concentrations. For commercially purchased solutions, the manufacturer's instructions for storage and shelf life apply.

Gravimetric control is recommended to obtain an accurate determination of the different stock and intermediate solutions.

When weighed in a vial, the solvent shall be added gravimetrically.

The stock solutions and the intermediate dilutions prepared from them should be in the same solvent, if possible.

For laboratory preparations, note the following information on storage and shelf life:

- Standard solutions in solvent (stock solutions, intermediate dilutions, calibration solutions) have a shelf life of at least one year if stored at $\leq -18\text{ °C}$ in the freezer.
- Standard solutions in pure water (intermediate dilutions and calibration solutions) are to be prepared fresh every measuring day and can be stored cooled in the refrigerator or autosampler at a maximum of $(5 \pm 3)\text{ °C}$ until measurement.

The accuracy of the solutions of reference substances shall be checked with an independent control standard ([6.31.10](#)), e.g. when performing calibration (see [10.3](#)).

6.31.2 Stock solutions (reference substances and internal standards).

Prepare solutions with a mass concentration of, for example, 0,1 mg/ml of each substance. For this, use, for example, a 5 mg amount of a substance ([6.30.1](#) or [6.30.2](#)) in separate 50 ml volumetric flasks ([7.3](#)), dissolve them in acetonitrile ([6.5](#)) or methanol ([6.4](#)), and then add solvent to solution until it reaches the mark.

Store the solutions at temperatures below -18 °C and in an area protected from light and evaporation. Under these conditions, the solutions are stable for one year.

Alternatively, commercially available (or custom made) stock solutions of individual reference substances (or internal standard substances) in organic solvent can be used for preparing further dilutions.

6.31.3 Intermediate dilution A (reference substances).

Prepare an intermediate solution with substance mass concentrations of, for example, 1 µg/ml each. This involves transferring, for example, 0,5 ml of each reference substance stock solution (see [6.31.2](#)) to a 50 ml volumetric flask ([7.3](#)) and then making the solution up to the mark with methanol ([6.4](#)) or acetonitrile ([6.5](#)).

Store the solution at temperatures below -18 °C and in an area protected from light and evaporation. Under these conditions, the solution is stable for one year.

6.31.4 Intermediate dilution B (reference substances).

Prepare an intermediate solution with substance mass concentrations of, for example, 50 ng/ml each. This involves transferring, for example, 0,5 ml of the intermediate dilution A (see [6.31.3](#)) to a 10 ml volumetric flask ([7.3](#)) and then making the solution up to the mark with methanol ([6.4](#)) or acetonitrile ([6.5](#)).

Store the solution at temperatures below -18 °C and in an area protected from light and evaporation. Under these conditions, the solution is stable for one year.

6.31.5 Intermediate dilution C (reference substances).

Prepare an intermediate solution with substance mass concentrations of, for example, 5 ng/ml each. This involves transferring, for example, 0,25 ml of the intermediate dilution A ([6.31.3](#)) to a 50 ml volumetric flask ([7.3](#)) and then making the solution up to the mark with methanol ([6.4](#)) or acetonitrile ([6.5](#)).

Store the solution at temperatures below -18 °C and in an area protected from light and evaporation. Under these conditions, the solution is stable for one year.

6.31.6 Intermediate dilution D (reference substances).

Prepare an intermediate solution with substance mass concentrations of, for example, 0,5 ng/ml each. This involves transferring, for example, 0,025 ml of the intermediate dilution A ([6.31.3](#)) to a 50 ml volumetric flask ([7.3](#)) and then making the solution up to the mark with methanol ([6.4](#)) or acetonitrile ([6.5](#)).

Store the solution at temperatures below -18 °C and in an area protected from light and evaporation. Under these conditions, the solution is stable for one year.

6.31.7 Intermediate dilution E (internal standards).

Prepare an intermediate solution with substance mass concentrations of, for example, 1 µg/ml each. This involves transferring, for example, 0,5 ml of each internal standard substance stock solution (6.31.2) to a 50 ml volumetric flask (7.3) and then making the solution up to the mark with methanol (6.4) or acetonitrile (6.5).

Store the solution at temperatures below –18 °C and in an area protected from light and evaporation. Under these conditions, the solution is stable for one year.

6.31.8 Intermediate dilution F (internal standards).

Prepare an intermediate solution with substance mass concentrations of, for example, 50 ng/ml each. This involves transferring, for example, 0,5 ml of intermediate dilution E (6.31.7) to a 10 ml volumetric flask (7.3) and then making the solution up to the mark with methanol (6.4) or acetonitrile (6.5).

Store the solution at temperatures below –18 °C and in an area protected from light and evaporation. Under these conditions, the solution is stable for one year.

6.31.9 Calibration solutions.

The calibration solutions are prepared by appropriate dilutions of the intermediate dilution-B (reference substances, 6.31.4) and the intermediate dilution-F (internal standards, 6.31.8).

Apply calibration solutions in such a way that:

- the mass concentration of the substances to be determined covers a calibration range of e.g. 0,001 ng/ml to 1 ng/ml (0,001 ng/l to 1 ng/l with enrichment by a factor of 1 000);
- the mass concentration of the internal standards is equal in each solution and lies in the lower calibration range, e.g. 0,1 ng/ml.

The solvent portion in the calibration solutions should be similar to the initial conditions of the chromatography for LC based methods analysis.

It should be the same in the samples to be analysed and shall not lead to any interfering peak broadening.

6.31.10 Control standard.

The control standard is a reference substances solution produced independently of the stock solutions, the deviation from the target value should be less than 15 %.

The solution should contain all of the substances to be determined.

The solution from an alternative batch or manufacturer shall be used.

7 Apparatus

Equipment or parts of equipment that come into contact with the water sample shall have no blank values for the substances to be measured within this method. All equipment used should preferably be made of glass. PTFE or HDPE may be used but are not recommended if very low concentration levels (< 0,1 ng/l) are to be measured.

Given the possible instability due to photodegradation of some estrogens, special caution is necessary when selecting of containers. The use of coloured glassware, dark storage and shipment is recommended.

7.1 Glass bottles, brown or green glass, narrow-necked, flat-bottomed, 250 ml to 1 000 ml, with PTFE cap liner.

All vessels should preferably be made of borosilicate glass.