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An isotope dilution gas chromatographic–mass spectrometric method for the simultaneous assay of estrogens and phytoestrogens in urine

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Abstract

The metabolism of endogenous estrogens is complicated and certain metabolic patterns may reflect an individual risk of estrogen-dependent diseases such as breast cancer. Since the 1960s we have been constantly involved in developing estrogen profiling methods, in the beginning using gas chromatography and later gas chromatography–mass spectrometry (GC–MS) in the selected ion monitoring mode (SIM) and finally utilizing isotope dilution (ID–GC–MS–SIM). The addition of the dietary phytoestrogens to the profile rendered the method even more complicated. The present work presents the final estrogen profile method for 15 endogenous estrogens, four lignans, seven isoflavonoids and coumestrol in one small urine sample (1/150th of a 24 h human urine sample, minimum 2.5–5 ml) with complete validation including investigations as to the precision, sensitivity, accuracy and specificity. The method does not include the minimal amounts of unconjugated estrogens in urine. It may also be used for animal (e.g. rat and mouse) urine using a minimum of 2 ml of usually pooled sample. Despite its complexity it was found to fulfill the reliability criteria, resulting in highly specific and accurate results.

Keywords: Estrogen; Phytoestrogen; Method; Urine; Gas chromatography–mass spectrometry

1. Introduction

Because of the great number of estrogen metabolites and their low concentration the determination of the whole estrogen profile in urine has been a great challenge. The first studies we carried out using gas-liquid chromatography (GC) [1,2]. The introduction of the first combined gas chromatography–mass spectrometry (GC–MS) instruments and the use of selected ion monitoring (SIM, also called mass fragmentography) considerably increased the sensitivity of the method and more estrogen metabolites could be determined [3,4].

Further development of the GC–MS instrumentation, the use of capillary columns and the introduction of ion exchange chromatographic procedures as well as the techniques developed to preserve during sample processing the highly labile α-ketolic and catecholic estrogens resulted in a completely new micromethodology with high potential [5]. At the same time it was found that in the earlier methods a group of compounds had been interfering with the estrogen analyses. These compounds were identified and found to be lignans [6,7] and soon many other phenolic compounds belonging to the group of the isoflavone series were identified in human urine [8–15]. Almost all of these new so-called phytoestrogens and both deuterated estrogen and phytoestrogen standards were synthesized and the isotope dilution gas chromatography–mass spectrometry technique in the selected ion monitoring mode (ID–GC–MS–SIM) was employed including both the estrogens and the phytoestrogens in the same method [15]. Since then new compounds have been identified in urine and included in the method. After 36 years of continuous work we

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now present the "final" estrogen profile method with all details and its complete evaluation.

2. Experimental

2.1. Instrumentation

A Fisons MD 1000 quadrupole mass spectrometer combined with a Fisons 8000 gas chromatograph combined with an autoinjector is used for the quantitative analyses. A 12.5 m (i.d. = 0.22 mm) BP-1 (SGE) capillary column with a stationary phase layer of 0.25 μm was used and helium served as the carrier gas. The temperatures for the transfer line, ion source and the analyzer were 270, 250 and 250 °C, respectively.

2.1.1. Standards

The following estrogens and phytoestrogens are used for the standard solution. All ketonic estrogens were first converted into their ethoximes.

Estrone-ethoxime (original compound from Makor Chemicals, Jerusalem, Israel), estradiol (Steraloids, Newport, USA), estriol (Sigma, St Louis, Missouri, USA), 2-methoxyestrone-ethoxime, 2-methoxyestradiol, 2-hydroxyestrone-ethoxime (Sigma), 4-hydroxyestrone-ethoxime (Sigma), 2-hydroxyestradiol (Sigma), 4-hydroxyestradiol (Sigma), 16-epiestriol (Sigma), 16-oxoestradiol-ethoxime, 16α-hydroxyestrone-ethoxime (Makor), 15α-hydroxyestrone-ethoxime (Schering).

Enterolactone (Enl), enterodiol (End), daidzein (Da) and equol (Eq) were synthesized as described [16]. *O*-desmethylangolensin (*O*-DMA) was synthesized from 2-(*p*-methoxyphenyl) propionic acid and 1,3-dimethoxybenzene in polyphosphoric acid followed by demethylation using 1.0 M boron tribromide in dichloromethane [10]. Dihydrodaidzein and dihydrogenistein were synthesized from the corresponding isoflavones daidzein and genistein by reducing selectively the carbon–carbon double bond in the C-ring by diisobutylaluminium hydride [17]. Matairesinol (Mat) was synthesized as described previously [18]. Genistein was also obtained from Karl Roth GmbH and Co., (Karlsruhe, Germany), coumestrol from Eastman Kodak Co., (Rochester, Minnesota, USA) and secoisolariciresinol from Plantech, UK.

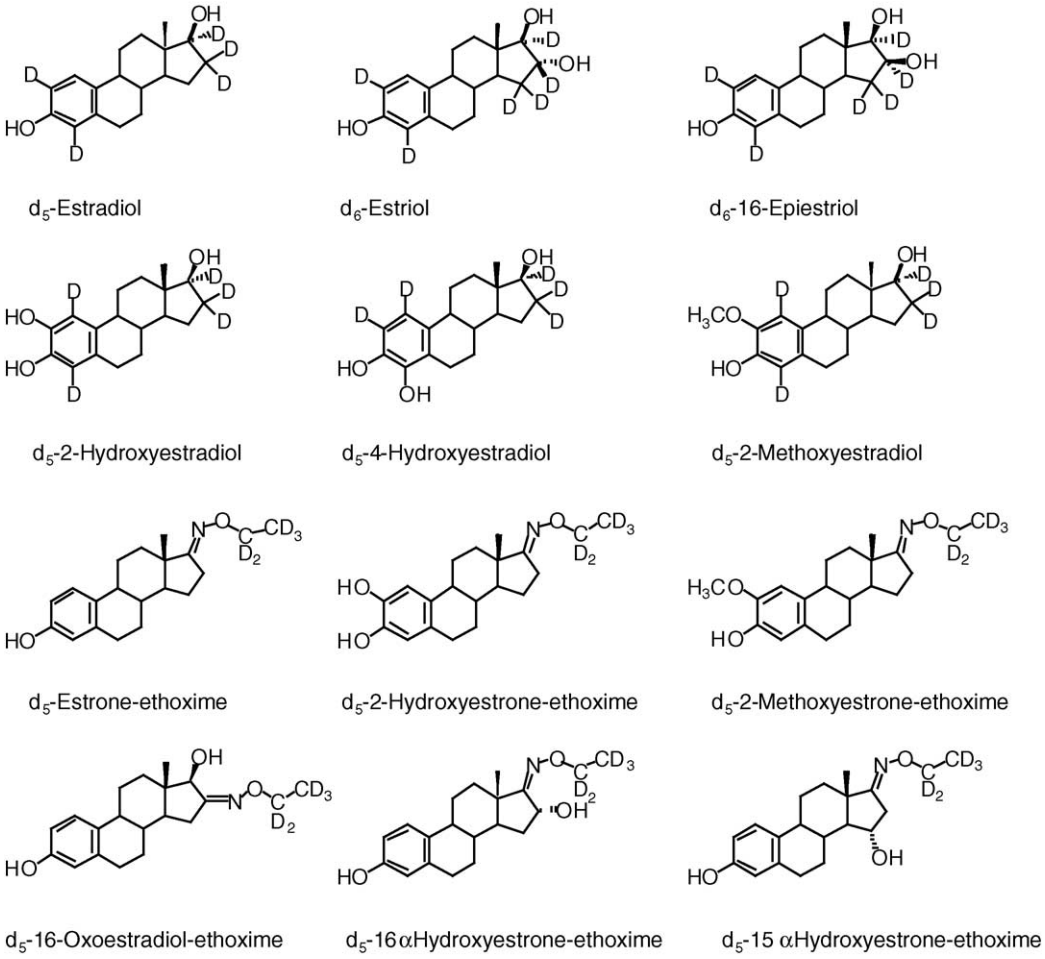

Fig. 1. Structure of deuterated estrogens.

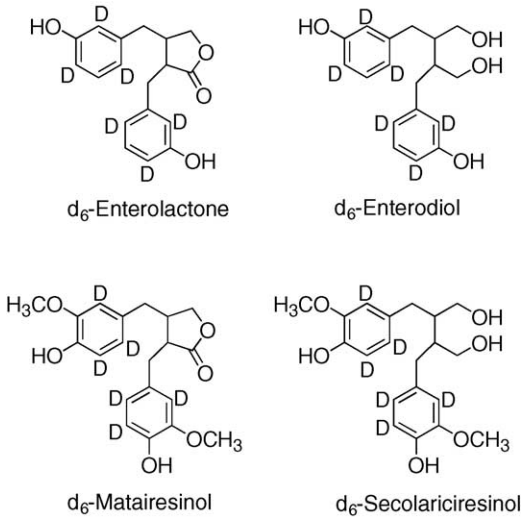

Fig. 2. Structure of deuterated lignans.

The following deuterated estrogens and phytoestrogens were synthesized and used in the standard solution. All compounds are shown in Figs. 1–3.

All compounds with an oxogroup were converted to ethoximes using a deuterated ethoxime reagent, d_5-ethoxyamine hydrochloride [19] (see Section 2.1.4.1): d_5-estrone-ethoxime, d_5-2-hydroxyestrone-ethoxime, d_5-4-hydroxyestrone-ethoxime, d_5-2-methoxyestrone-ethoxime, d_5-16-oxoestradiol-ethoxime, d_5-15α-hydroxyestrone-ethoxime, and d_5-16α-hydroxyestrone-ethoxime. d_5-estradiol, d_5-2-hydroxyestradiol, d_5-4-hydroxyestradiol, d_5-2-methoxyestradiol, d_6-16-epiestriol, and d_6-estriol were synthesized as described [20]. The following deuterated phytoestrogen standards were synthesized as described [21]: d_6-Enl, d_6-End, d_4-Da, d_4-Eq, and d_5-*O*-Dma. The synthesis of a stable deuterated genistein (d_4-Gen) has also been described [22] as well as the synthesis of d_6-Matairesinol [15] and d_6-secoisolariciresinol which was obtained by $LiAlH_4$ reduction from d_6-matairesinol [23]. Dihydrodaidzein, dihydrogenistein, and coumestrol were deuterated by $D_3PO_4{\cdot}BF_3$ in D_2O [24] to give d_4-dihydrodaidzein, d_4-dihydrogenistein, and d_4-coumestrol.

2.1.2. Chemicals

Methanol, *n*-hexane of HPLC grade and analytical-grade ethyl acetate were purchased from Rathburn Chemicals Ltd. (Walkerburn, Peeblesshire, Scotland). Pyridine, glacial acetic acid of suprapure quality, sodium hydroxide, sodium bicarbonate, diethyl ether, ascorbic acid, boric acid, lithium chloride and hydrochloric acid were obtained from E. Merck AG (Darmstadt, Germany). Trimethylchlorosilane (TMCS) and hexamethyldisilazane (HMDS) were from Pierce Chemical Co., (Rockford, Ill. USA). Dimethylchlorosilane (DMCS) was obtained from Fluka AG (Buchs, Switzerland). *Helix pomatia* was from Biosepra Division of Cipbergen (Cergy-Saint-Christophe, France). The ion exchanger materials DEAE-Cl- and QAE-Cl- were from Pharmacia (Uppsala, Sweden), the ethoxyamine hydrochloride from Eastman Kodak, USA and Lipidex-5000 from Packard, PerkinElmer Life and Analytical Sciences, (Boston, MA, USA).

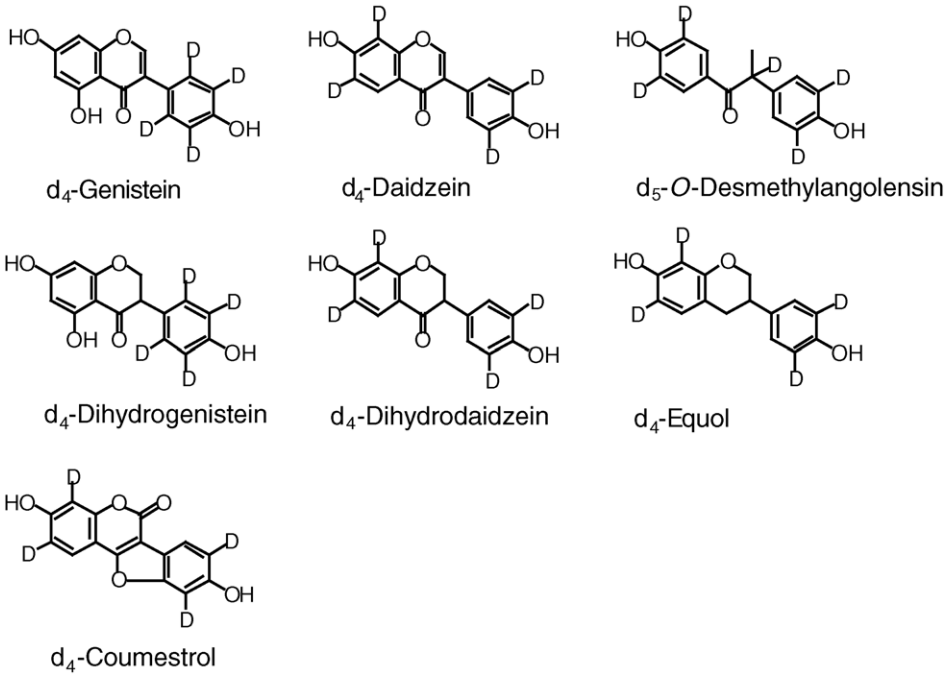

Fig. 3. Structure of deuterated isoflavones and coumestrol.

2.1.3. Preparation of reagents, buffers and anion exchangers

2.1.3.1. Ethoximation. The ethoxymation reagent (1.5 M pyridinium acetate buffer pH 5.0) is prepared by mixing 79.2 ml of distilled water, 12 ml of pyridine, 8.4 ml of glacial acetic acid, 792 mg of ascorbic acid and 1200 mg of ethoxyamine.

2.1.3.2. Ascorbate buffer. The buffer is prepared by dissolving 500 mg of ascorbic acid, 750 mg of sodium bicarbonate and 400 mg of sodium hydroxide in 10 ml of distilled water.

2.1.3.3. Conversion of ion exchangers. DEAE-Cl- and QAE-Cl-: Both gels are washed before use subsequently with 20% ethanol, 50% ethanol and ethanol (1 l/50 g of gel) for one hour at room temperature with occasional agitation. After washing the gels are suspended in methanol and stored at +4 °C. Both gels are stable for several months.

DEAE-Ac- and QAE-Ac-: DEAE-Cl- and QAE-Cl- are converted to the acetate form by washing them subsequently with 10 bed volumes of 0.1 M NaOH in 70% MeOH, 70% MeOH, 0.5 M acetic acid in 70% MeOH, and 70% MeOH. The pH should be approximately neutral after both 70% MeOH washings. DEAE-Ac- is suspended in 70% MeOH and QAE-Ac- in MeOH and stored at +4 °C, both gels are stable for several months.

QAE-Bor-: QAE-Cl- is converted to the borate form by washing with 10 bed volumes of 0.1 M NaOH in 70% MeOH and 70% MeOH, 0.5 M boric acid in 70% MeOH and 70% MeOH, respectively. QAE-Bor- is suspended in 70% MeOH and stored at +4 °C, stable about three weeks.

QAE-Carb-: QAE-Ac- is converted to the carbonate form by washing subsequently with 10 bed volumes of 0.2 M $NaHCO_3$ in 40% MeOH, and 40% MeOH and washed thoroughly with about 15 bed volumes of MeOH. QAE-Carb- should be prepared just before use, because it decomposes rapidly.

DEAE-OH-: DEAE-Cl- is converted to the hydroxyl form by washing subsequently with 10 bed volumes of 0.1 M NaOH in 70% MeOH, 70% MeOH, and MeOH. DEAE-OH- should be prepared just before use, because it decomposes rapidly.

Lipidex-5000: Lipidex-5000 is delivered as a methanol suspension and has to be washed with n-hexane and then suspensed in n-hexane:chloroform 1:1 by volume.

3. Method

3.1. Purification with a Sep-pak column

To the urine samples (1/150th of the 24 h volume, minimum 5 ml for a postmenopausal sample) an equal volume of ethoximation reagent is added and the sample incubated over night at room temperature (RT). The sample is applied to a primed Sep-pak cartridge (primed by washing with 6 ml of MeOH and 10 ml of H_2O) and allowed to drip through, the cartridge is washed with 5 ml of acetate buffer 0.15 M, pH 3.0 and the sample is eluted with 3 ml of MeOH, and 1.2 ml of H_2O added to the methanol eluate.

3.2. DEAE-Ac- ion exchange chromatography

A 3 cm column of DEAE-Ac- is prepared in a Pasteur pipette in 70% MeOH. The column has a small piece of cotton in the lower end.

Fraction (*FR*) *1* (*free FR*): The sample is applied in 4.2 ml of 70% MeOH and eluted with 4 ml of 70% MeOH. Total eluents = FR 1 (discarded)

FR 2 (*acidic FR*): The column is eluted with 10 ml of 0.2 M CH_3COOH in 70% MeOH (discarded)

FR 3 (*conjugate FR*): The column is eluted with 10 ml of 0.3 M LiCl in 70% MeOH To FR 3 (the conjugate fraction), appropriate amounts of the deuterated standards (deuterated ethoximes for the ketonic estrogens and deuterated unmodified compounds for the other estrogens and for all lignans and isoflavones) are added and the fraction is evaporated until only water is left. The fraction volume is supplemented with H_2O to 9 ml and the pH adjusted to 3.0 by adding 1/10th of the sample volume of acetate buffer 1.5 M pH 3.0.

The sample is applied to a primed Sep-pak column and allowed to drip through and eluted with 3 ml of MeOH which is evaporated to dryness.

3.3. Hydrolysis

The dry sample is dissolved in 5 ml of hydrolysis buffer containing 25 mg of ascorbic acid and 50 μl of *Helix pomatia* juice in 5 ml of 0.15 M acetate buffer pH 4.1. The sample is then incubated over night at +37 °C.

After incubation the sample is applied to a primed Sep-pak, and allowed to drip through. The cartridge is washed with 5 ml of water and eluted with 3 ml of MeOH and subjected to chromatography in QAE-Ac-.

3.4. QAE-Ac- ion exchange chromatography

A 5 cm column of QAE-Ac- is prepared in a Pasteur pipette in MeOH.

FR 1 (*estrogen and lignan FR*): The sample is applied in 3 ml of MeOH and, eluted with 4 ml of MeOH. Total eluents = FR 1.

FR 2 (*phytoestrogen FR*): The column is eluted with 9 ml of 0.2 M CH_3COOH in MeOH Both FRs are evaporated to dryness and dissolved in 0.5 ml of MeOH. FR 1 (estrogen and lignan FR) is subjected to chromatography in QAE-Bor-. FR 2 (phytoestrogen FR) is stored in the freezer until silylated.

3.5. QAE-Bor- ion exchange chromatography

A 3 cm column of QAE-Bor- in a Pasteur pipette is prepared in methanol.

FR 1 (*estrogen and lignan FR*): The sample is applied in 0.5 ml of MeOH and eluted with 2 ml of MeOH followed by 4 ml of 0.05 M CH_3COOH in MeOH. Total eluents = FR 1.

FR 2 (*catechol estrogen FR*): The column is eluted with 4 ml of 0.05 M CH_3COOH in MeOH.

Both FRs are evaporated to dryness. FR 1 is dissolved in 0.5 ml of methanol and subjected to chromatography in QAE-Carb- (see below).

FR 2 is dissolved in 5 ml of ethyl acetate and washed with 0.5 ml of 0.5 M HCl, 0.5 ml of ascorbate buffer, 0.5 ml of 10% CH_3COOH and 0.5 ml of H_2O. The ethyl acetate extract is evaporated to dryness and the sample dissolved in 0.5 ml of MeOH and stored in the freezer until silylated before GC–MS.

3.6. QAE-Carb- ion exchange chromatography

A 4 cm column of QAE-Carb- in a Pasteur pipette is prepared in methanol.

Fr 1 (*estrogen FR*): The sample is applied in 0.5 ml of MeOH and eluted with 1.5 ml of MeOH followed by 6 ml of 80% MeOH. Total eluents = FR 1.

Fr 2 (*lignan FR*): The column is eluted with5 ml of 0.1 M CH_3COOH in 80% MeOH.

Both FRs are evaporated to dryness and dissolved in 0.5 ml of MeOH. FR 1 is subjected to chromatography in DEAE-OH- (see below). FR 2 is stored in the freezer until silylated.

3.7. DEAE-OH- ion exchange chromatography

A 1.5 cm column of DEAE-OH- in a Pasteur pipette is prepared in methanol.

FR 1 (*neutral steroid FR*): The sample is applied in 0.5 ml of MeOH and eluted with 3 ml of MeOH (discarded).

FR 2 (*estrogen FR*): The column is eluted with 5 ml of 0.1 M CH_3COOH in MeOH.

FR 2 is evaporated to dryness, dissolved in 0.5 ml of n-hexane:chloroform 1:1 by volume and subjected to chromatography in Lipidex-5000.

3.8. Lipidex-5000 chromatography

A 3 cm column of Lipidex-5000 in Pasteur pipette in n-hexane:chloroform 1:1 is prepared.

FR 1 (*oxoestrogen FR*): The sample is applied in 0.5 ml of n-hexane:chloroform 1:1 and eluted with 7.5 ml of n-hexane:chloroform 1:1

FR 2 (*estriol FR*): The column is eluted with 4 ml of 5% MeOH in chloroform. Both FRs are evaporated to dryness, dissolved in 0.5 ml of MeOH and stored in the freezer until silylated.

The estriol fraction is separated from the other estrogens only if pregnancy urine is investigated. For all other samples the estriol fraction is combined with the oxoestrogen fraction.

3.9. Silylation

The sample is evaporated to dryness and dissolved in 100 (estrogens) or 1000 (phytoestrogens) μl of silylation reagent, containing pyridine:HMDS:TMCS 9:3:1, and incubated for at least 30 min at RT. The estrogen fractions are evaporated to dryness and 100 μl of n-hexane is added and the samples are ready for GC–MS. The phytoestrogen-containing samples are directly transferred to the closed vials used for automatic injection of the samples.

The compositions of the standard mixtures are shown in Table 1 including the predicted retention times of each compound, which may change a little when changing the column. For 16α-hydroxyestrone two peaks are obtained. For quantification the compound with two silylated hydroxyl groups and one ethoxime group is used (ion pair *m*/*z* 473/478).

4. Injection volumes and oven temperature programs

4.1. Catechol and cis-glycolic estrogens fraction

One microliter of the silylated sample is injected. Dwell time for all compounds is 0.08 and span time 0.10 s. The initial temperature is kept at 150 °C for one min, and then increased by 40 °C/min to 240 °C and further increased by 3 °C/min to 274 °C and then increased by 50 °C/min to 280 °C and kept at that temperature for 2 min.

4.2. Lignan and equol fraction

One microliter of the silylated sample is injected. Dwell time for all compounds is 0.08 s and span time 0.10 s. The initial temperature is kept at 150 °C for one min, and is increased by 40 °C/min to 240 °C and further increased by 2.6 °C/min to 270 °C and kept at that temperature for 2 min.

4.3. Phytoestrogen fraction

One microliter of the silylated sample is injected. Dwell time for all compounds is 0.08 and Span time 0.10 s. The initial temperature is kept at 150 °C for 1 min, then increased by 40 °C/min to 230 °C and further by 2 °C/min to 262 °C and finally by 50 °C/min to 290 °C where it is kept for 1 min.

4.4. Estrogens and secoisolariciresinol

One microliter of the silylated sample is injected. Dwell time for all compounds is 0.08 s and span time 0.10 s. Initial temperature at 150 °C is kept for 1 min and then increased by 40 °C/min to 240 °C and further increased by 2.6 °C/min to 270 °C, and finally by 40 °C/min to 280 °C where it is kept for 2 min.

Table 1
List of predicted retention time and ions used for quantification

Compound	Predicted retention time (min)	Ions used for quantification (*m*/*z*)	Other ions used (*m*/*z*)
Catechol- and cisglycolic estrogen fraction			
d_5-2-Hydroxyestrone-ethoxime	9.160	478	
2-Hydroxyestrone-ethoxime	9.250	473	
d_5-4-Hydroxyestrone-ethoxime	9.460	478	
4-Hydroxyestrone-ethoxime	9.520	473	
d_5-4-Hydroxyestradiol	9.127	509	
4-Hydroxyestradiol	9.160	504	
d_5-2-Hydroxyestradiol	8.660	509	
2-Hydroxyestradiol	8.690	504	
d_6-16-Epiestriol	9.836	510	
16-Epiestriol	9.840	504	
17-Epiestriol	9.319	504	
Lignans and equol fraction			
d_4-Equol	6.532	390	
Equol	6.552	386	
d_6-Enterodiol	8.464	416	
Enterodiol	8.494	410	
d_6-Enterolactone	9.034	448	
Enterolactone	9.064	442	
d_6-Matairesinol	13.711	508	212[a]
Matairesinol	13.745	502	209[a]
Phytoestrogen fraction			
d_5-*O*-desmethylangolensin	6.524	464	283[a]
O-desmethylangolensin	6.524	459	281[a]
d_4-Daidzein	12.206	402	
Daidzein	12.241	398	
d_4-Genistein	13.175	475	
Genistein	13.164	471	
d_4-Dihydrodaidzein	8.860	404	
Dihydrodaidzein	8.873	400	
d_4-Dihydrogenistein	9.541	477	
Dihydrogenistein	9.554	473	
d_4-Coumestrol	15.208	416	
Coumestrol	15.243	412	
Glycitein	15.850	428	
Estrogen and secoisolariciresinol fraction			
d_5-Estradiol	7.576	419	
Estradiol	7.619	416	
d_5-Estrone-ethoxime	7.901	390	
Estrone-ethoxime	7.945	385	
d_5-2-Methoxyestrone-ethoxime	10.288	420	
2-Methoxyestrone-ethoxime	10,352	415	
d_5-2-Methoxyestradiol	9.073	451	
2-Methoxyestradiol	9.116	446	
d_5-16α-Hydroxyestrone-ethoxime	9.746	478	
16α-Hydroxyestrone-ethoxime	9.811	473	
d_5-15α-Hydroxyestrone-ethoxime	10.006	478	
15α-Hydroxyestrone-ethoxime	10.071	473	
d_5-16-Oxoestradiol-ethoxime	10.679	478	
16-Oxoestradiol-ethoxime	10.744	473	
16ß-Hydroxyestrone-ethoxime	10.325	428	
d_6-Secoisolariciresinol	12.297	566	
Secoisolariciresinol	12.343	560	
d_6-Estriol	10.288	510	
Estriol	10.332	504	

[a] These ions are used if there are some problems when quantifying the analyte with the first ion.

4.5. Quantification

Standard for 16β-hydroxyestrone is not available anymore. The values are calculated using the 16α-hydroxyestrone standard curve. Deuterated glycitein is also not available and this analyte is quantified using the deuterated daidzein. The same is true for 17-epiestriol, which is quantified using the deuterated 16-epiestriol. The amount of standards for the standard curves are the following (approximately) when analyzing non-pregnancy samples: 2-hydroxyestrone-ethoxime 3–120 ng, 4-hydroxyestrone-ethoxime 0.5–30 ng, 4-hydroxyestradiol 0.60–30 ng, 2-hydroxyestradiol 0.8–50 ng, 17-epiestriol 0.3–20 ng, 16-epiestriol 0.3–25 ng, estradiol 1.4–50 ng, estrone-ethoxime 5–150 ng, 2-methoxyestrone-ethoxime 1–35 ng, 16α-hydroxyestrone-ethoxime 0.6–20 ng, 15α-hydroxyestrone-ethoxime 0.30–8.5 ng, 16-oxoestradiol-ethoxime 0.80–30 ng, and 2-methoxyestradiol 0.30–9 ng. For the isoflavones and lignans the amounts depend so much on the concentration of the compounds in urine due to highly variable intake in different subjects that these values are not presented. All standard curves show good linearity (results not shown).

Calibrators with the same amount of added deuterated internal standards as originally added to the samples were evaporated to dryness as well as the samples. To the estrogen-containing samples 100 μl of the silanization reagent pyridine/HMDS (hexamnethyldisilazane)/TMS (trimethylchlorosilane) 9:3:1, v/v was added and incubated either for 30 min or overnight. To the lignan- and isoflavone-containing fractions 1 ml of the reagent was added and the tubes were incubated for 30 min at room temperature and 1–2 μl was injected (without evaporation) into the GC–MS instrument. The estrogen fractions were, however, evaporated after incubation and thereafter the samples were dissolved in 100 μl was n-hexane and 1–2 μl was injected into the GC–MS instrument.

The calculation of the results was carried out by comparing the ratios of the ions for the urinary compounds and deuterated internal standards with the same ratios of the standards used for preparing the standard curve.

5. Results

5.1. Accuracy

Because the isotope dilution technique is used all losses during the procedure are corrected for from the point of addition of the deuterated internal standards after the DEAE-Ac- chromatography. No deuterated conjugated internal standards are available. If the unconjugated standards are added earlier, they will be lost as only conjugated compounds are isolated and purified at the very beginning of the method. Urine contains normally very little unconjugated estrogens and phytoestrogens and they are not measured with this

method. By studying separately the Sep-pak purification procedure and the DEAE chromatography with tritiated estrogen conjugates we have arrived to the conclusion that there is a maximum loss of about 10% in the steps before the addition of the labeled compounds. Because recovery experiments have been carried out previously using most of the estrogens [5] and phytoestrogens [15] and the method modifications are relatively small and have been mainly done to include the many new compounds, we did not find it necessary to repeat the recovery experiments for all compounds. The results for the new compounds are shown in Table 3. They show results similar to those previously obtained [5,15] indicating that the accuracy of the method is satisfactory.

5.2. Sensitivity

Due to improvements of the GC–MS systems and the data programs the sensitivity of the method has improved during the 17 years since its original publication [5]. Calculating the sensitivity from 5 ($n = 4$)–20 ($n = 5$) repeated analyses on different days of nine estrogens having usually very low concentrations (means 0.13–1.50 nmol/24 h) by multiplying the standard deviation of the means by 3 we obtained sensitivity limits between 0.01 and 0.27 nmol/24 h (mean 0.10 nmol/24 h). The mean is about four to five times times lower than we found in the original publication [5]. The concentrations of the phytoestrogens in urine are considerably higher than those of the estrogens and the method is sensitive enough to measure the phytoestrogens even in 1–2 ml of urine samples in Western populations. The lowest points of the phytoestrogen standard curves are between 2 and 2000 ng/100 μl. (results for the total of 26 standard curves are not shown).

5.2.1. Imprecision

The imprecision of the method was evaluated by calculating the coefficients of variation (CV) as percentage of the mean value (CV%) of repeated assays of the same sample on the same day (intra-assay imprecision) or on different days (inter-assay imprecision). Our control samples (low and high values) were used for this purpose. The results are shown in Tables 2 and 3. At very low levels the CV% may exceed 10%, but in general the values are below 10%.

5.2.2. Specificity

Methods utilizing isotope dilution gas chromatography–mass spectrometry in the selective ion-monitoring mode (ID–GC–MS–SIM) are usually regarded as reference methods due to their high specificity. In the present method the ions used for quantification are the molecular ions (M^+) or large fragments in the higher molecular range. This reduces background and increases sensitivity and specificity. All compounds have been carefully identified using authentic refer-

Table 2
Intra-assay coefficient of variation (CV%) of estrogens and phytoestrogens in urine

Compound	Concentration in low control (nmol/24 h)	Intra-assay CV% Low control sample ($n = 5$)	Concentration in high control (nmol/24 h)	Intra-assay CV% High control sample ($n = 5$)
Estrone	6.15	8.9	n.d.	n.d.
Estradiol	3.73	12.6	n.d.	n.d.
Estriol	3.78	3.3	n.d.	n.d.
2-Hydroxyestrone	0.69	3.6	n.d.	n.d.
2-Hydroxyestradiol	0.18	11.2	n.d.	n.d.
4-Hydroxyestrone	0.23	4.6	16.8	4.8
4-Hydroxyestradiol	0.19	1.7	17.3	6.4
2-Methoxyestrone	0.79	11.1	n.d.	n.d.
2-Methoxyestradiol	0.48	5.0	n.d.	n.d.
15α-Hydroxyestrone	0.36	5.2	n.d.	n.d.
16α-Hydroxyestrone	0.80	7.3	n.d.	n.d.
16ß-Hydroxyestrone	0.84	9.9	n.d.	n.d.
16-Ketoestradiol	1.50	6.0	n.d.	n.d.
16-Epiestriol	?	[a]	n.d.	n.d.
17-Epiestriol	0	[b]	n.d.	n.d.
Genistein	23.3	7.6	528	5.4
Daidzein	26.4	6.8	265	5.0
Glycitein	2.62	15.0	411.0	4.0
Dihydrogenistein	6.42	6.8	4165	1.9
Dihydrodaidzein	20.8	5.8	4828	1.1
O-Desmethylangolensin	3.62	18.6	24.6	3.8
Equol	39.6	9.0	39.6	9.0
Coumestrol	4.52	11.1	2245	4.5
Matairesinol	n.d.	n.d.	n.d.	n.d.
Secoisolariciresinol	n.d.	n.d.	n.d.	n.d.
Enterodiol	n.d.	n.d.	n.d.	n.d.
Enterolactone	n.d.	n.d.	n.d.	n.d.

n.d.: not determined, because published previously [15].
[a] Interfering compound in the sample with very low concentratioins of estrogens.
[b] No values obtained because below sensitivity limit.

Table 3
Interassay coefficient of variation (CV%), and recovery of standards added to urine (%)

	Concentration in low control nmol/24 h	Inter-assay CV% Low control sample (n = 6–8)	Concentration in high control nmol/24 h	Inter-assay CV% High control sample (n = 6–8)	Accuracy % recovery (N = 3)
Estrone	15.1	9.3	20.2	4.4	
Estradiol	5.4	11.1	9.95	7.1	
Estriol	6.14	5.8	11.30	5.3	
2-Hydroxyestrone	6.58	5.1	9.30	6.9	
2-Hydroxyestradiol	4.92	12.2	4.51	8.2	
4-Hydroxyestrone	1.94	13.0	1.97	12.1	88.9–97.7
4-Hydroxyestradiol	0.53	4.1	1.03	12.9	96.1–98.0
2-Methoxyestrone	1.87	13.2	3.40	10.3	
2-Methoxyestradiol	0.73	6.5	0.86	8.7	89.5–98.4
15α-Hydroxyestrone	0.32	12.9	0.42	14.8	
16α-Hydroxyestrone	0.91	11.7	2.11	9.1	
16β-Hydroxyestrone	0.96	11.4	1.89	6.7	
16-Oxoestradiol	1.39	9.2	2.45	8.2	
16-Epiestriol	1.85	8.0	2.22	5.5	
17-Epiestriol	0.42	16.3	0.51	8.7	
Genistein	20.0	10.9	574	4.5	
Daidzein	17.0	8.7	1919	5.4	
Glycitein	5.95	10.8	923	9.3	88.9–98.8
Dihydrogenistein	5.54	12.0	282	9.6	96.0–101.1
Dihydrodaidzein	5.36	10.8	1805	9.5	96.0–99.2
O-Desmethylangolensin	3.99	12.4	156	4.7	
Equol	56.8	7.1	22.4	9.1	
Coumestrol	2.14	7.8	2.41	6.2	88.1–95.8
Matairesinol	21.4	7.3	19.7	6.9	
Secoisolariciresinol	120	13.0	125	8.8	
Enterodiol	332	11.4	763	5.4	
Enterolactone	5060	7.3	2260	9.7	

ence standards. To show the high specificity of the method even at low levels chromatograms from urine samples with low estrogen and phytoestrogen concentrations are shown in Figs. 4–6.

Three analytes are sometimes difficult to quantify specifically: 16-epiestriol because of an occasionally occurring unknown compound eluting close to this estrogen, 17-epiestriol and 15α-hydroxyestrone because they are minor metabolites and the concentrations may be too low for reliable quantification.

In Figs. 4–6 some chromatograms for estrogens with low concentrations are shown. As can be seen the chromatograms are practically free from extra peaks.

6. Discussion

Looking at the history of estrogen profile method development the first attempts to measure more than the three "classical" estrogens was made by Givner et al. [25,26], Hobkirk and Nilsen [27] and Breuer [28] utilizing column and/or paper chromatography and in some studies separating ketonic estrogens from non-ketonic ones by Girard separation. The final determination was made by the Kober reaction or modifications thereof. All results presented were obtained using late pregnancy urine as the sensitivity was not sufficient for non-pregnancy urine. Breuer [28] was able to measure 16 estrogens in three late pregnancy urine samples, the identity of which was established for 14 compounds. Three of the compounds have not been measured by the method presented here (6α-hydroxyestradiol, 6α-hydroxyestrone and 6ß-hydroxyestrone), because we have not applied our method to pregnancy urine.

The chemical assay using the Kober reaction was followed by gas chromatography (GC), a blind detection system by which the estrogens could be faster separated from each other [1,2,29]. The former group used trimethylsilyl ether derivates, the latter acetates. However, the methods needed a lot of purification because of the non-specific detection system and many mistakes were done when believing that certain peaks belonged to a specific estrogen. Extremely sensitive methods could be developed using e.g. electron capture detectors and the resolution was improved when capillary columns were developed. However, at the same time the first GC–MS instruments were developed and further equipped with techniques allowing measurement of single ions. When these became available it was found that much of the earlier work done had little to do with reality because one peak could contain many compounds. The monitoring of single ions called selected (selective) ion monitoring (GC–MS–SIM) resulted in more specific assays. Still the purification needed was extensive in order to achieve sufficient specificity and sensitivity for those estrogens with low concentrations but still of great biological interest, like some catechol estrogens. Purifica-

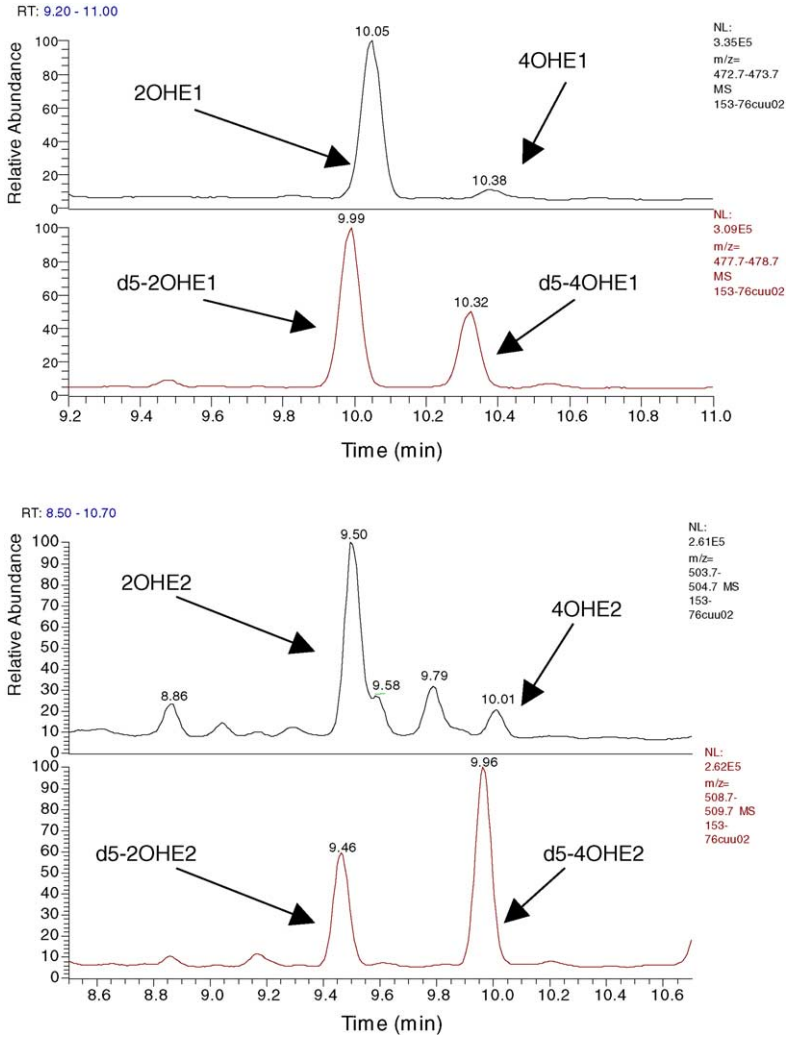

Fig. 4. GC–MS–SIM chromatograms of catecholestrogens in urine. The oxoestrogens are in the form of ethoximes.

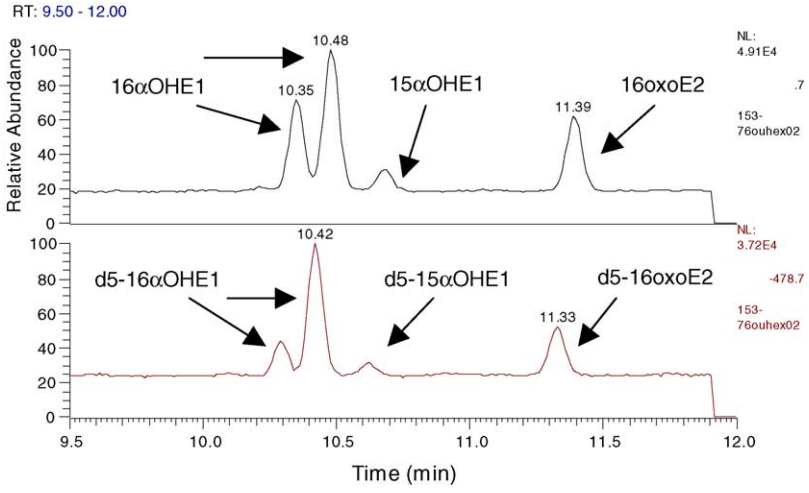

Fig. 5. GC–MS–SIM chromatograms of some ring D-ketolic estrogen ethoximes.

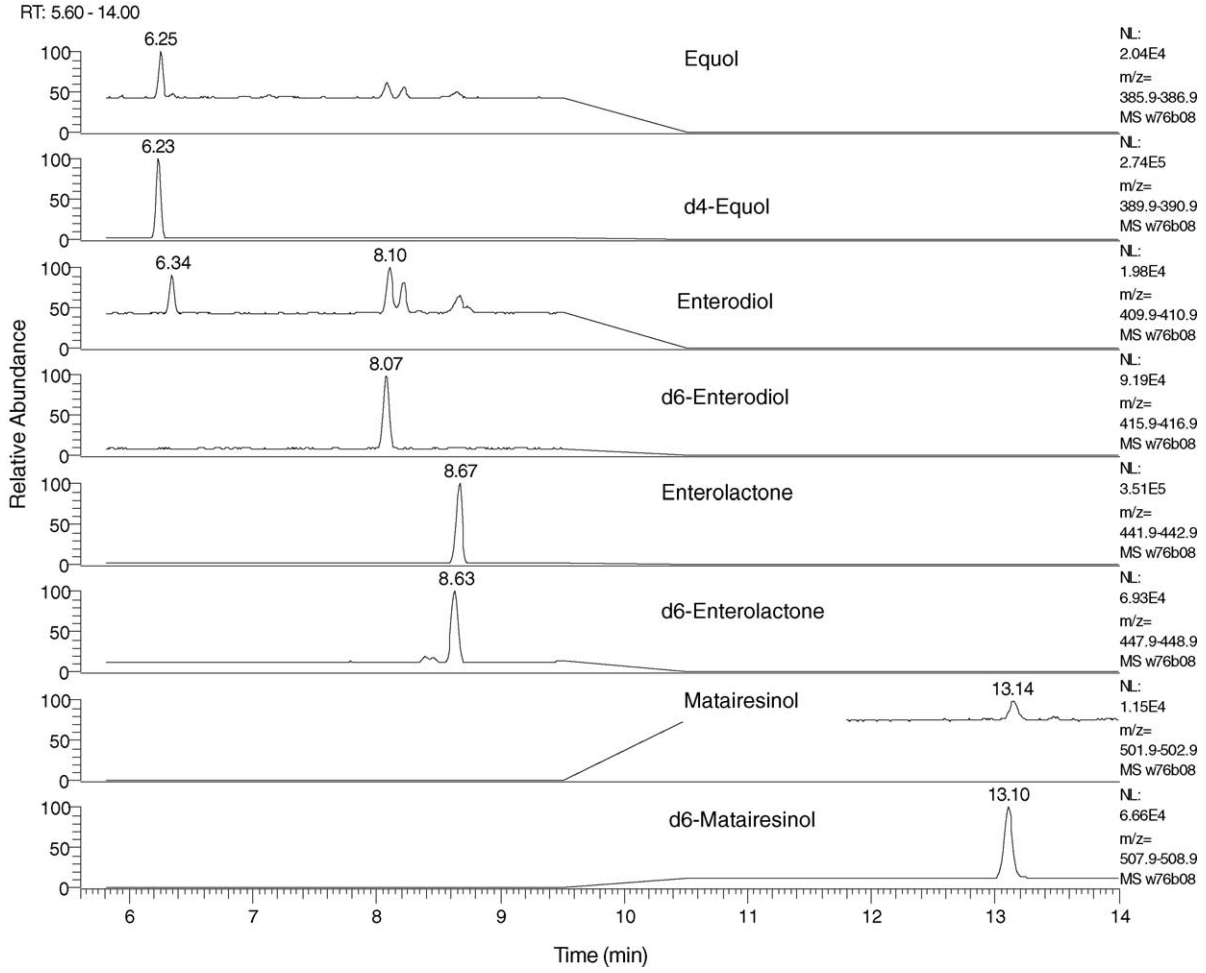

Fig. 6. GC–MS–SIM results for some lignans and equol.

tion was also necessary in order to eliminate the interference by neutral steroids and phytoestrogens. The mammalian lignan enterolactone interfered particularly with the α-ketolic estrogens. Another problem was the extreme instability of the last-mentioned estrogens making various purifying procedures involving alkaline reagents impossible.

Both the specificity and instability problems were solved by ethoximation of all ketonic estrogen conjugates directly in urine before initiating the extraction procedure. [5,30]. The corresponding deuterated ethoximated internal standards, deuterated in the ethoxime group, were then added to the sample before hydrolysis. These novel steps solved the main problems: the stability of the labile estrogens and the final correction of the values of these compounds for losses during the procedure. The accuracy for the other compounds was secured by synthesizing deuterated internal standards for most of the non-ketonic estrogens.

Because of improvement of the sensitivity of the GC–MS instruments, more and more estrogens could be added to the method and finally a number of isoflavones and lignans, both plant lignans and mammalian lignans were included [15]. In the present, final version of the method 27 compounds are measured in one small urine sample (1/150th of a 24 h sample). Minimum used has been 2.5 ml for an overnight sample or 5 ml for a postmenopausal sample and for rat and mouse urine 2 ml of pooled urine. The results show that the method is highly sensitive and specific because of the extensive purification of the urine before GC–MS and the internal deuterated standards cover all losses except those in the first steps of the method. Further experiments have shown that these losses are about 10% but they are not corrected for.

A large review on the measurement of endogenous estrogens has recently been published [31] discussing all the main problems involved. In agreement with the review HPLC methods even using coulometric electrode array detection (CEAD) have, in our hands, been found to be too insensitive for many of the important metabolites of estrogens in urine. Only after estrogen load in animal studies HPLC has been used. However, HPLC–CEAD is excellent for the assay of phytoestrogens both in plasma and urine [32,33]. In his review Giese [31] techniques as the one presented here using isotope dilution are just mentioned but he indicates that such methods are not always accurate. In our opinion this is due mainly to insufficient purification of the samples.

Without deuterated or ^{13}C internal standards or using only one deuterated standard with only two deuterium atoms for a great number of estrogens combined with insufficient purification erroneous results are obtained [34,35]. The availability of ^{13}C labeled phytoestrogens has made it possible to develop a relatively simple method for the most abundant phytoestrogens in urine [36]. The results obtained with our method and its earlier versions in Finnish women [37–39] agree well with those obtained with the method of Grace et al. Recently time-resolved fluorescence immunoassay for enterolactone, genistein, daidzein, equol and *O*-desmethylangolensin in plasma and urine were developed They are very convenient provided that an automatic instrument for the TR-FIA assay is available.

More recent techniques like electrospray mass spectrometry (ESI–MS) [40] and various developments of atmospheric pressure chemical ionisation–mass spectrometry (APCI–MS) have not been used for measuring estrogen profiles in urine. Gas chromatography with electron capture detection (GHC–ECD) and gas chromatography–electron capture–mass spectrometry (GC–EC–MS) has been used only for single or a few estrogens. A method based on GC-negative chemical ionization mass spectrometry for the classical estrogens, the synthetic estrogen ethynylestradiol and 3 catecholestrogens (2-hydroxyestrone, 4-hydroxyestrone and 2-hydroxyestradiol) has been presented and validated [41]. The sensitivity of the method is similar to that described here. Furthermore, the three "classical" estrogens and their conjugates have been measured in serum by sodium cholate micelle capillary electrophoresis [42]. However, the procedure has very low sensitivity and seems, therefore, not to be applicable to urine analyses.

The method presented here has been used in several metabolic studies in its previous version including a smaller number of estrogens and phytoestrogens [43–46] and in two methodological studies to establish the specificity of a radioimmunoassay method [47,48].

Zhu and Conney [49] have reviewed the important topic of functional role of estrogen metabolism in target cells. An example of the role of the metabolism is the stimulation of SHBG production in HepG2 cells. The catechol estrogen 2-hydroxyestradiol is first converted to 2-methoxyestradiol before it stimulates SHBG production [50]. 2-Hydroxyestradiol is an important metabolite of estradiol in the liver.

We have in this presentation given all details, including minor ones, of the method so that anyone can reproduce it. The laboratory description for the technicians has been distributed to several laboratories and will be sent on request. It is concluded that the method, despite its complicated nature, fulfills the reliability criteria of a research method.

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