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RESEARCH PAPER

Receptor-based high-throughput screening and identification of estrogens in dietary supplements using bioaffinity liquid-chromatography ion mobility mass spectrometry

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Abstract A high-throughput bioaffinity liquid chromatography-mass spectrometry (BioMS) approach was developed and applied for the screening and identification of recombinant human estrogen receptor α (ERα) ligands in dietary supplements. For screening, a semi-automated mass spectrometric ligand binding assay was developed applying $^{13}C_2$,^{15}N-tamoxifen as non-radioactive label and fast ultra-high-performance–liquid chromatography–electrospray ionisation–triple-quadrupole-MS (UPLC-QqQ-MS), operated in the single reaction monitoring mode, as a readout system. Binding of the label to ERα-coated paramagnetic microbeads was inhibited by competing estrogens in the sample extract yielding decreased levels of the label in UPLC-QqQ-MS. The label showed high ionisation efficiency in positive electrospray ionisation (ESI) mode, so the developed BioMS approach is able to screen for estrogens in dietary supplements despite their poor ionisation efficiency in both positive and negative ESI modes. The assay was performed in a 96-well plate, and all these wells could be measured within 3 h. Estrogens in suspect extracts were identified by full-scan accurate mass and collision-cross section (CCS) values from a UPLC-ion mobility-Q-time-of-flight-MS (UPLC-IM-Q-ToF-MS) equipped with a novel atmospheric pressure ionisation source. Thanks to the novel ion source, this instrument provided picogram sensitivity for estrogens in the negative ion mode and an additional identification point (experimental CCS values) next to retention time, accurate mass and tandem mass spectrometry data. The developed combination of bioaffinity screening with UPLC-QqQ-MS and identification with UPLC-IM-Q-ToF-MS provides an extremely powerful analytical tool for early warning of ERα bioactive compounds in dietary supplements as demonstrated by analysis of selected dietary supplements in which different estrogens were identified.

Keywords Bioaffinity mass spectrometry · Ligand binding assay · Estrogen receptor · Dietary supplements · High-throughput screening · Ion mobility mass spectrometry

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Introduction

In 2006, the nutritional supplement market was worth US$ 60 billion and is growing continuously [1]. Supplements are widely available in local stores and on the Internet and easy to order for (professional) athletes to enhance their performance and for ordinary people with certain life styles. However, several investigations have shown the deliberate and unintentional addition of (pro)hormones to supplements [2–7]. Labels of these supplements are often incomplete or incorrect [4, 7]. The

presence of anabolic steroids in supplements is banned in the European Union (EU) and USA. Despite this, various supplements are easily accessible to consumers. Geyer et al. described that 21 % of supplements purchased in the EU contained anabolic steroids [7]. Generally, they assumed that the presence of steroids in supplements is a result of accidental cross-contamination during either manufacturing or packaging [7]. As an example of deliberate addition of steroids to herbal supplements, Toorians et al. presented in their study the presence of diethylstilbestrol (DES) in a supplement marketed on the Internet for prostate problems [4]. Due to the high intake of DES (4.1 ± 0.1 mg g^{-1}) through these pills, the male consumer of this herbal supplement developed abnormally large mammary glands. In modern laboratories, gas chromatography–mass spectrometry (GC-MS) and liquid chromatography (LC)-tandem mass spectrometry (MS/MS) are used for the fast, sensitive and specific detection of steroids [8–10], appetite suppressors [11], mycotoxins [12] and pharmaceuticals [13] in dietary supplements. In order to achieve the highest sensitivity and selectivity, both GC-MS and LC-MS/MS are set up in pre-selected ion or ion transition acquisition modes [9, 14–17]. In this way, only known compounds can be measured, and new estrogenic compounds may escape from routine testing and remain undetected. Alternatively, biorecognition-based assays, using, e.g. the estrogen receptor (ER), are used for rapid screening of estrogenic compounds. However, non-immobilised ER tends to be very sensitive to slight changes in, e.g. temperature, salt concentration and pH [18]. Usami et al. [19] developed a surface plasmon resonance (SPR)-based biosensor assay in which 17β-estradiol (E2) was used as a ligand, human recombinant ERα for biorecognition and test chemicals as competitors. By means of this biosensor assay, dissociation constants for the binding of estrone (E1), β-E2, estriol (E3), tamoxifen (Tamo), DES, bisphenol A (BPA) and 4-nonylphenol were determined. Blair et al. [20] determined the relative binding affinity for a large group of chemicals by using an ERα competitive ligand binding assay. In this radio receptor assay, ER was obtained from rats, and [^{3}H]-E2 was used as the competing label. The obvious disadvantages of this assay include the use of a radiolabel and the long assay time of 24 h. The combination of a bioaffinity extraction with MS detection could serve as a powerful tool for the identification of known and unknown estrogenic compounds. Choi et al. developed a screening assay for ligands of the estrogen receptor based on magnetic microparticles and LC-MS [21]. Although this method was capable of screening genistein and daidzein in botanical extracts, the throughput of this method was low, and a high amount of the costly estrogen receptor was required due to the low affinity of the phytoestrogens [22, 23]. De Vlieger et al. [24] developed an online dual post-column estrogen receptor affinity assay based on fluorescence (limit of detection (LOD) 4.7 nM) and parallel detection by MS (LOD 40 nM) for quantification and identification purposes of estrogenic compounds. However, in order not to decrease receptor activity by the LC mobile phase gradient, a make-up gradient had to be added post-column in order to dilute the organic solvent content, thus complicating the setup. To decrease protein consumption and to omit the make-up gradient, pre-column bioaffinity MS methods have been described in literature as well [25–28]. Niessen et al. developed an off-line competitive MS binding assay for determining the binding affinity of dopamine receptor ligands using spiperone as a label [27]. That binding assay was presented as a possible alternative to radiolabeled assays; however, since only the unbound fraction of the marker was measured, at best, indirect information was obtained about the bound ligands. Moreover, because of the use of a non-volatile buffer, an additional solid-phase extraction (SPE) step was required prior to LC-MS detection. Due to the SPE step and the absence of microtiter plates, high-throughput screening was not feasible. Zepperitz et al. described a competitive MS binding assay in which the γ-aminobutyric acid transporter-bound fraction of the label was measured after elution with methanol [25]. Although that method had the potential for high-throughput characterisation of new drug candidates, the format was used for kinetic measurements in buffer only, and no screening in real samples was performed. By the lengthy (30–60 min) filtration steps during wash and dissociation steps, the method became longer and less straightforward. In general, these off-line pre-column bioaffinity MS methods are focused on determining affinities, and no screening or identification is performed for food or environmental contaminants [25–28]. In a previous bioaffinity MS (BioMS) study, a mass spectrometric ligand binding assay was presented in pre-column format in which recombinant human sex hormone-binding globulin (rhSHBG) and LC-MS were used for screening and identification of androgenic and estrogenic (designer) steroids in dietary supplements [29]. Although that method was rapid and able to identify (un)known rhSHBG binders in supplements, the screening of estrogens other than β-E2 was not feasible due to the low affinity; moreover, identification of this estrogen could only be achieved following derivatisation and GC-MS/MS. In the present work, a semi-automated high-throughput BioMS method is presented in which the recombinant ligand binding domain (LBD) of human ERα and dedicated modes of fast ultra-high performance liquid chromatography (UPLC)-MS are used for screening and identification of estrogenic steroids in dietary supplements. Since estrogens have poor ionisation efficiencies in both positive and negative electrospray ionisation (ESI) modes [30, 31], a label having excellent ionisation efficiency in ESI is required for the rapid screening of estrogens. Therefore, a mass spectrometric ligand binding assay was developed based on $^{13}C_2$,^{15}N-tamoxifen as label, which has a high ionisation efficiency in positive ESI and fast UPLC-electrospray ionisation-triple-quadrupole-MS (UPLC-QqQ-MS), operated in the single reaction monitoring (SRM) mode, as readout system for the detection and

quantification of the non-radioactive label. The LBD of ERα was immobilised covalently and non-covalently onto paramagnetic microbeads using two different surface chemistries. To identify the estrogens, a UPLC-ion mobility-Q-ToF-MS equipped with a novel atmospheric pressure ion source [32] was used to obtain adequate ionisation efficiency, retention time, collision cross-section (CCS) values and high-resolution full-scan accurate mass data. This novel ion source was evaluated for the first time in negative ion mode. Several dietary supplements were screened for ER binders, and, in suspect samples, different estrogens were identified to demonstrate the applicability of this newly developed ERα-based BioMS approach.

Experimental

Materials

Tamoxifen (2-{4-[(1Z)-1,2-diphenylbut-1-en-1-yl] phenoxy}-N,N-dimethylethanamine, Tamo), $^{13}C_2$,^{15}N-tamoxifen (tamoxifen-^{15}N,N,N-dimethyl-$^{13}C_2$ ($^{13}C_2$,^{15}N-Tamo)), zearalenone (Zon), BPA, naringenin (Nar), β-sitosterol (β-Sito), 2-(*N*-morpholino) ethanesulfonic acid (MES) and *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC) were purchased from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands). 17β-Estradiol (β-E2), 17α-estradiol (α-E2), 17α-ethinyl estradiol (EE2), estrone (E1) and diethylstilbestrol (DES) were purchased from Steraloids (Newport, RI, US). Acetonitrile (ACN) and methanol (MeOH) were from Biosolve (Valkenswaard, The Netherlands) and formic acid (HCOOH), EDTA (Triplex), HEPES, sodium azide (NaN_3), imidazole and sodium chloride (NaCl) from Merck (Whitehouse Station, NJ, USA). The purified recombinant his-tagged wild-type ligand binding domain (LBD) of the human estrogen receptor α (ERα, 25 kD) was kindly provided by Dr. Luca Varani of the Institute for Research in Biomedicine (Bellinzona, Switzerland). The LBD was over-expressed in *Escherichia coli*, refolded from inclusion bodies and purified with His-tag affinity and size exclusion chromatography. LoBind tubes were provided by Eppendorf (Hamburg, Germany) and LoBind 96-well micro flat-bottom plates (655161) and 96-well micro V-shaped bottom plates (651201) by Greiner Bio-One B.V. (Alphen a/d Rijn, The Netherlands). Water purification was performed using a Milli-Q system (Milipore, Bedford, MA, USA). SiMAG-Carboxyl microbeads (product number 1402–1, 1 μm diameter) and SiMAG-IDA/Nickel microbeads (product number 1512–1, 1 μm diameter) were supplied by Chemicell GmbH (Berlin, Germany). The Milliplex magnet Handheld Magnetic Separator Block for 96-well flat-bottom plates was purchased from Milipore, the microtiter plate vari-shaker from Dynatech (Alexandria, VI, USA), the REAX2 head-over-head shaker from Heidolph (Schwabach, Germany) and the automatic magnetic wash station BioPlex™ pro II from BioRad Laboratories B.V. (Veenendaal, The Netherlands).

Instrumentation

Ultra-high performance liquid chromatography–triple-quadrupole mass spectrometry A Waters (Milford, MA, USA) Acquity Ultra-Performance LC (UPLC) system, consisting of a degasser, a binary gradient pump, an auto sampler (at 10 °C) and a column oven (at 50 °C), was used. The injection volume was 10 μL and the analytical column an Acquity UPLC™ BEH 1.7 μm C18, 50×2.1 mm I.D. from Waters. The UPLC system was coupled to a Micromass (Manchester, UK) Quattro Platinum tandem mass spectrometry (QqQ) system equipped with an ESI source used in positive ion mode. The two mobile phases consisted of (A) H_2O/HCOOH (99.9/0.1 %, *v*/*v*) and (B) ACN/HCOOH (99.9/0.1 %, *v*/*v*), and the flow rate was 0.3 mL min^{-1}. The gradient started at 30 % B for 0.30 min, increased sharply to 95 % B in 0.10 min with a hold for 1.10 min and returned to the initial state in 0.05 min with a final hold of 0.45 min. The total run time was 2 min only. The UPLC was interfaced with the ESI MS/MS instrument without a flow split. The ESI capillary voltage was +2.7 kV, and the cone voltage was 30 V. The desolvation gas was nitrogen (700 L/h) with a temperature of 350 °C; the source temperature was 120 °C, and the collision-induced dissociation gas was argon at a pressure of 2.5×10^{-3} mbar. Data acquisition for $^{13}C_2$,^{15}N-Tamo, which was used as label in this study, was performed in SRM mode at transition m/z 375.2→m/z 75.3 using collision energy 25 eV.

Ultra-high performance liquid chromatography–ion mobility–quadrupole–time-of-flight mass spectrometry Chromatography was performed on an Acquity UPLC system consisting of a degasser, a binary gradient pump, an auto sampler (at 10 °C) and a column oven at 50 °C. The UPLC system was coupled to a Synapt G2-S (Waters) ion mobility-quadrupole-time-of-flight-MS (IM-Q-ToF-MS) system equipped with a novel atmospheric pressure ionisation (API) source. In this API source design [32], a high-velocity droplet stream is impacting on a high-voltage electrode (see also Fig. S1 in the Electronic supplementary material). The injection volume of the sample from a 384-well plate was 10 μL, and the analytical column was an Acquity UPLC™ BEH 1.7 μm C18, 50×2.1 mm I.D. from Waters. The mobile phase consisted of (A) H_2O/NH_4OH (99.9/0.1 %, *v*/*v*) and (B) MeOH/NH_4OH (99.9/0.1 %, *v*/*v*), in order to support negative ion formation for estrogens. After injection, separation was done using gradient elution at a constant flow rate of 0.6 mL min^{-1}. The gradient started at 30 % B for 0.3 min followed by a linear increase to 95 % B in 0.1 min with a hold of 1.1 min and from 95 % to 30 % B in 0.05 min with a hold of 0.45 min, prior to the next injection.

The prototype ion source was operated in the negative ion mode at 150 °C and a voltage of −4 kV with a cone voltage of −40 V. In order to get extra structural information, MS^e was applied, which means simultaneous scans at low (20 eV) and high collision energy (ramped from 20 to 55 eV) in separated data acquisition functions. Additionally, in order to measure specific product ions at the highest sensitivity, the Q-ToF-MS was also operated in accurate mass product ion scanning mode. The ion mobility separation was performed under the following conditions: trap collision energy −4.0 V, transfer collision energy −2.0 V, IM gas N_2, IMS gas flow 90 mL min^{-1}; IM wave height −40 V; IM wave velocity 800 m/s. As lock mass, a solution of leucine-enkephalin was continuously introduced by a separate lock mass probe at a flow rate of 10 μL min^{-1}. For accurate mass calibration of the *m/z* axis, *m/z* 554.2615 was used as a lock mass in negative ion mode.

Methods

The protocols for immobilisation of the ERα LBD on the two types of paramagnetic beads are described in the Electronic supplementary material.

Sample preparation The primary extraction of steroids from dietary supplements was based on the procedure described by Rijk et al. [33], which was simplified by omitting the SPE and evaporation steps. The dietary supplements were ground, and 0.05 gram was weighed into a 10 mL plastic tube. To this tube, 2 mL MeOH and 2 mL H_2O were added for extraction of steroids. First, the tubes were placed in an ultra-sonic bath for 10 min, followed by 15 min head-over-head mixing. The tubes were centrifuged at 3,000×*g* for 10 min; the supernatant was transferred into glass vials and diluted five times with PBST buffer (9 g NaCl, 0.76 g Na_2HPO_4, 0.17 g KH_2PO_4, 2 mL Tween-20 25 % in 1 L H_2O) from which 100 μL was used in the BioMS assay for screening and identification purposes.

BioMS screening First, 100 μL of PBST was added to each well of a 96-well flat-bottom plate, followed by the addition of 6 μL of ERα-coated Ni^{2+}- or COOH-beads. Then, the Milliplex magnetic plate (Millipore, Bedford, MA, USA) was used to magnetise the beads against the well wall in 1 min after which the supernatants were removed. Next, 0.5 ng of label ($^{13}C_2$,^{15}N-Tamo) in 100 μL PBST (with or without addition of competing estrogens) was added to the wells. To construct dose–response curves, various competitors were added between 0 and 1,000 ng. In case of screening of supplement extracts, 0.5 ng of label in 100 μL supplement extract (five times diluted) was added to the beads in the wells. Following the addition of the label, the mixture was incubated for 15 min on a shaker at 500 rpm (Dynatech Alexandria, VI, USA). To remove the unbound label and unbound competitors, the automatic magnet wash station was used to wash the 96-well plate three times with PBST buffer. After the wash cycle, 70 μL PBST buffer were added to each well, and the content of each well was transferred by an eight-channel pipette into a new 96-well flat-bottom plate. This step was done prior to the elution step, in order to exclude non-specific binding of competitors to the surface of the well-plate. The well-plate was placed on the magnetic plate, and after 1 min, the supernatants were removed. To elute all bound label and bound competitors, 50 μL of elution solution (H_2O/ACN/HCOOH, 49/50/1 %, *v/v/v*) was added to the wells, and the plate was shaken for 2 min (500 rpm). With the help of the magnetic plate, the supernatants with all the eluted compounds were transferred to a 384-well plate for rapid UPLC-QqQ-MS screening. The total duration of this BioMS assay procedure was 30 min for 96 samples. Dose–response curves were fitted using the five-parameter curve fitting in the GraphPad Prism software of GraphPad Software Inc. (La Jolla, CA, USA).

Bioaffinity isolation prior to chemical identification The bioaffinity isolation procedure deviates from the BioMS screening by the use of five times more ERα-coated beads without label. So, 30 μL of ERα-coated beads (either Ni^{2+} or COOH) was added to the plate. After diluting the primary supplement extract in PBST, 100 μL were added to the plate, incubated for 15 min, and the same procedure was followed as described for the screening, except that the UPLC-IM-Q-ToF-MS was used for identification. The total duration of this bioaffinity purification procedure was <30 min for 96 samples.

Results and discussion

BioMS screening assay The principle of the BioMS screening assay is based on competition between the $^{13}C_2$,^{15}N-Tamo label and any known or unknown estrogenic compound in a sample for binding to the LBD of the ERα coupled to paramagnetic microbeads. The amount of label recovered by the elution solution decreases if a competing ERα binder, e.g. β-E2, is present in a sample. The limit of detection (LOD) of the $^{13}C_2$,^{15}N-Tamo label in the UPLC-QqQ-MS was determined as 500 fg on-column (S/N>6). This estrogenic compound was chosen because of its very high ionisation efficiency in ESI+ due to the high proton affinity of its tertiary amine substructure. This is in sharp contrast to other estrogens, of which most are phenolic and show poor ionisation in both ESI+and ESI−. During the development of the semi-automated BioMS

screening assay, various parameters were optimised, such as LC conditions for the label, relative amounts of label and receptor required for reproducible measurements, influence of buffer composition and incubation, wash and elution conditions. After testing different amounts of label with different amounts of beads and elution solvents (data not shown), the optimum amount of added label was 0.5 ng per test when using H_2O/ACN/HCOOH (49/50/1 %, *v/v/v*) as the elution solvent. The optimum amounts of the two types of beads, oriented non-covalent immobilised His-tagged ERα onto Ni^{2+}-coated paramagnetic beads versus non-oriented covalent coupled ERα onto COOH-coated paramagnetic beads, were investigated (Fig. S2 in the Electronic supplementary material). The absolute eluted amounts of label increased with increasing amounts of the two types of ERα-coated beads (from 1 to 45 pg using 2 to 10 μL of ERα-coated Ni^{2+}-beads and from 2 to 55 pg using 2 to 10 μL of ERα-coated COOH-beads). The amount of label found in a negative control without any ERα-coated beads was close to the LOD, which proved that only the ER was responsible for capturing the label. The results in Fig. S2 (Electronic supplementary material) demonstrate that, when ERα is immobilised in a non-oriented covalent manner, slightly higher label yields are obtained. It is important to note that the immobilisation procedure of Ni^{2+}-beads is shorter than with COOH-beads (1 versus 3 h, see the Electronic supplementary material). The stability of ERα-coated beads stored at 4 °C was determined to be 2 weeks for both bead types, which is in sharp contrast to non-immobilised ERα, which is stable for <1 day at 4 °C [18]. Further experiments proved that 6 μL of both types of ERα-coupled beads yielded reproducible label recoveries and, in the presence of estrogens, good competition. This means that, with each immobilised bead stock, 83 tests can be performed; however, multiple bead stocks can easily be prepared in parallel.

Typical reconstructed UPLC-QqQ-MS chromatograms of the eluted label from non-oriented covalently coupled ERα-coated beads (Fig. 1) illustrate the fast analysis, with a total run time of 2 min, and the effect of a competitor on the amount of label measured with the BioMS screening assay. Dose–response curves were constructed with ERα-coated COOH-beads for different estrogenic compounds in buffer (Fig. 2). Sensitivities at 50 % inhibiting concentration (IC50) were found to range from 4 ng mL^{-1} (0.4 ng absolute) for EE2 as the strongest binder to 550 ng mL^{-1} (55 ng absolute) for BPA as the weakest binder, and all curves illustrate good precision of the method. For clarity, the dose–response curves of E1, E3, Tamo, Zon and Nar are not shown in this figure, but the calculated IC_{50} values are given in Table 1. The same dose–response curves were made with Ni^{2+}-beads and in Table 1, all IC_{50} values are summarised. In dietary supplements, intentionally added estrogenic compounds are typically present at high levels (60 to 1,800 μg g^{-1}) [4, 9] which would correspond to final concentrations in the BioMS screening assay between 0.15 and 4.5 μg mL^{-1} which is far above the IC50 values obtained with the estrogens in the BioMS screening. The developed BioMS assay showed in general lower sensitivities compared with alternative receptor-based assays in the literature, such as the yeast estrogen bioassay (YEB), the tritium-label displacement method and the fluorescent polarisation (FP) competitive inhibition assay [20, 22, 34–36]. However, the BioMS sensitivities are more than adequate for the screening of estrogenic compounds in dietary supplements. Relative binding (RB) values for various ERα competitors (see Table 1) were calculated by dividing the IC_{50} value of β-E2 (RB=1) by those of other competitors and RB values <1 represent binders having lower affinity towards ERα. In literature, affinities of ERα binders were investigated by the FP competitive inhibition assay, the SPR-based biosensor assay, the YEB and tritium-labelled displacement approaches [20, 22, 34–36]. The calculated RB values from our BioMS screening method and the values obtained from literature compare generally well (Table 1): Similar weak and strong binders are found in the BioMS assay (EE2>β-E2>DES=E1>E3>BPA). The slightly lower IC_{50} values obtained with COOH-beads than those with the Ni^{2+}-beads are possibly due to the influence of the different immobilisation approaches.

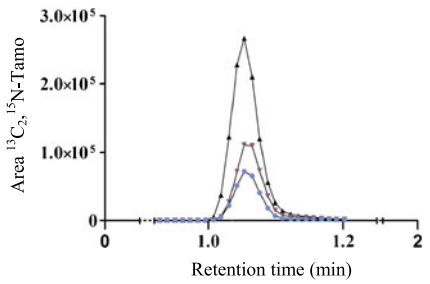

Fig. 1 Reconstructed UPLC-QqQ-MS SRM chromatograms showing the peak areas and retention time of the label ($^{13}C_2$,^{15}N-Tamo) eluted from 6 μL of ERα-coated COOH beads in the absence (*black upright triangle*) and in the presence of 1 ng (*black inverted triangle*) and 10 ng (*black circle*) of β-E2 as the competitor

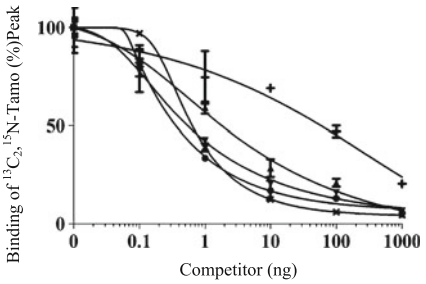

Fig. 2 Normalised average dose–response curves ($n=2$) for α-E2 (*black upright triangle*), β-E2 (*black inverted triangle*), EE2 (*black circle*), DES (*x* mark) and BPA (*plus sign*) in PBST buffer obtained by the BioMS screening assay using 6 μL of ERα-coated COOH beads and UPLC-QqQ-MS

Table 1 IC_{50} values and relative binding (RB) of various ERα competitors in the BioMS assay as measured by $^{13}C_2$,^{15}N-Tamo label response in the UPLC-QqQ-MS compared with literature data from other assays (FP competitive inhibition assay [34], SPR-based biosensor [19], the YEB [22] and radiolabel receptor assay (RRA) [20, 35, 36]

	BioMS				FP [34]		SPR [19]	YEB [22]		RRA [20]	
	Ni^{2+} beads		COOH beads								
Competitor	IC_{50} (ng mL^{-1})	RB	IC_{50} (ng mL^{-1})	RB	IC_{50} (ng mL^{-1})	RB	K_D[a] (nM)	EC_{50} (ng mL^{-1})	REP[b]	IC_{50} (ng mL^{-1})	RB
EE2	8	1.25	4	1.25				0.15	1.2	0.15	1.7
β-E2	10	1	5	1	3.6	1	0.4	0.14-0.28	1.0	0.25	1
α-E2	60	0.17	20	0.25				1.96	0.07	8.1	0.03
E1	20	0.5	6	0.83	146	0.02	4.29	0.76	0.2	3.0	0.08
E3	55	0.18	30	0.17			0.8	35	0.01	2.7	0.09
DES	14	0.71	7	0.7	2.9	1.18	0.1	0.16	1.0	0.05	5, 2,4[35]
Tamo	45	0.22	15	0.33	100	0.03	22				
Zon	70	0.14	12	0.42				33	5E-3		
BPA	400	3E-3	550	9E-3	14E3	4E-4	13.5E3			5E3	5E-5
Nar	250	4E-3	30	0.17				2.5E4	<1E-5		0.01-0.07 [36]
β-Sito	–	–	–	–				–[23]	–		–[36]

En dash means no IC_{50} value obtained as no binding was observed within the concentration range tested

[a] Dissociation constants (K_D) of ERα and estrogens obtained with SPR biosensor (the lower the K_D value, the higher the affinity)

[b] Relative estrogenic potency (REP) of compounds with ERα

BioMS screening of estrogens in dietary supplements To investigate the performance of our new BioMS screening, 13 different dietary supplements, previously analyzed for the presence of estrogens by another BioMS method using sex hormone-binding globulin (SHBG) as a biorecognition element [29] and LC-MS/MS [9] or LC-Q-ToF-MS [4], were screened again using the newly developed BioMS screening method based on ERα (Fig. 3). From the 13 dietary supplements, samples 1–10 were previously screened as blanks, and samples 11–13 were expected to contain estrogenic compounds. Sample 11 is a herbal food supplement marketed as 'a non-estrogenic mixture' for the treatment of mild prostate cancer and is described by its manufacturer as a pharmaceutical that is tested for toxicity in a trial with prostate cancer patients. However, this supplement showed a strong effect in the yeast estrogen bioassay [4], and by using LC-ToF-MS method, this was caused by very high levels of DES (4.1±0.1 mg g^{-1}). Sample 12 is a suspect dietary supplement that was ordered via the Internet, intercepted by the Belgian inspection at the post-office, and when analyzed with conventional LC-MS/MS, the results revealed the presence of several steroids including β-E2 [9]. Indeed that steroid was also screened suspect with the SHBG-based BioMS approach [29]. The third suspect sample (sample 13) was a birth control pill containing 30 μg EE2 per pill. Figure 3 shows the screening results of the BioMS using both ERα-coated Ni^{2+}- and COOH-beads. The BioMS assay results show that the blanks gave average eluted amounts of label of 51±10 and 42±7 pg using ERα-coated COOH- and Ni^{2+}-beads, respectively. Decision levels calculated from these data (average minus 3 times SD) indicate that label levels below 21 pg pinpoint to suspect samples for both types of beads. Indeed, the known suspect samples gave eluted amounts of label below that decision level (7±4 and 6±2 pg for the ERα COOH- and Ni^{2+}-beads, respectively). Since phytosterols and phytoestrogens may occur in sports supplements, the potential binding of these compounds to ERα was investigated. The labels of supplements 3 and 8 declared the presence of 10 mg of phytosterols β-sitosterol, campesterol and stigmaterol. As these supplements were screened as blanks, no false-positive results are obtained in the BioMS screening by the presence of 10 mg/pill phytosterol in

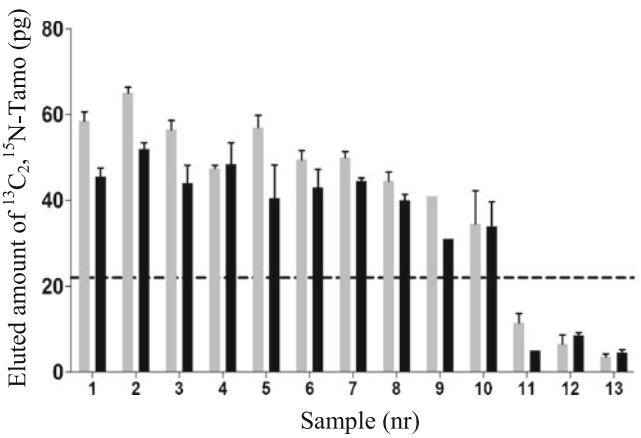

Fig. 3 BioMS screening results of blank (samples 1 to 10) and positive (samples 11 to 13) dietary supplements (using ERα-coated Ni^{2+}- (*black bars*) and COOH-beads (*grey bars*)). Data are the mean of duplicate analysis (dash line shows the level of decision for both bead types)

dietary supplements. The affinities of phytoestrogens such as daidzein, genistein, enterolactone and equol are reported to range from very low affinity to no affinity towards ERα [22, 23] causing no false-positive results in the BioMS screening. Phytoestrogens such as genistein and daidzein bind to ERα when high amounts of ERα are used (e.g. 20 μg) [21]; however, in the BioMS screening, <0.6 μg was used. The results show that the BioMS screening assay using $^{13}C_2$,^{15}N-Tamo as a label and fast UPLC-QqQ-MS as a read-out system is fit for purpose of screening any (un)known estrogens in dietary supplements.

BioMS chemical identification of estrogens In this study, a UPLC-IM-Q-ToF-MS system equipped with a novel API source was used. The ionisation mechanism is based on a high-velocity droplet stream impacting on a high-voltage electrode. An image of the novel ion source is included in the Electronic supplementary material (Fig. S1). A major advantage of such a "multi-source" is that it ionises a wide range of polarities in a single chromatographic run without switching between ESI and APCI. Compared with state-of-the-art ESI, higher ionisation efficiencies were obtained in both negative (see Fig. S3, in the Electronic supplementary material) and positive ion modes [32]. The UPLC-IM-Q-ToF-MS system also acquires ion mobility drift time data from which CCS values can be derived, which offer an additional orthogonal identification point next to retention time, accurate mass and MS/MS data. The experimental CCS values can be compared with the theoretical CCS values from modelling software (e.g. MOBCAL). As an example, Fig. 4 shows typical UPLC-IM-Q-ToF-MS reconstructed accurate mass ion chromatograms, highlighting the fast and good separation, even for isobaric estrogen isomers, including the accompanying ion mobility drift times. Additionally, Fig. 5 illustrates a 3D view of retention times and ion mobility drift times of a standard mixture of DES, EE2 and β-E2.

In Table 2, all identification results for estrogens in the suspect dietary supplements obtained by UPLC-IM-Q-ToF-MS are summarised and compared with literature data [4, 9]. All suspect samples were subjected to identification using both ERα-coated Ni^{2+}- and COOH-beads, and two different biopurified extracts were prepared: "screening extracts" (using the normal amount of beads plus label) and "dedicated identification extracts" (i.e. five times more beads without the addition of label). The screening extracts were used for rapid identification, and the identification extracts were prepared to increase the chance to detect compounds having very low RB values in the BioMS approach. In the three positive samples of Fig. 3, β-E2, EE2 and DES were identified on basis of retention time, accurate mass and MS/MS spectra (in accurate product ion scanning mode), ion mobility drift times and CCS values. Table 2 demonstrates that both in screening and identification extracts, β-E2 (in sample 12), EE2 (in sample 13) and DES (in sample 11) were identified. This means that, for

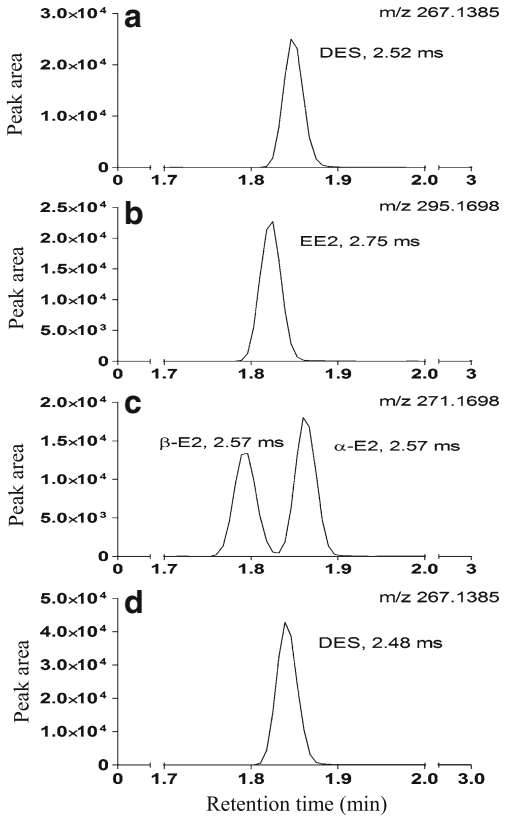

Fig. 4 Reconstructed UPLC-IM-Q-ToF-MS ion chromatograms (within± 0–2 ppm window) of **a** DES, **b** EE2 and **c** the isomers β-E2 and α-E2 (all standards, 1 ng on-column) and of DES (**d**) in the extract of suspect sample nr 11

screening and identification of strong ERα-binders, the leftover from the screening extract is sufficient for unambiguous identification. Only a slight difference was observed between the results obtained with screening and identification extracts. Measuring the screening extracts in MS^e mode, only the most abundant product ion was detected for β-E2, EE2 and DES

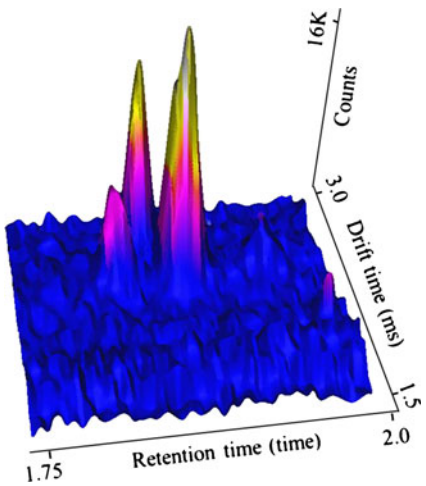

Fig. 5 3D view of retention and ion mobility drift times (within 200–350 *m/z*) of a standard mixture of 10 ng mL^{-1} β-E2, EE2 and DES analyzed by the UPLC-IM-Q-ToF-MS system

Table 2 Theoretical exact masses, elemental composition and experimental accurate mass, mass errors, retention times, drift times and CCS values of EE2, β-E2 and DES in standard solutions and in dietary supplement, affinity extracted with two BioMS protocols and identified using UPLC-IM-Q-ToF-MS

Measurement	Type of ERα	Extract	t_R	Experimental	Theoretical	Mass error	Elemental composition	Accurate mass product ion scanning	Drift times	CCS_{Exp}	CCS_{Theory}
(Sample nr)	Beads		(min)	$[M-H]^-$	$[M-H]^-$	(ppm)	$[M–H]^-$	(*m/z*)	(ms)	($Å^2$)	($Å^2$)
EE2											-
Standard	N.A.	N.A.	1.81	295.1669	295.1669	0	$C_{20}H_{23}O_2$	145.071, 159.086	2.75	96.36	
EE2 pill (13)	Ni^{2+}	Screening extract	1.81	295.1669		0	$C_{20}H_{23}O_2$	145.071, 159.085	2.84	98.56	
EE2 pill (13)	COOH	Screening extract	1.81	295.1669		0	$C_{20}H_{23}O_2$	145.065, 159.083	2.84	98.56	
EE2 pill (13)	Ni^{2+}	Identification extract	1.82	295.1668		0.4	$C_{20}H_{23}O_2$	145.071, 159.085	2.79	97.46	
EE2 pill (13)	COOH	Identification extract	1.82	295.1669		0	$C_{20}H_{23}O_2$	145.065, 159.084	2.79	97.46	
β-E2											99.38[a]
Standard	N.A.	N.A.	1.79	271.1696	271.1698	0.7	$C_{18}H_{23}O_2$	145.065, 183.080	2.57	92.27	
β-E2 pill (12)	Ni^{2+}	Screening extract	1.79	271.1696		0.7	$C_{18}H_{23}O_2$	145.065, 183.081	2.57	92.27	
β-E2 pill (12)	COOH	Screening extract	1.79	271.1696		0.7	$C_{18}H_{23}O_2$	145.065, 183.081	2.57	92.27	
β-E2 pill (12)	Ni^{2+}	Identification extract	1.79	271.1696		0.7	$C_{18}H_{23}O_2$	145.071, 183.082	2.52	91.13	
β-E2 pill (12)	COOH	Identification extract	1.79	271.1696		0.7	$C_{18}H_{23}O_2$	145.065, 183.079	2.57	92.27	
DES											-
Standard	N.A.	N.A.	1.85	267.1384	267.1385	0.3	$C_{18}H_{19}O_2$	251.113, 237.087	2.52	91.20	
DES pill (11)	Ni^{2+}	Screening extract	1.85	267.1384		0.3	$C_{18}H_{19}O_2$	251.112, 237.086	2.48	90.06	
DES pill (11)	COOH	Screening extract	1.85	267.1384		0.3	$C_{18}H_{19}O_2$	251.113, 237.086	2.52	91.20	
DES pill (11)	Ni^{2+}	Identification extract	1.85	267.1389		1.4	$C_{18}H_{19}O_2$	251.113, 237.087	2.57	92.34	
DES pill (11)	COOH	Identification extract	1.85	267.1384		0.3	$C_{18}H_{19}O_2$	251.112, 237.086	2.57	92.34	

N.A. not applicable

[a] Theoretical value of CCS from Shimizu et al. [39] calculated using MOBCAL and Trajectory Method

(*m/z* 145.065, 145.071 and 251.113, respectively). However, when the identification extracts were measured in MS^e mode, two specific ion products were detected for β-E2 (*m/z* 145.065, 183.080), EE2 (*m/z* 145.071, 159.086) and DES (*m/z* 251.113, 237.087) [37, 38]. In contrast to the MS^e results, in accurate mass product ion scanning mode, the two specific product ions for each estrogen were measured in both screening and identification extracts. Furthermore, no significant difference was observed between the identification results obtained with ERα-coated Ni^{2+}- or COOH-beads. By using the drift times, experimental CCS values were calculated based on polyalanine calibration and compared with theoretical CCS value for β-E2 (Table 2). The latter was adapted from Shimizu et al. [39] who used MOBCAL with Trajectory Method to calculate a theoretical CCS value of 99.38 $Å^2$, only 7 % higher than the experimentally determined CCS value for β-E2 (92.27 $Å^2$) in this work. It is important to note that the theoretical CCS values are significantly affected by error ratios in the modelling, especially for small molecules [40, 41]. The experimental CCS values of the estrogens in standard solutions were similar to the experimental CCS values in the suspect samples (only 1–2 % deviation). Increasing CCS values were calculated for increasing larger molecules: DES (*m/z* 267.1385: 91.20 $Å^2$), β-E2 (*m/z* 271.1698: 92.27 $Å^2$) and EE2 (*m/z* 295.1169: 96.36 $Å^2$). Although CCS values for DES and β-E2 are close to each other, further discrimination between these estrogens was easily achieved with the help of retention time and accurate mass acquired in full-scan mode with UPLC-IM-Q-ToF-MS.

Conclusions

In this study, we demonstrated that, by using $^{13}C_2$,^{15}N-Tamo as ESI+label in a mass spectrometric ligand binding assay, the developed BioMS approach is able to screen (un)known estrogens despite their poor ionisation efficiency in ESI [30, 31]. For rapid screening, ERα was successfully immobilised using either oriented or non-oriented approaches onto paramagnetic microbeads, and the BioMS assay was successfully demonstrated by screening for the ERα ligands DES, EE2 and β-E2 in dietary supplements. The method features ultrahigh-throughput (the entire sample treatment, BioMS assay and measuring time was <3 h for 96 tests) and the possibility to use even the leftover from the BioMS screening extract for subsequent rapid (<5 h for 96 tests) identification using UPLC-IM-Q-ToF-MS. This instrument provided excellent sensitivity using a novel API source and an additional identification point (experimental CCS value) next to retention time, accurate mass and MS/MS data. Thus, the combination of rapid bioaffinity screening using UPLC-QqQ-MS and identification with UPLC-IM-Q-ToF-MS is an extremely powerful analytical tool for early warning of ERα bioactive steroids in dietary supplements.

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References

1. Crowley R, FitzGerald LH (2006) The impact of cGMP compliance on consumer confidence in dietary supplement products. Toxicology 221(1):9–16
2. Kohler M, Thomas A, Geyer H, Petrou M, Schänzer W, Thevis M (2010) Confiscated black market products and nutritional supplements with non-approved ingredients analyzed in the cologne doping control laboratory 2009. Drug Test Anal 2(11–12):533–537
3. Plotan M, Elliott CT, Scippo ML, Muller M, Antignac J-P, Malone E, Bovee TFH, Mitchell S, Connolly L (2011) The application of reporter gene assays for the detection of endocrine disruptors in sport supplements. Anal Chim Acta 700(1–2):34–40
4. Toorians AWFT, Bovee TFH, de Rooy J, Stolker LAAM, Hoogenboom RLAP (2010) Gynaecomastia linked to the intake of a herbal supplement fortified with diethylstilbestrol. Food Addit Contam A 27(7):917–925
5. Maughan RJ (2005) Contamination of dietary supplements and positive drug tests in sport. J Sports Sci 23(9):883–889
6. Judkins CMG, Teale P, Hall DJ (2010) The role of banned substance residue analysis in the control of dietary supplement contamination. Drug Test Anal 2(9):417–420
7. Geyer H, Parr MK, Koehler K, Mareck U, Schänzer W, Thevis M (2008) Nutritional supplements cross-contaminated and faked with doping substances. J Mass Spectrom 43(7):892–902
8. Becue I, Van Poucke C, Van Peteghem C (2011) An LC-MS screening method with library identification for the detection of steroids in dietary supplements. J Mass Spectrom 46(3):327–335
9. Van Poucke C, Detavernier C, Van Cauwenberghe R, Van Peteghem C (2007) Determination of anabolic steroids in dietary supplements by liquid chromatography–tandem mass spectrometry. Anal Chim Acta 586(1–2):35–42
10. Van Thuyne W, Delbeke FT (2004) Validation of a GC-MS screening method for anabolizing agents in solid nutritional supplements. Biomed Chromatogr 18(3):155–159
11. Park S, Lee JG, Roh SH, Kim G, Kwon CH, Park HR, Kwon KS, Kim D, Kwon SW (2012) Determination of PDE-5 inhibitors and appetite suppressants in adulterated dietary supplements using LC/PDA and LC/MS. Food Addit Contam B 5(1):29–32
12. Di Mavungu JD, Monbaliu S, Scippo ML, Maghuin-Rogister G, Schneider YJ, Larondelle Y, Callebaut A, Robbens J, van Peteghem C, de Saeger S (2009) LC-MS/MS multi-analyte method for mycotoxin determination in food supplements. Food Addit Contam A 26(6):885–895
13. Liang Q, Qu J, Luo G, Wang Y (2006) Rapid and reliable determination of illegal adulterant in herbal medicines and dietary supplements by LC/MS/MS. J Pharmaceut Biomed 40(2):305–311
14. Hansen M, Jacobsen NW, Nielsen FK, Björklund E, Styrishave B, Halling-Sørensen B (2011) Determination of steroid hormones in blood by GC-MS/MS. Anal Bioanal Chem 400(10):3409–3417
15. Rambaud L, Monteau F, Deceuninck Y, Bichon E, André F, Le Bizec B (2007) Development and validation of a multi-residue method for the detection of a wide range of hormonal anabolic compounds in hair using gas chromatography-tandem mass spectrometry. Anal Chim Acta 586 (1–2 SPEC. ISS.):93–104.

16. Salvia MV, Vulliet E, Wiest L, Baudot R, Cren-Olivé C (2012) Development of a multi-residue method using acetonitrile-based extraction followed by liquid chromatography-tandem mass spectrometry for the analysis of steroids and veterinary and human drugs at trace levels in soil. J Chromatogr A 1245:122–133
17. Thevis M, Kuuranne T, Geyer H, Schänzer W (2011) Annual banned-substance review: analytical approaches in human sports drug testing. Drug Test Anal 3(1):1–14
18. Jonker N, Kretschmer A, Kool J, Fernandez A, Kloos D, Krabbe JG, Lingeman H, Irth H (2009) Online magnetic bead dynamic protein-affinity selection coupled to LC−MS for the screening of pharmacologically active compounds. Anal Chem 81(11):4263–4270
19. Usami M, Mitsunaga K, Ohno Y (2002) Estrogen receptor binding assay of chemicals with a surface plasmon resonance biosensor. J Steroid Biochem 81(1):47–55
20. Blair RM, Fang H, Branham WS, Hass BS, Dial SL, Moland CL, Tong W, Shi L, Perkins R, Sheehan DM (2000) The estrogen receptor relative binding affinities of 188 natural and xenochemicals: structural diversity of ligands. Toxicol Sci 54(1):138–153
21. Choi Y, Van Breemen RB (2008) Development of a screening assay for ligands to the estrogen receptor based on magnetic microparticles and LC-MS. Comb Chem High Throughput Screen 11(1):1–6
22. Bovee TFH, Helsdingen RJR, Rietjens IMCM, Keijer J, Hoogenboom RLAP (2004) Rapid yeast estrogen bioassays stably expressing human estrogen receptors α and β, and green fluorescent protein: a comparison of different compounds with both receptor types. J Steroid Biochem 91(3):99–109
23. Bovee TFH, Schoonen WGEJ, Hamers ARM, Bento MJ, Peijnenburg AACM (2008) Screening of synthetic and plant-derived compounds for (anti)estrogenic and (anti)androgenic activities. Anal Bioanal Chem 390(4):1111–1119
24. de Vlieger JSB, Kolkman AJ, Ampt KAM, Commandeur JNM, Vermeulen NPE, Kool J, Wijmenga SS, Niessen WMA, Irth H, Honing M (2010) Determination and identification of estrogenic compounds generated with biosynthetic enzymes using hyphenated screening assays, high resolution mass spectrometry and off-line NMR. J Chromatogr B 878(7–8):667–674
25. Zepperitz C, Hofner G, Wanner KT (2006) MS-binding assays: kinetic, saturation, and competitive experiments based on quantitation of bound marker as exemplified by the GABA transporter mGAT1. ChemMedChem 1(2):208–217
26. Hess M, Hofner G, Wanner KT (2011) (S)- and (R)-fluoxetine as native markers in mass spectrometry (MS) binding assays addressing the serotonin transporter. ChemMedChem 6(10):1900–1908
27. Niessen KV, Hofner G, Wanner KT (2005) Competitive MS binding assays for dopamine D2 receptors employing spiperone as a native marker. Chembiochem 6(10):1769–1775
28. Hofner G, Wanner KT (2003) Competitive binding assays made easy with a native marker and mass spectrometric quantification. Angew Chem 42(42):5235–5237
29. Aqai P, Cevik E, Gerssen A, Haasnoot W, Nielen MWF (2013) High-throughput bioaffinity mass spectrometry for screening and identification of designer anabolic steroids in dietary supplements. Anal Chem 85(6):3255–3262
30. Athanasiadou I, Angelis YS, Lyris E, Georgakopoulos C (2013) Chemical derivatization to enhance ionization of anabolic steroids in LC-MS for doping-control analysis. TrAC-Trends Anal Chem 42: 137–156
31. Wong CHF, Leung DKK, Tang FPW, Wong JKY, Yu NH, Wan TSM (2012) Rapid screening of anabolic steroids in horse urine with ultra-high-performance liquid chromatography/tandem mass spectrometry after chemical derivatisation. J Chromatogr A 1232:257–265
32. Major M, Bajic S, Bristow A, Ray A (2012) A novel source design for the analysis of both polar and non-polar species. Poster presentated at the 60th American Society for Mass Spectrometry conference, Vancouver, http://www.waters.com/waters/library.htm?locale=en_US&lid=134684811, accessed on 01-03-2013
33. Rijk JCW, Bovee TFH, Wang S, Van Poucke C, Van Peteghem C, Nielen MWF (2009) Detection of anabolic steroids in dietary supplements: the added value of an androgen yeast bioassay in parallel with a liquid chromatography-tandem mass spectrometry screening method. Anal Chim Acta 637(1–2):305–314
34. Bolger R, Wiese TE, Ervin K, Nestich S, Checovich W (1998) Rapid screening of environmental chemicals for estrogen receptor binding capacity. Environ Health Perspect 106(9):551–557
35. Kuiper GGJM, Lemmen JG, Carlsson B, Corton JC, Safe SH, Van Der Saag PT, Van Der Burg B, Gustafsson JÅ (1998) Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. Endocrinology 139(10):4252–4263
36. Matthews J, Celius T, Halgren R, Zacharewski T (2000) Differential estrogen receptor binding of estrogenic substances: a species comparison. J Steroid Biochem 74(4):223–234
37. Shao B, Zhao R, Meng J, Xue Y, Wu G, Hu J, Tu X (2005) Simultaneous determination of residual hormonal chemicals in meat, kidney, liver tissues and milk by liquid chromatography–tandem mass spectrometry. Anal Chim Acta 548(1–2):41–50
38. Guo F, Liu Q, Qu G-b, Song S-j, Sun J-t, Shi J-b, Jiang G-b (2013) Simultaneous determination of five estrogens and four androgens in water samples by online solid-phase extraction coupled with high-performance liquid chromatography–tandem mass spectrometry. J Chromatogr A 1281:9–18
39. Shimizu A, Ohe T, Chiba M (2012) A novel method for the determination of the site of glucuronidation by ion mobility spectrometry-mass spectrometry. Drug Metab Dispos 40(8):1456–1459
40. Campuzano I, Bush MF, Robinson CV, Beaumont C, Richardson K, Kim H, Kim HI (2012) Structural characterization of drug-like compounds by ion mobility mass spectrometry: comparison of theoretical and experimentally derived nitrogen collision cross sections. Anal Chem 84(2):1026–1033
41. Knapman TW, Berryman JT, Campuzano I, Harris SA, Ashcroft AE (2010) Considerations in experimental and theoretical collision cross-section measurements of small molecules using travelling wave ion mobility spectrometry-mass spectrometry. Int J Mass Spectrom 298(1–3):17–23