Solid-surface fluorescent properties of estrogens: green analytical applications

Rocío L. Pérez, Graciela M. Escandar*

Instituto de Química Rosario (CONICET-UNR), Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531 (2000) Rosario, Argentina. E-mail: escandar@iquir-conicet.gov.ar

Abstract

For the first time, the fluorescent signals produced by 17β-estradiol (E2), estradiol valerate (E2V) and 17α-ethinylestradiol (EE2) were investigated after being retained in selected solid surfaces. Among different studied supports, both C18 and nylon membranes demonstrated to be able to induce intense signals over their surface, after aqueous solutions of the analytes were retained via a solid-phase extraction procedure. Physicochemical variables which affect the quality of the fluorescence signals were optimized, and the results obtained in the studied solid supports were compared and discussed. While nylon successfully faced the trouble of spectrofluorimetrically quantifying estrogens in pharmaceuticals with unbalanced estrogen/progestagen ratios, C18 membranes showed very good qualities for second-order spectrofluorimeric measurements, allowing the determination of E2 (the most active estrogen) in potentially contaminated fish and chicken samples. The fluorescence excitation-emission matrices, directly measured on a C18 surface, were processed using appropriate chemometric algorithms in order to efficiently quantify E2 in the presence of natural matrix constituents. The present strategy avoids the elution step, considerably decreasing the use of organic solvents, the analysis time and the experimental errors. In addition, the instrumental involved is nonsophisticated and, therefore, the experiments could be carried out in routine laboratories.

Keywords: Solid-surface spectrofluorimetry; C18 membranes; Nylon membranes; Second-order calibration; Endocrine disruptors

1. **Introduction**

Estrogens are steroidal sex hormones produced by the ovaries, the placenta during pregnancy and in smaller amounts by the adrenal glands. These hormones are usually prescribed in replacement therapies, for the treatment of menopausal symptoms, and for contraception to prevent pregnancy. Both the natural 17β-estradiol (E2) and the synthetic estradiol valerate (E2V) are the main estrogens applied in hormonal replacement therapy [1]. On the other hand, the synthetic estrogen ethinylestradiol (EE2) and progestagens of different generations are present in most oral contraceptives.

While estrogens exhibit weak fluorescence in aqueous solution, more intense signals are obtained in organic solvents and, thus, most current spectrofluorimetric methods for estrogen quantification are developed in the latter media. Although several decades ago the fluorimetric determination of estrogens were usually based on the signals produced in hot concentrated sulfuric acid [2], due to these drastic experimental conditions, the latter practices became unpopular. As part of a plan committed to the development of green analytical methods, the present paper evaluates the fluorescent properties of selected estrogens adsorbed in solid surfaces, in order to assess the usefulness of the signal for analytical purposes. To the best of our knowledge, this is the first report about the fluorescence signals produced from estrogens adsorbed on solid supports.

Solid-surface fluorescence (SSF) analysis is very sensitive for the determination of a great variety of organic compounds, and is especially valuable for the development of green analytical techniques [3]. In fact, with the purpose of demonstrating the usefulness of SSF for the development of green methods, two novel strategies for estrogen determination in different types of samples are implemented.

One of them involves the use of a nylon membrane as a support for a solid-phase extraction (SPE) procedure, combined to univariate fluorescence calibration for estrogens determination in various commercial pharmaceuticals, with especial interest in those containing a significantly large amount of progestagens in relation to the estrogen content. Progestagens do not emit natural fluorescence, but produce a serious interference through the inner filter effect, if they are simultaneously retained with the estrogen in the solid support.

In a second example, the most active estrogen E2 is retained from both fish and chicken tissue extracts on a C18 membrane, and then determined by excitation-emission fluorescence matrices (EEFMs), directly recorded on the surface of the solid substrate. Subsequently, three chemometric algorithms which achieve the second order advantage, namely, parallel factor analysis (PARAFAC) [4], unfolded partial least-squares coupled to residual bilinearization (U-PLS/RBL) [5], and multivariate curve resolution-alternating least-squares (MCR-ALS) [6] are applied to process the EEFMs, and their prediction capabilities are discussed. The term "second-order advantage" refers to the capacity of certain second-order algorithms to predict concentrations of sample components in the presence of any number of unsuspected constituents [7]. All results obtained with the proposed strategies are discussed and compared with those provided by a chromatographic reference method.

2. Materials and methods

2.1. Reagents and solutions

All reagents were of high-purity grade and used as received. E2, E2V, EE2, levonorgestrel (LNG), desogestrel (DSG), norgestimate (NGM), norethisterone acetate (NTA) and cyproterone acetate (CTA) were purchased from Sigma-Aldrich (Milwaukee, WI, USA).

Methanol (MeOH) and acetonitrile (ACN) were obtained from Merck (Darmstadt, Germany), while trichloroacetic acid (TCA) was obtained from Anedra (San Fernando, Argentina).

Different lots of nylon membranes of 0.2 µm pore size were tested: Varian (Seattle, USA), Scleicher-Schuell (Dassel, Germany), GE Osmonics (Trevose, USA), and Whatman (Sigma-Aldrich, USA). No significant differences were observed among these membranes, either in the background emission or in the luminescence properties of the retained estrogens. Two C18 solid-phase-extraction disks were tested: ENVI-18 DSK and Empore Octadecyl C18, both purchased from Supelco (Bellefonte, PA, USA). The surface-modified silica is contained in a glass fiber matrix in the former disks and in Teflon in the latter ones. Although the obtained signals are similar in both disks, the experiments were carried out using ENVI-disk because they are thicker, more rigid and less expensive than disks based on Teflon.

MeOH stock solutions of E2, E2V and EE2 of about 2500 µg mL^{-1} were prepared and stored in dark flasks at 4 °C. From these solutions, more diluted methanol solutions were obtained. Working aqueous solutions were prepared immediately before their use by taking appropriate aliquots of methanol solutions, evaporating the organic solvent by use of dry nitrogen and diluting with ultrapurified water from a Millipore system (Massachusetts, USA) to the desired concentrations.

2.2. Instrumentation

Fluorescence measurements were done on an Aminco Bowman (Rochester, NY, USA) Series 2 luminescence spectrometer equipped with a 7W pulsed xenon lamp. Specific wavelengths for the different analyses will be discussed below. The slit widths were both of 4 nm. All fluorescence measurements were performed at 20 °C using the thermostated cell holder and a Lauda (Frankfurt, Germany) RM6T thermostatic bath. The pH of solutions was

measured with a Metrohm (Herisau, Switzerland) 713 pH meter equipped with a combined glass electrode. Absorbance data were obtained with a Beckman 120 (Fullerton, CA, USA) DU 640 spectrophotometer. High-pressure liquid chromatography (HPLC) was carried out on an Agilent 1200 (Agilent Technologies, Waldbronn, Germany) liquid chromatograph with an Agilent 1260 Infinity diode array detector. The system was controlled by the ChemStation software.

2.3. SPE-solid-surface fluorescence

The employed 47-mm C18 membranes were dissected into 13-mm disks. The disk was loaded into a stainless steel filter syringe kit (Alltech, Deerfield, IL, USA), and was conditioned with 0.5 mL of MeOH followed by 0.5 mL of water. With the purpose of concentrating the analyte into a restricted area of the solid surface, a Teflon ring was fitted over the membrane before the extraction. Thus, an extractive surface with final diameter of 5 mm was exposed to the flowing solution. A 5 mL syringe was coupled to the filter holder and positive pressure was used to force the solution through the membrane in approximately 30 s per sample. This flow rate is in the optimum range for maximum breakthrough volume (10–30 mL min^{-1}) [8]. Following the estrogen extraction, the sample was partially dried by forcing air through the disk using a 25 mL syringe. The disk was then removed from the holder, it was placed in a laboratory-made SPE membrane holder [8] and the fluorescence spectra were measured. The angle formed between the excitation and emission beams was 90°, with an incident angle of 45°.

The procedure performed with nylon membranes was similar to that carried out with C18, but in this case the membrane did not need conditioning with MeOH. Before the extraction procedure the nylon disk was dried on a heating plate for 10 min at about 100 °C.

2.4. Zeroth-order calibration for pharmaceutical analysis

E2, E2V and EE2 solutions for the calibration curves were obtained by performing convenient dilutions of standard aqueous solutions with water. The former were prepared from the corresponding MeOH standards. Then, SPE (both C18 and nylon) and fluorescence measurements were carried out by the procedure described above, setting the excitation and emission wavelengths at 280 and 310 nm, respectively.

The analysed pharmaceutical preparations were Lindisc 50 (Bayer, Buenos Aires, Argentina), Rontagel (Ferring, Argentina), Ciclocur (Bayer, São Paulo, Brazil), April (Gador, Buenos Aires, Argentina), Marvelon (Organon, Buenos Aires, Argentina), Evra (LTS Lohmann Therapie-Systeme AG, Andernach, Germany), Cilest (Cilag International, Zug, Switzerland), Diane 35 (Schering, Buenos Aires, Argentina), Primosiston (Bayer, São Paulo, Brazil), Ginelea (Elea, Montevideo, Uruguay).

2.4.1. Estradiol and ethynylestradiol (tablets)

Ten tablets of the corresponding preparation were weighed in order to find the average tablet mass, triturated and mixed. Alkaline solutions of each commercial sample were prepared by weighing about 100.0 mg of the latter mixture, treating them with 3.00 mL of NaOH solution (final pH ≈ 13) and stirring the mixture during 20 min. The mixture was then centrifuged for 10 min at 12,000 rpm. Aliquots of 200 or 250 µL of supernatant were treated with HCl and water in order to obtain an aqueous solution of pH about 7 and 5.00 mL of final volume.

2.4.2. Estradiol (patch)

One square centimeter of Lindisc 50 patch was treated with 1.50 mL of methanol, sonicated during 10 min, and centrifuged during ten additional minutes. An aliquot of 200 µL of supernatant was diluted with 1.00 mL of MeOH. From this latter solution, an aqueous solution was prepared by taking an aliquot of 100.0 µL, evaporating the solvent with nitrogen, and diluting with water to 5.00 mL.

2.4.3. Estradiol (gel)

A mass of 0.400 g of gel was dissolved in 10 mL of methanol, sonicated during 10 min, and centrifuged during ten additional minutes. An aqueous solution was prepared by taking 100 uL of supernatant, evaporating the solvent with nitrogen, and diluting with water to 5.00 mL.

2.4.4. Estradiol valerate

Ten tablets of Ciclocur (brown pills) were weighed, triturated and mixed, and the same procedure was separately carried out with ten tablets of Ciclocur (white pills). An amount of 20.0 mg of the mixture was treated with 1.00 mL of MeOH and sonicated by 10 min. The mixture was then centrifuged, and 100 µL of the supernatant was diluted with 1.00 mL of MeOH. From this latter solution, an aqueous solution was prepared by taking an aliquot of 50.0 µL, evaporating the MeOH by use of nitrogen and diluting with water to 5.00 mL.

2.4.5. Ethynylestradiol (patch)

One square centimeter of Evra patch was treated with 1.00 mL of MeOH, sonicated during 10 min, and centrifuged during ten additional minutes. Fifty microliters of the supernatant were transferred into a 5.00 mL flask, the solvent was evaporated with a nitrogen stream, and water was added to the mark.

2.5. Second-order calibration for tissue analysis

A calibration set of five samples by triplicate (15 samples) was prepared taking appropriate volumes of E2 aqueous solution and diluting with water until 10 mL, in order to obtain concentrations in the range 0–200 ng mL^{-1}. Each sample was subjected to an SPE procedure described above when using C18 disks but treating, in this case, 10 mL of solution. The EEFMs were collected at 90°, irradiating the disk surface in the range 250-286 nm (each 2 nm) and obtaining the corresponding emission in the range 290-350 nm (each 1 nm). The resulting EEFMs were then subjected to second-order data analysis.

A set of nine validation samples, different from the calibration ones, was prepared and processed in a similar way as the calibration samples. Fish and chicken were purchased from local commercial markets. Because all analyzed samples did not contain E2 at levels higher than the attained detection limit, a recovery study was carried out by spiking them with the analyte at different concentration levels. Tissue samples were chopped, triturated and then lyophilized in a Liotop L101 Liobras dryer (San Carlos, Brazil), and standard solution of E2 was added to the lyophilized samples in order to obtain concentrations between approximately 0.1 and 2 µg g^{-1}. Tissue samples generally contain large amounts of lipids and proteins and, therefore, ACN was selected as solvent for their removal [9]. ACN (5-10 mL) was added to the sample and the mixture was ultrasonically extracted for 20 min at room temperature. The sample was then centrifuged in a high speed centrifuge (Sigma 3-18 KH, Osterode am Harz, Germany) at 10500 rpm for 10 min. A portion of the supernatant was dried and, in order to achieve better protein removal, TCA solution was added. After centrifugation, a volume of supernatant was placed into a 10 mL volumetric flask, the solution was neutralized with NaOH solution, and water was added to the mark. This solution

was subjected to the SPE with a C18 membrane and the EEFM was measured and chemometrically processed as described above. Each measurement was performed in duplicate.

2.6. High-performance liquid chromatography analysis

The method proposed for pharmaceuticals was validated by HPLC, following the procedures of both extraction and determination suggested by the United States Pharmacopeia and the National Formulary (USP 35-NF 30) [10]. Separations were performed on an Agilent Eclipse XDB-C18 4.6×150 mm (5 µm particle size) analytical column. For E2 and E2V, the mobile phase was a mixture of ACN:water (55:45, v/v), and the flow rate was 1 mL min^{-1}. For EE2, the mobile phase was a mixture of ACN and 20 mM potassium phosphate buffer, pH = 6.0 (50:50, v/v) flowing at 2 mL min^{-1}. In all cases UV detection proceeded at 280 nm.

The E2 concentrations in fish and chicken samples were also corroborated by HPLC, analyzing portions of the same fortified samples. In this case, a more rigorous clean-up procedure for interferences removal had to be applied. Briefly, after addition of ACN (3-8 mL) to the sample, the mixture was ultrasonically extracted for 20 min at room temperature, and centrifuged at 10500 rpm for 10 min. A portion of the supernatant (2-6 mL) was added with the same volume (2-6 mL) of hexane and, after 15 min of agitation, an aliquot of the ACN phase (1.5-5.5 mL) was measured and evaporated. The residue was reconstructed with water and TCA acid and, after the system was centrifuged, a volume (4-4.5 mL) of the supernatant was placed in a 10.00 mL volumetric flask, neutralized with NaOH and diluted with water to the mark. Then, this solution was subjected to an SPE using a C18 membrane previously conditioned with methanol. The membrane was eluted with 1 mL of MeOH,

collecting the eluate in a 2 mL volumetric flask. Water was added to the mark, and the solution was filtered through a 0.2 µm pore size nylon disk and injected into the HPLC apparatus. In this case, the mobile phase was a mixture of MeOH:water (70:30, v/v), and the flow rate was 0.8 mL min^{-1}.

2.6. Chemometric algorithms and software

The theory of the applied algorithms is well documented and a brief description can be found in the Electronic Supplementary Material. The routines employed for PARAFAC, U-PLS, U-PLS/RBL and MCR-ALS are written in MATLAB 7.6 [11]. All algorithms were implemented using the graphical interface of the MVC2 toolbox, which is available on the Internet [12].

3. Results and discussion

3.1. Fluorescence properties of E2, E2V and EE2 in solid-surfaces

The fluorescence properties of E2, E2V and EE2 in filter paper, cellulose acetate and cellulose nitrate membranes, octadecyl C18 disks, and nylon membranes were probed following two experimental approaches. One of them involved a classical practice of depositing on the solid support a few microliters of the solution of the analyte (dissolved in either water or an organic solvent) [13]. The second one consisted in an SPE procedure using an aqueous solution of the analyte [8,14]. Among the investigated solid supports, only nylon and C18 membranes were able to produce intense fluorescence signals from the studied analytes when the extraction procedure was applied (Figs. 1A and 1B).

The comparison of the SSF signals of E2, E2V and EE2 with those in aqueous solution (Fig. 1C) shows that the employed membranes did not promote significant shifts in neither excitation nor the emission wavelengths of the analysed estrogens. Furthermore, interferences from Raman bands which appear in water solutions are avoided in the solid-surface systems. It can also be appreciated that, as in solution, estrogens have very similar spectral shapes when they are adsorbed in the studied surfaces. The relative intensities of the SSF spectra follow this order: EE2 > E2 > E2V.

Before analyzing the characteristics of each solid support, issues common to both membranes will be discussed.

3.2. Effect of experimental variables in the SSF signals

The syringe extractive procedure was carried out testing volumes of aqueous estrogen solutions from 2 to 20 mL. Although in all cases good quality signals were obtained, a value of 5 mL showed to be suitable for observing proper signals for concentrations until about 1 µg mL^{-1}, without involving a long experimental time. However, sensitivity can be improved, if required, by employing higher sample volumes of more diluted solutions.

It is important to notice that when using the continuous lamp of the spectrofluorimeter for irradiating the samples, the emission intensity from the estrogens adsorbed in the evaluated surfaces decreased during successive scans from the same membrane. It is apparent that the fluorescence of estrogens retained in the membrane surface is susceptible to lamp irradiation. Fortunately, irradiation with the flash lamp led to stable signals, and therefore this lamp was employed in all measurements. As an example, Fig. S1 (Electronic Supplementary Material) shows the measured intensities for the EE2 system as a function of the irradiation time when both types of lamps are applied.

The study of the influence of the temperature on the fluorescence profiles of the investigated systems in both membranes showed a behavior similar to that found in solution: a temperature decrease of the reading chamber does not significantly improve the fluorescence intensity. Therefore, the experiments were conducted at 20 °C.

Finally, the effect of auxiliary reagents on the fluorescence intensity of the retained estrogens was checked. Thus, in different experiments, 5 µL of cyclodextrins solutions, surfactants solutions, and organic solvents (cyclohexane, hexane, chloroform, cyclohexanol, butanol, pentanol, isopropanol, and acetone) were deposited on the surfaces previously treated with the estrogens. No significant improvements were observed in the fluorescence intensity to justify the use of these reagents.

3.2.1. C18 membrane surface

Due to the apolar nature of the C18 reversed-phase membrane, neutral structures of the compounds are required for their complete adsorption in this type of support through hydrophobic interactions. The acidity constant values of the evaluated estrogens (pKa > 10) suggest that below pH 9 these molecules remain in their uncharged structures and should be retained on the C18 membrane. Fortunately, the fluorescence intensities for the evaluated estrogens in solution are maxima under their neutral structures, and this property is preserved when they are adsorbed over the C18 surface (Fig. 1A).

The complete extraction of the estrogens in C18 during the SPE procedure was confirmed by comparing the fluorescence intensity of the solution before and after extraction. In all cases, the read signals in solution after extraction were undistinguishable from the blank (water), suggesting a complete adsorption of the estrogens in the membrane.

As will be demonstrated below, C18 is also able to adsorb other uncharged compounds which could interfere in the determination, for example, through an inner-filter effect. This

fact makes C18 membrane less attractive for the determination of the studied estrogens through univariate calibration.

3.2.2. Nylon membrane surface

As established in previous works [13–16], nylon membrane (a polyamide microporous film made from nylon 6,6) proved to be an outstanding solid-support for the quantification through luminescence signals of a variety of organic and inorganic compounds retained in its surface.

There are literature precedents for the retention of E2 in a nylon filter membrane [17]. The excitation and emission fluorescence spectra of the studied estrogens on a nylon membrane (Fig. 1B) are similar to those obtained in C18 surface. In this case, both hydrophobic and hydrophilic forces could be implied in the adsorption process [17]. In fact, in addition to the hydrophobic interactions between the sterane nucleus of estrogens and the methylene chains of nylon, a possible explanation for the retention is the formation of a hydrogen bond between the phenolic group of the analytes and the amide group of the support. As will be demonstrated below, the presence of the phenolic group in estrogens is crucial for their determination on nylon in the presence of progestagens. The latter compounds are not retained (or only partially retained) in nylon, presumably due to the lack of a phenolic group in their structures. Therefore, their interference in the analysis is negligible.

It is also important to remark that the amide groups of nylon play an important role in the extraction procedure from aqueous solutions, such as those presently discussed. Amide groups are hydrophilic, and are expected to enhance the motion of water molecules into the sorbent, improving mass transfer and making it more effective.

To confirm the complete extraction of the estrogens on the nylon membrane, a similar procedure to that carried out with the C18 disk was performed (see above).

Exploratory experiments demonstrated that the heating of the nylon membranes in a plate at about 100 °C before the E2, E2V and EE2 extractions improved the obtained fluorescence signals (see Fig. S2 of the Electronic Supplementary Material). This was the only treatment applied to the nylon membrane before its use. Fig. S2 shows that upon heating, the estrogen fluorescence increases by more than 10 % of the initial value until ca. 10 min. This behavior is ascribed to the loss of water molecules absorbed in nylon, which compete for the binding sites. The decrease of the fluorescence emission after about 10 min could be originated by changes in the extractive properties due to prolonged heating. It is important to point out that the blank signal decreases by about 18 % with 10 min of heating, and then remains practically constant, increasing the measured net signal.

3.3. Analytical applications

3.3.1 Pharmaceuticals

Because medical studies have found that significant amount of estrogens in pharmaceuticals may cause serious health complications, such as stroke, heart attack, and pulmonary embolism [18,19], modern contraceptive pills contain no more than 35 micrograms of estrogen, while the progestagen is present at levels 5–150 times higher. Besides, some drugs used for hormone replacement therapy do also have progestagens in varying proportions in relation to the estrogen amount. The determination of small amounts of estrogen in the presence of large amounts of progestagen represents an analytical challenge, especially when trying to work under the green-analytical principles [3].

The knowledge gained on SSF properties of E2, E2V and EE2 was applied in the development of methods for their determinations in pharmaceutical preparations. The solid-surface spectrofluorometric determination of estrogens in both types of membranes involved

the construction of the corresponding univariate calibration curves. Table 1 summarises the analytical figures of merit (AFOM) calculated for these estrogens on the investigated membranes.

The linearity for the corresponding calibration curves was tested by an ANOVA method [20]. No significant differences were observed in the figures of merit of the analytes in both assayed membranes. The achieved limits of detection are acceptable, considering that they are not a limiting factor when analyzing pharmaceutical preparations, because the concentration of analyte available and the amount of sample are generally large. The relative standard deviations (in the order of 1 % or lower) express the good repeatability of the assays.

The analysed pharmaceuticals included selected oral contraceptives and drugs usually used in hormone replacement therapy, all containing an estrogen (E2, E2V or EE2) alone or in the presence of progestagens of first (*e.g.* NTA), second (*e.g.* LNG, NGM), and third (*e.g.* DSG, GSD) generation, or an anti-androgen agent such as CTA.

With the purpose of decreasing the use of organic solvents, NaOH solution was attempted rather than MeOH for sample preparation from the pharmaceutical formulation. At pHs higher than 11, estrogens release the phenolic proton, remaining in their anionic structures and, in principle, they should be soluble in an aqueous alkaline solution. With the exception of patches, gel and the tablet containing E2V, where the estrogens were dissolved with MeOH, the remaining assayed pharmaceuticals were successfully extracted with NaOH solution. These solutions were then neutralized before SPE. In these latter cases, in addition to the advantage of using an aqueous solution, the procedure avoids the transfer of a large amount of progestagens to the extract, since they have low water solubility, even in alkaline media. However, despite this fact, in most investigated systems part of the original progestagen contained in the sample is present in the solutions subjected to the SPE

procedure. On the other hand, progestagens are also present when the commercial drug was treated with MeOH, as expected.

Since progestagens are not fluorescent, their interference in the spectrofluorimetric method, if any, is due to inner-filter effect on the estrogen signals. The inner-filter effect is produced by the presence of species able to absorb a fraction of the excitation light or the analyte emission energy (or both), and it is frequent in luminescence techniques, especially if they are carried out in solid surfaces. In these SSF methods, the support concentrates in its surface all retained compounds, both desired and undesired ones, leading to potential inner-filter effects on the analyte signals.

In Fig. 2, the absorption spectra of the progestagens are shown. For comparison, the spectra of estrogens were also included in this figure. It is clear that the evaluated progestagens absorb, in different degrees, in the regions corresponding to both excitation and emission fluorescence wavelengths of estrogens. Therefore, the former are potentially able to produce inner-filter effect in the fluorescence of the estrogen if they are simultaneously adsorbed in the membrane.

As was previously corroborated (see above), estrogens are totally adsorbed in both types of membranes after filtration. However, the studied progestagens are adsorbed in C18 membranes, while in nylon they are not retained. These conclusions were reached by measuring the UV spectra of the neutral solutions of the pharmaceutical preparation extracts, before and after the solutions were filtered through the investigated disks.

Fig. 3 shows the recoveries obtained for E2, E2V and EE2 in selected pharmaceuticals applying the proposed SPE methodology in both types of membranes, as discussed below.

In analyzing the estrogen recoveries in C18 membrane, it can be concluded that the values are good for those pharmaceuticals without progestagen (e.g. Lindisc 50, Rotangel, Ciclocur white pill) and for Diane 35 tablet. This last result can be justified considering the absence of

CTA in the original alkaline extract, because, if present, it would produce inner-filter effect (Fig. 2). To confirm this fact, the same experiment but treating the Diane 35 tablet with MeOH was carried out. In this case, the recovery of EE2 in C18 membrane was poor (lower than 30 %), demonstrating the inner-filter effect produced by CTA on the fluorescence of EE2 when that progestagen is simultaneously retained in the surface.

A rapid inspection of Fig. 3 seems to indicate that the method carried out in nylon yields much better results than those performed in C18 membranes. These results are in accordance to the above discussion about the different selectivity of nylon and C18 for progestagens. In fact, all recoveries using the nylon approach were within the limits recommended by the United States Pharmacopeia and the National Formulary (USP 35-NF 30): estrogens tablets must contain 90.0–115.0 % of the labeled amounts while in intradermal delivery patches values between 85.0–120.0 % are admitted [10].

In order to test the accuracy of the proposed method on nylon concerning the content of estrogens in the studied pharmaceuticals, the samples were also assayed by HPLC (Fig. 3), and the paired Student *t*-test was applied [20]. The experimental *t* values obtained were in the range 0–1.68, whereas the critical $t_{crit(0.05,4)}$ is 2.78. Therefore, since the experimental *t* values are lower than the critical one, both methods give results which are not statistically different.

The good results obtained employing nylon as support suggest that the different excipients of the analyzed pharmaceuticals, which may probably be present in some working extracts, do not interfere in the proposed method.

In comparing the proposed strategy with usual HPLC methods, it is important to remark that the latter involve a significant amount of organic solvents, and require longer total analysis times than the one presently proposed .

3.3.2. Fish and chicken

A second example for the applicability of the SSF approach here developed was the determination of E2 in fish and chicken tissues. The most active estrogen E2 has been found in many aquatic environments and, because its anabolic effects, it has been used in farm animal fattening, with the concomitant negative impact in the health of humans exposed via the food chain [21]. Through different directives, the EU banned the use of this estrogen and compounds with hormonal action in husbandry and aquaculture [22], highlighting the need of developing useful methods for estrogen detection in fishery and farm products [21].

It is very likely that real samples, such as those here analyzed, contain constituents able to interfere in the fluorimetric analysis. Therefore, the selectivity of the method was improved through a second-order calibration using EEFMs and applying algorithms which achieve the so-called second-order advantage [7]. In this case, C18 membrane was selected as solid support because the nylon background signal is very similar to that from the analyte (see Fig. 1A).

After the extraction procedure, EEFMs of E2 were recorded on the C18 surface for calibration and validation samples (Fig. 4A), and were then subjected to chemometric analysis. It is known that a set of EEFMs can be arranged as a three-way array, which in general complies with the trilinearity conditions [23] and, therefore, the first option when choosing the algorithm for data processing should be PARAFAC [24]. Fig. S3 (Electronic Supplementary Material) shows the spectral profiles retrieved by PARAFAC for a typical validation sample, where the signals corresponding to E2 and C18 membrane background are clearly distinguished, and Fig. S4 (Electronic Supplementary Material) displays the successful predictions obtained with this algorithm for validation samples. If the elliptical joint confidence region (EJCR) is analyzed for the slope and intercept of the above plot, we conclude that the ellipse includes the theoretically expected values of (1,0), indicating the accuracy of the used methodology [25]. The statistical results shown in Table 2 for 10 mL of

treated sample, with a root-mean-square error of prediction (RMSEP) and relative error of prediction (REP) values of 5 ng mL^{-1} and 5 % respectively, suggest a very good precision.

However, the PARAFAC results were poor when real samples were processed. This fact may be explained considering the spectral similarity among the analyte and interferences, which precludes the decomposition of the three-way data in physically reasonable profiles and scores [23]. In this latter case, appropriate options are U-PLS/RBL and MCR-ALS, which are, in principle, able to circumvent the spectral similarity problem.

In a first phase, the same set of 9 validation samples examined by PARAFAC was processed by both U-PLS (RBL is not required in the absence of interferents) and MCR-ALS. The number of latent variables for U-PLS calibration was estimated, as detailed in the Electronic Supplementary Material, by leave-one-out cross validation [26]. This leads to the conclusion that this number is 2, ascribed to the analyte and to a background signal, and was employed for U-PLS modeling and prediction.

The MCR-ALS model was then applied as described in the Electronic Supplementary Material. Specifically for the present system, an augmented data matrix was built with the investigated sample data and the calibration data matrices. Augmentation was performed along the excitation spectral mode, since spectral similarity is observed between the excitation spectra for E2 and interferences (see below). In the case of the validation samples, two MCR-ALS components were considered, ascribed to the analyte and the background.

Validation samples were successfully resolved by both U-PLS and MCR-ALS algorithms (Fig. S4 of the Electronic Supplementary Material), and the resulting ellipses and the statistical results (Table 2) denote accurate and precise approaches.

The usefulness of the proposed approach coupled to either U-PLS/RBL or MCR-ALS to quantify E2 in real samples was then evaluated. Different authors have evaluated a varying range of E2 concentrations (e.g. 0.01 µg g^{-1} [9], 0.35 µg g^{-1} [27], 0.5-25 µg g^{-1} [28], 10-100

µg g^{-1} [21]) in fish and chicken tissues. In the present work E2 concentrations in the range 0.1-1.8 µg g^{-1} were checked. However, lower concentrations can be attained working with a higher weight of tissue.

Ten samples, five of them corresponding to fish tissue and the other five to chicken muscle, were prepared according to the procedure presented in the experimental section and were evaluated with U-PLS/RBL and MCR-ALS algorithms. A typical EEFM and the corresponding contour plot of a chicken sample are shown in Fig. 4B. When U-PLS/RBL was applied to real samples, in addition to the number of latent variables estimated for the calibration set, these samples required the introduction of the RBL procedure with two unexpected components in most cases. Adding more unexpected components did not improve the fit, indicating that U-PLS/RBL models the profiles of the interferences using two principal components.

On the other hand, when MCR-ALS was applied to real samples, the number of components was three. As an example, Fig. 5 shows the good quality of the MCR-ALS recovered profiles for a selected chicken sample. The recovery values, in µg of E2 per gram of investigated tissues, are summarized in Table 3.

The statistical equivalence among the recoveries demonstrates the capacity of both U-PLS/RBL and MCR-ALS to cope with interferences from concomitants in the real samples. The obtained values are statistically comparable to those provided by an HPLC reference method when a paired Student *t*-test is applied at a 95% confidence level [20]. The experimental *t*-coefficients for U-PLS/RBL (t = 0.17) and MCR-ALS (t = 0.43) approaches favorably compare with the tabulated value for n – 1 degrees of freedom [$t_{crit(0.05,9)}$ = 1.83], suggesting that both algorithms are adequate for the E2 determination. Finally, the good analytical performances for both selected algorithms applied to tissue samples can be appreciated from the statistical results shown in Table 2.

A comparison with common chromatographic methods employed for this type of samples [9,21,27,28] allows us to state several advantages of the present approach. Because the chemometric analysis is directly performed in the sample extracts, rigorous clean-up and elution steps are not needed. As a consequence, the experimental time, the amount of organic solvents involved in the analysis and the error associated with multiple working steps are substantially diminished.

4. Conclusions

After a solid-phase extraction procedure, both C18 and nylon membranes proved to be excellent materials for the emission of fluorescence of estrogens. In the solid supports, both the excitation and emission spectra of the studied analytes are similar to those corresponding to water solutions, with the advantage that Raman bands, which overlap with the weak analytes signals in water, are absent in the solid-surface systems. The fluorescence signals read in the solid surface are susceptible to lamp irradiation, and therefore the procedure was optimized by using a flash lamp. On the other hand, slight changes in the reading chamber temperature and auxiliary reagents did not significantly modify the fluorescence intensity. In nylon systems, an improvement of the signal intensities is verified when heating the nylon disk at about 100 °C during 10 minutes before the estrogen extractions. A very simple zeroth-order calibration using nylon membranes and aqueous solutions of estrogens allowed their successful spectrofluorimetric determination in challenging samples containing large amounts of progestagen. On the other hand, a second-order calibration through the measurement of excitation-emission fluorescence matrices of 17β-estradiol on the C18 surface was implemented for the quantification of this estrogen in fish and chicken tissues. In this regards, the use of large

volumes of organic solvents and tedious preparation process, generally required in the analysis of bio-samples matrices, are avoided. In sum, it was demonstrated that solid-surface fluorescence gave rise to the development of green and straightforward approaches for the estrogen determination, providing an effective alternative to the existing chromatographic methods.

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References

[1] P.H.M. van de Weijer, L.Å. Mattsson, O. Ylikorkala, Benefits and risks of long-term low-dose oral continuous combined hormone therapy, Maturitas, 56 (2007) 231-248.

[2] W.R. Jr. Slaunwhite, L. L. Engel, J. F. Scott, C. L. Ham, Fluorescence and absortion of estrogens heated in sulfuric acid, J. Biol. Chem. 201 (1953) 615-620.

[3] A. Molina-Díaz, J.F. García-Reyes, B. Gilbert-López, Solid-phase spectroscopy from the point of view of green analytical chemistry, Trends Anal. Chem. 29 (2010) 654-666.

[4] R. Bro, PARAFAC. Tutorial and applications, Chemom. Intell. Lab. Syst. 38 (1997) 149-171.

[5] A.C. Olivieri, On a versatile second-order multivariate calibration method based on partial least-squares and residual bilinearization: Second-order advantage and precision properties, J. Chemom. 19 (2005) 253-265.

[6] R. Tauler, Multivariate curve resolution applied to second order data, Chemom. Intell. Lab. Syst. 30 (1995) 133-146.

[7] K.S. Booksh, B.R. Kowalski, Theory of analytical chemistry, Anal. Chem. 66 (1994) 782A-791A.

[8] G.M. Escandar, A.J. Bystol, A.D. Campiglia, Spectrofluorimetric method for the determination of piroxicam and pyridoxine, Anal. Chim. Acta, 466 (2002) 275-283.

[9] Y. Hu, Y. Wang, X. Chen, Y. Hu, G. Li, A novel molecularly imprinted solid-phase microextraction fiber coupled with high performance liquid chromatography for analysis of trace estrogens in fishery samples, Talanta 80 (2010) 2099-2105.

[10] United States Pharmacopeia and National Formulary, USP 35-NF 30, The United States Pharmacopeial Convention Inc., Rockville, USA, 2 (2012).

[11] MATLAB R2011b, The MathWorks Inc, Natick, MA, USA.

[12] www.iquir-conicet.gov.ar/descargas/mvc2.rar. Accessed July 2014.

[13] J.P. Chiarandini, G.M. Escandar, Nylon membrane as a fluorimetric probe for the herbicide bentazone, Anal. Bioanal. Chem. 402 (2012) 2221-2225.

[14] S.A. Bortolato, J.A. Arancibia, G.M. Escandar, Chemometrics-assisted excitation−emission fluorescence spectroscopy on nylon membranes. Simultaneous determination of benzo[*a*]pyrene and dibenz[a,h]anthracene at parts-per-trillion levels in the presence of the remaining EPA PAH priority pollutants as interferences, Anal. Chem. 80 (2008) 8276-8286.

[15] S.A. Bortolato, J.A. Arancibia, G.M. Escandar, A novel application of nylon membranes to the luminescent determination of benzo[*a*]pyrene at ultra trace levels in water samples, Anal. Chim. Acta 613 (2008) 218-227.

[16] V.A. Lozano, G.M. Escandar, M.C. Mahedero, A. Muñoz de la Pena, A novel nylon membrane-rhodamine 6G spirocyclic phenylthiosemicarbazide derivative system as a fluorimetric probe for mercury(II) ion, Anal. Methods 4 (2012) 2002-2008.

[17] M. Liu, E.P.C. Lai, Y. Yang, Removal of 17β-estradiol by nylon filter membrane: adsorption kinetics and thermodynamics, Int. J. Res. Rev. App. Sc. 11 (2012) 67-73.

[18] F.Z. Stanczyk, D.F. Archer, B.R. Bhavnani, Ethinyl estradiol and 17β-estradiol in combined oral contraceptives: pharmacokinetics, pharmacodynamics and risk assessment, Contraception, 87 (2013) 706-727.

[19] M.P. Warren, A comparative review of the risks and benefits of hormone replacement therapy regimens, Am. J. Obstet. Gynecol. 190 (2004) 1141-1167.

[20] W.P. Gardiner, Statistical analysis methods for chemists. A sofware-based approach, Royal Society of Chemistry, Cambridge, 1997.

[21] T. Jiang, L. Zhao, B. Chu, Q. Feng, W. Yan, J.M. Lin, Molecularly imprinted solid-phase extraction for the selective determination of 17β-estradiol in fishery samples with high performance liquid chromatography, Talanta, 78 (2009) 442-447.

[22] J. Aufartová, C. Mahugo-Santana, Z. Sosa-Ferrera, J.J. Santana-Rodríguez, L. Nováková, P. Solich, Determination of steroid hormones in biological and environmental samples using green microextraction techniques: An overview, Anal. Chim. Acta, 704 (2011) 33-46.

[23] A.C. Olivieri, G.M. Escandar, A. Muñoz de la Peña, Second-order and higher-order multivariate calibration methods applied to non-multilinear data using different algorithms, Trends Anal. Chem. 30 (2011) 607-617.

[24] A.C. Olivieri, G.M. Escandar, Practical three-way calibration, Elseiver, Amsterdam, 2014.

[25] A.G. González, M.A. Herrador, A.G. Asuero, Intra-laboratory testing of method accuracy from recovery assays, Talanta, 48 (1999) 729-736.

[26] D.M. Haaland, E.V. Thomas, Partial least-squares methods for spectral analyses. 1. Relation to other quantitative calibration methods and the extraction of qualitative information, Anal. Chem. 60 (1988) 1193-1202.

[27] P. Navarro, J. Bustamante, A. Vallejo, A. Prieto, A. Usobiaga, S. Arrasate, E. Anakabe, E. Puy-Azurmendi, O. Zuloaga, Determination of alkylphenols and 17β-estradiol in fish homogenate. Extraction and clean-up strategies, J. Chromatogr. A, 1217 (2010) 5890-5895.

[28] B. Chen, Y. Huang, M. He, B. Hu, Hollow fiber liquid-liquid-liquid microextraction combined with high performance liquid chromatography-ultraviolet detection for the determination of various environmental estrogens in environmental and biological samples, J. Chromatogr. A, 1305 (2013) 17-26.

[29] L.A. Currie, Nomenclature in evaluation of analytical methods including detection and quantification capabilities: (IUPAC Recommendations 1995), Anal. Chim. Acta 391(1999)105-126.

[30] G.M. Escandar, H.C. Goicoechea, A. Muñoz de la Peña, A.C. Olivieri, Second- and higher-order data generation and calibration: A tutorial, Anal. Chim. Acta 806 (2014) 8-26.

Figure Captions

Fig. 1 Excitation and emission solid-surface fluorescence (SSF) spectra on C18 (A) and nylon (B) membranes treated with 5 mL of 0.8 µg mL^{-1} EE2 (blue), E2 (black), and E2V (red) solutions. (C) Excitation and emission spectra for aqueous solution of 0.5 µg mL^{-1} EE2 (blue), E2 (black), and E2V (red) at neutral pH. Dashed-black lines correspond to background signals. In (A) and (B), λex = 280 nm and the photomultiplier tube (PMT) sensitivity = 355 V. In (C), λex = 270 nm and PMT = 650 V.

Fig. 2 UV-visible absorption spectra for aqueous solution of 17β-estradiol (E2, black), estradiol valerate (E2V, red), 17α-ethynylestradiol (EE2, blue), levonorgestrel (LNG, cyan), desogestrel (DSG, green), norgestimate (NGM, gray), norethisterone acetate (NTA, pink) and cyproterone acetate (CTA, orange). The dashed lines mark the excitation and emission wavelengths of the studied estrogens. $C_{EE2} = C_{E2} = C_{E2V} = C_{LNG} = C_{DSG} = C_{NGM} = C_{NTA} = 0.5$ µg mL^{-1}.

Fig. 3 Recoveries of estrogens in pharmaceuticals (as indicated), based on the amount reported by the manufacturing laboratory, using the fluorescence methodology in C18 (black bar) and nylon (red bar) membranes, and HPLC (green bar). Concentrations (indicated between parenthesis) in tablets, patches and gel are given in µg/pill, µg/cm^2, and µg/g respectively. Error bars correspond to triplicates. CTA, cyproterone acetate; DSG, desogestrel; GSD, gestodene; LNG, levonorgestrel; NTA, norethisterone acetate; NGM, norgestimate.

Fig. 4 Three-dimensional and contour plots for excitation-emission fluorescence matrices corresponding to C18 membranes treated with 10 mL of a typical validation sample containing 30 ng mL^{-1} of E2 (A), and of a chicken sample fortified with 57 ng mL^{-1} of E2 (B).

Fig. 5 Spectral profiles retrieved by MCR-ALS in the augmented mode (the dotted vertical lines separate, from left to right, a selected chicken sample and the successive calibration samples) (A), and spectral profiles in the augmented (B) and non-augmented (C) modes when processing a selected chicken sample. In all plots, green, red and blue lines indicated E2, interferent and background signals, respectively.

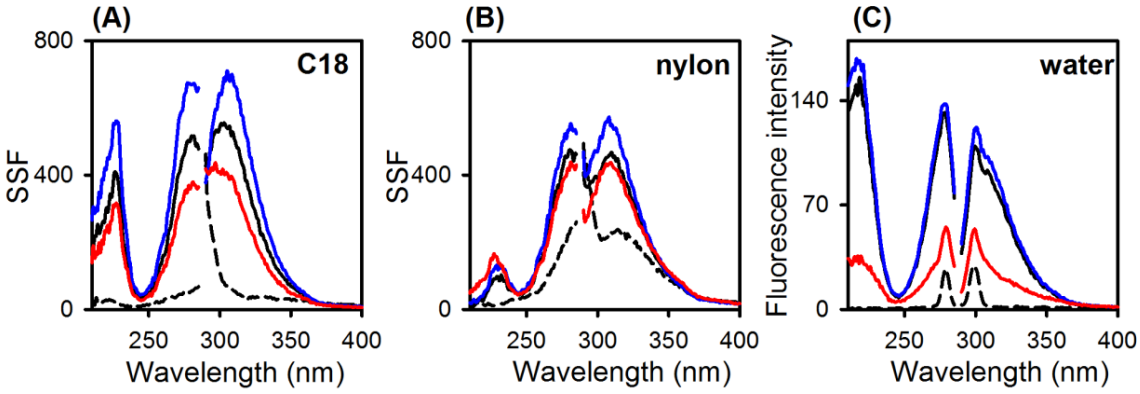


Figure 1

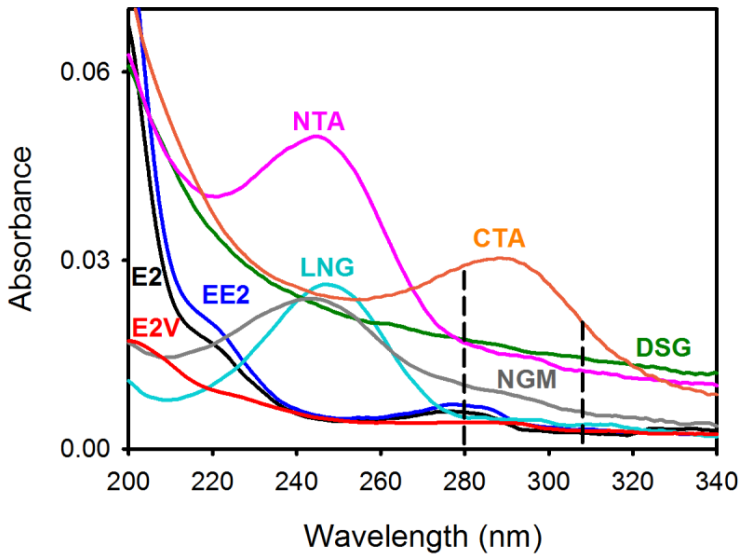
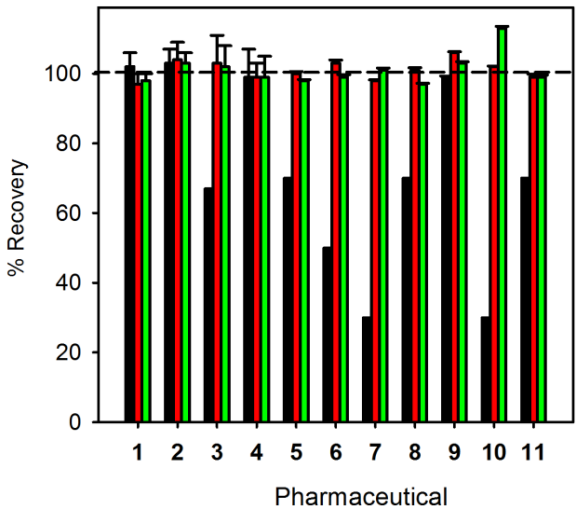


Figure 2



1 Lindisc 50 (patch): E2 (304)
2 Rontagel (gel): E2 (600)
3 Ciclocur brown pill (tablet): E2V(2000)/LNG (250)
4 Ciclocur white pill (tablet): E2V (2000)
5 April: EE2 (tablet) (20) LNG (100)
6 Marvelon (tablet): EE2 (30)/DSG (150)
7 Evra (patch): EE2 (30)/NGM (300)
8 Cliest (tablet): EE2 (36.75)/NGM (262.5)
9 Diane 35 (tablet): EE2 (35)/CTA (2000)
10 Primosiston (tablet): EE2 (10)/NTA (2000)
11 Ginelea (tablet): EE2 (30)/GSD (75)

Figure 3

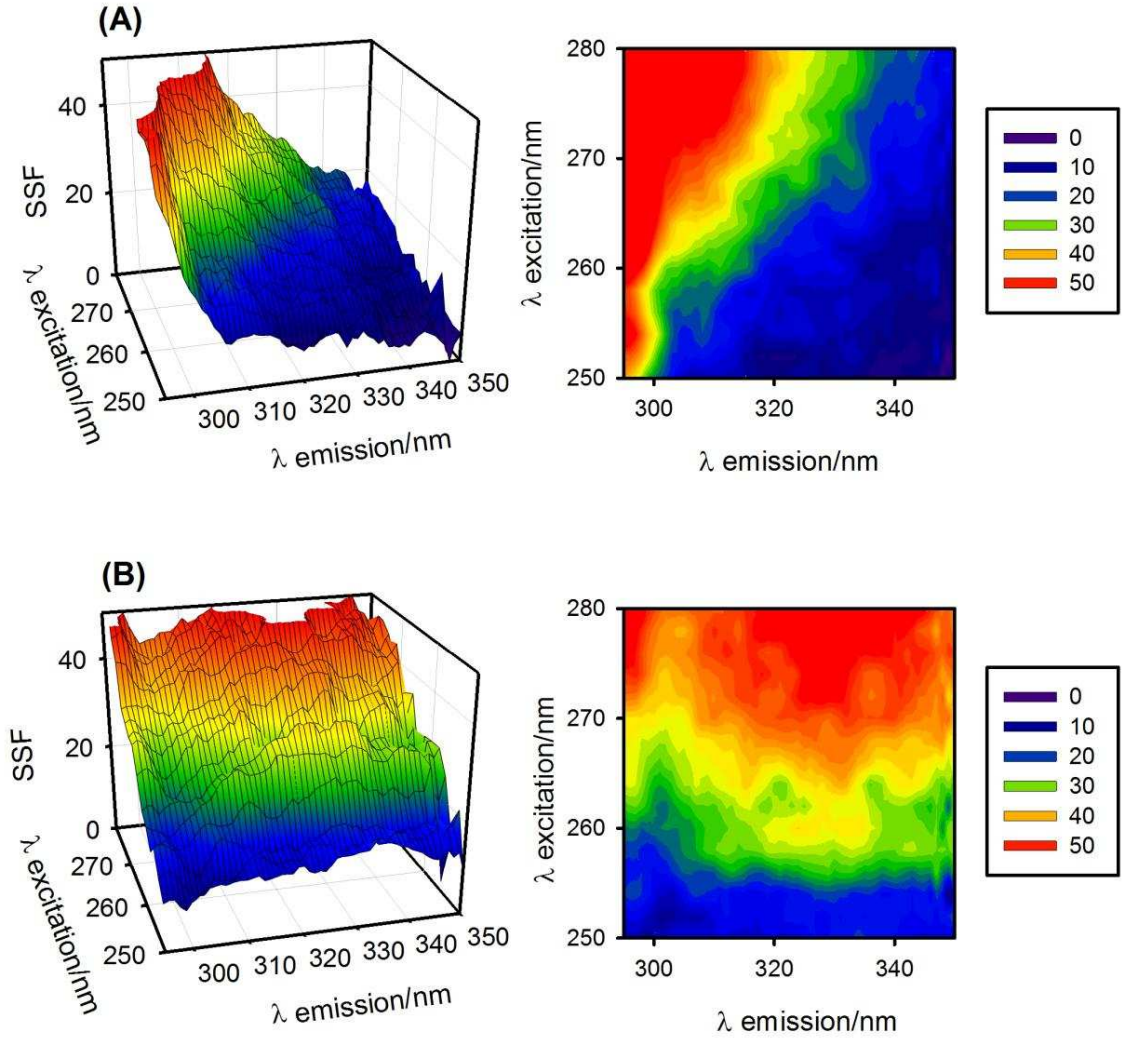


Figure 4

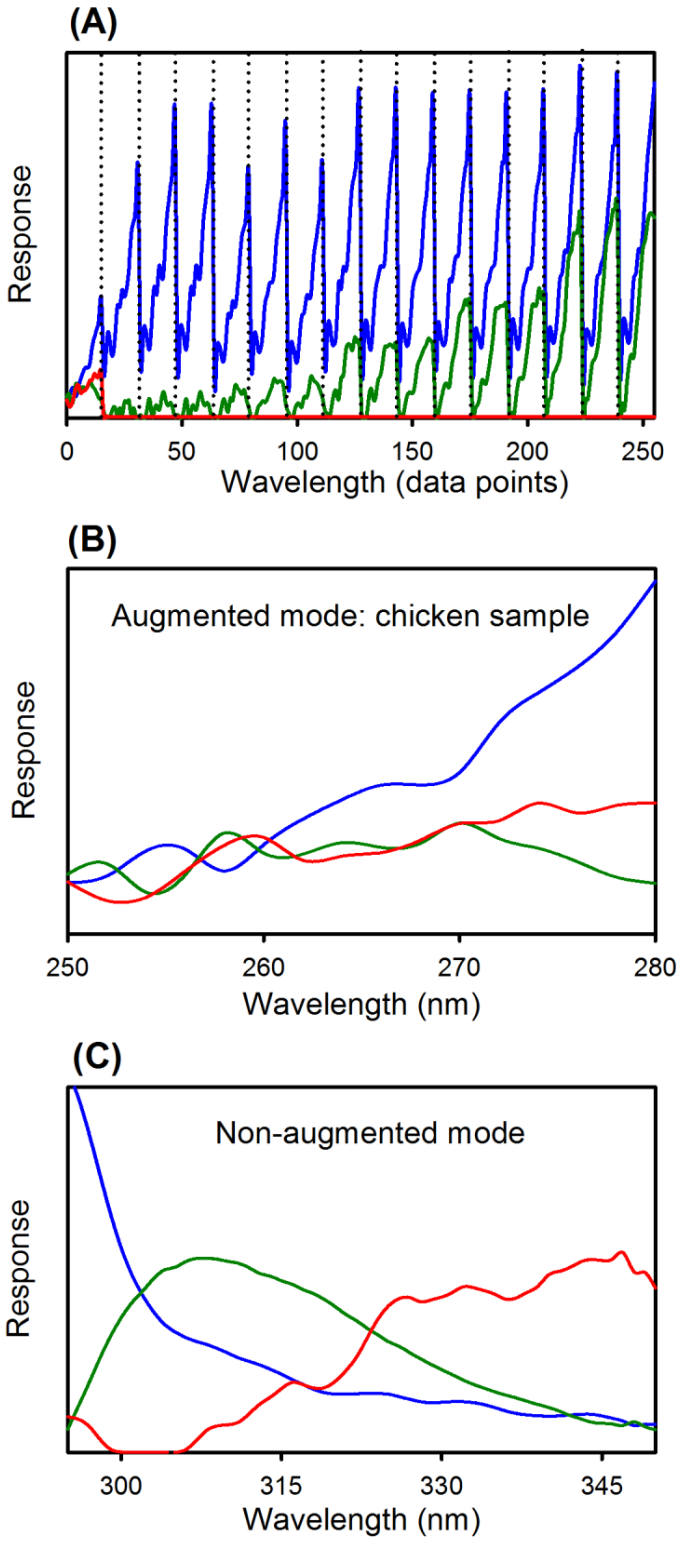


Figure 5

Table 1
Solid-surface fluorescence analytical figures of merit for E2, E2V and EE2[a]

	C18 membrane			Nylon membrane		
	E2	E2V	EE2	E2	E2V	EE2
Linear range ($\mu g\ mL^{-1}$)	0.15-1.0	0.10-0.8	0.15-1.0	0.21-1.0	0.12-0.8	0.15-1.0
Slope[b]	560 (8)	400 (5)	740 (10)	267 (5)	277 (5)	387 (6)
Intercept[b]	63 (5)	61 (2)	78 (6)	254 (3)	220 (2)	241 (3)
R[c]	0.998	0.999	0.998	0.996	0.998	0.998
(γ^{-1})[d] ($\mu g\ mL^{-1}$)	0.02	0.01	0.02	0.03	0.02	0.02
LOD[e] ($\mu g\ mL^{-1}$)	0.05	0.04	0.05	0.07	0.04	0.05
LOQ[f] ($\mu g\ mL^{-1}$)	0.15	0.10	0.15	0.21	0.12	0.15
RSD[g] (%)	0.5	0.8	0.6	1.2	0.5	1.1

[a] Values obtained when 5 mL of sample solution are extracted on membranes of 5 mm exposed diameter size. The number of data for each calibration curve corresponds to six different concentration levels, with three replicates for each level (n = 18)
[b] The corresponding standard deviations are given between parenthesis
[c] Correlation coefficient
[d] The inverse of analytical sensitivity (γ) represents the minimum concentration difference which can be measured
[e] LOD, limit of detection calculated according to IUPAC [39]
[f] LOQ, limit of quantification calculated as (10/3.3)×LOD
[g] RSD, relative standard deviation. In all cases four replicates were measured (estrogen amount = 0.4 $\mu g\ mL^{-1}$)

Table 2
Statistical results for E2 in samples without unexpected constituents (validation set) and in spiked real samples using EEFMs and different chemometric algorithms[a]

	PARAFAC	U-PLS/RBL	MCR-ALS
Validation set			
RMSEP (ng mL^{-1})[b]	5	5	5
REP (%)[c]	5	5	5
LOD (ng mL^{-1})[d]	6	2	8
LOQ (ng mL^{-1})[e]	18	6	24
Fish samples			
RMSEP (ng mL^{-1})[b]		5	5
REP (%)[c]		5	5
LOD (ng mL^{-1})[d]		7	8
LOQ (ng mL^{-1})[e]		21	24
Chicken samples			
RMSEP(ng mL^{-1})[b]		2	2
REP (%)[c]		2	2
LOD (ng mL^{-1})[d]		7	8
LOQ (ng mL^{-1})[e]		21	24

[a] Values obtained when 10 mL of sample solution are extracted on a C18 membrane of 5 mm exposed diameter size. Final E2 concentrations up to 200 ng mL^{-1} were included in the known linear range and no attempts were made to establish the upper concentration of the linear range since the goal was to detect low concentrations of E2.
[b] RMSEP, root-mean-square error of prediction
[c] REP, relative error of prediction
[d] LOD, limit of detection calculated according to ref. 40
[e] LOQ, limit of quantification calculated as (10/3.3)×LOD

Table 3
Recovery study of 17-β-estradiol in fish and chicken using solid-phase (C18) EEFMs and selected second-order algorithms[a]

	Added	Found[b]		
		U-PLS/RBL	MCR-ALS	HPLC
Fish				
Sample 1	0	–	–	–
Sample 2	0.12	0.10 (0.01) [83]	0.14 (0.09) [116]	0.12 (0.01) [100]
Sample 3	0.25	0.24 (0.04) [96]	0.21 (0.04) [84]	0.24 (0.05) [96]
Sample 4	0.69	0.7 (0.2) [101]	0.65 (0.01) [94]	0.71 (0.03) [103]
Sample 5	1.10	1.08 (0.01) [98]	1.04 (0.01) [95]	1.06 (0.03) [96]
Sample 6	1.84	1.98 (0.03) [107]	1.57 (0.03) [87]	1.81 (0.09) [98]
Chicken				
Sample 1	0	–	–	–
Sample 2	0.10	0.11 (0.01) [110]	0.10 (0.01) [100]	0.10 (0.01) [100]
Sample 3	0.20	0.20 (0.01) [100]	0.18 (0.02) [90]	0.22 (0.02) [110]
Sample 4	0.33	0.31 (0.04) [94]	0.36 (0.09) [109]	0.32 (0.03) [97]
Sample 5	0.52	0.52 (0.03) [100]	0.49 (0.03) [94]	0.51 (0.02) [98]
Sample 6	1.00	0.93 (0.01) [93]	0.92 (0.01) [92]	0.95 (0.08) [95]

[a] Concentrations are given in $\mu g\ g^{-1}$.
[b] Experimental standard deviations of duplicates are given between parentheses. The recoveries (in square brackets) are based on the added amounts.

Highlights

- Nylon and C18 membranes successfully extract estrogens from aqueous solutions

- Estrogens fluorescence zeroth- and second-order data are obtained from the surfaces

- MCR-ALS and U-PLS/RBL allow the quantification in very interfering media

- Simple and safe analysis of estrogens are developed using green-chemistry principles