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A green extraction method based on carbon nitride sorbent for the simultaneous determination of free and conjugated estrogens in milk

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ABSTRACT

This study proposes a quick and green method based on carbon nitride sorbent to extract six free and five conjugated estrogens from milk samples simultaneously. The adsorption process was studied in detail via adsorption isotherms, while the rate-limiting steps were investigated using adsorption kinetics. An experimental design was carried out for the elution step optimization regarding elution time, pH, and percentage of organic solvent. The best elution condition was 40 min at alkaline pH without the use of dichloromethane. After the matrix solid phase extraction optimization, extraction was carried out using this magnetic material after a previous deproteinization step. Separation, determination, and quantification of the target analytes were achieved by ultra-high-performance liquid chromatography coupled with triple quadrupole-tandem mass spectrometry. The methodology was validated in 6 milk samples.

The LODs and LOQs for free and conjugated estrogens investigated were in the range of 0.01–0.1 ng mL^{-1}. The recoveries of estrogens (concentration range of 0.5–10 ng mL^{-1}) from milk samples were in the range of 89–100%, with standard deviations ranging between 1 and 3%. The method was successfully applied to milk samples leading to the identification of estrone, 17-α-estradiol, and 17-β-estradiol and four forms of conjugated estrogens (17β-estradiol-3-glucuronide; estrone-3-sulfate; 17β-estradiol-3,17β-disulfate; 17β-estradiol- 3-sulfate).

1. Introduction

Endocrine-disrupting chemicals (EDCs) are defined by the Environmental Protection Agency (EPA) as exogenous substances that can interfere with the production and regulation processes, causing disorders in development, reproduction, metabolism, and behavior in various animal species, including humans [1,2]. Among EDCs, an important class consists of estrogens which can be of natural or synthetic origin. Endogenous estrogens such as estrone (E1), 17-α-estradiol (αE2), 17-β-estradiol (βE2), and estriol (E3), and their glucuronide and/or sulfate metabolites are formed naturally by humans and wildlife in ovaries and partially in the adrenal glands and adipose tissue [3,4]; exogenous estrogens are foreign compounds, either naturally or synthetically produced, including 17-α-ethinylestradiol (EE2), which has been widely used as a growth promoter for livestock or as a treatment for estrogen-deficiency disorders in veterinary medicine [5,6]. The occasional presence of relatively high amounts of natural estrogens in milk and milk derivatives and the abusive or illegal use of synthetic estrogens in dairy practices have become causes for concern since the intake of these hormones is associated with illnesses or disorders [7]. It is thus necessary to develop a sensitive and reliable analytical method for monitoring trace residues of estrogens in light of the complexity of milk matrixes. In recent years, several analytical approaches have been developed for the determination of estrogens, including high-performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC–MS), and enzyme-linked immunosorbent assay [5]. However, due to the complexity of the milk matrixes and the low concentrations, it is mandatory to develop sample pretreatment techniques with excellent clean-up and preconcentration efficiency for the selective extraction of estrogens before instrumental analysis. Currently, reported extraction approaches for estrogens include solid-phase extraction (SPE) [7–10], solid-phase microextraction (SPME) [11], and QuEChERS [12–14]. Magnetic SPE (MSPE) has recently received considerable attention due to its phase separation more conveniently by facile magnetic decantation than traditional SPE [15,16].

Moreover, MSPE possesses many advantages since it can increase the contact surface and improve the diffusion and mass transfer rate [17,18]. In addition, MSPE protocols involve shorter extraction time using a low amount of sorbent material and organic solvents, thus preventing the generation of toxic and dangerous wastes per the principles

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of green chemistry. [19,20]. In this scenario, carbon nitride (g-C_3N_4), a material composed of tri-s-triazine, represents a green and efficient sorbent featuring biocompatibility and high stability [21]. Its good extraction efficiency is due to its richness of nitrogen-containing functional groups and extensive π-conjugated system. It interacts favorably with analytes via complexation, hydrophobic effects, hydrogen bonding, electrostatic attraction, and π-interaction, making it one of the most promising candidates to complement carbonaceous materials in sample preparation [22]. Different synthesis approaches based on solid-state reactions, electrochemical deposition, solvothermal reactions, and thermopolymerization have been developed for bulk-g-C_3N_4 [23,24].

Magnetic g-C_3N_4 has excellent potential and has been used to enrich several compounds, such as phenolic acids, phthalate esters, polycyclic aromatic hydrocarbons, and brominated flame retardants in water samples, as described in the recent review [21]. A recent work applied magnetic g-C_3N_4 to enrich four free estrogens in milk powder [15].

The present work reported the application of g-C3N4/Fe3O4 nanocomposites to the simultaneous enrichment of five free and five conjugated estrogens from milk samples.

A synthesis starting from a green precursor such as melamine was carried out by an in situ growth method. The thermodynamics and absorption kinetics were studied to evaluate the interaction of the analytes of interest with the synthesized material. Furthermore, an experimental design was implemented to evaluate the elution conditions, the percentage of dichloromethane, the elution time, and the pH of the elution phase. The results showed that the best conditions were an elution of 40 min at alkaline pH without using dichloromethane, with recoveries higher than 97% for all the analytes. The optimized method was validated and used to quantify free and conjugated estrogens in 6 different milk samples. Three free estrogens (E1, αE2, and βE2) and four forms of conjugated estrogens, such as 17-β-estradiol-3-β-D-glucuronide (βE2–3G), 17-β-estradiol-3-sulfate (βE2–3S), estrone-3-sulfate (E1–3S), 17-β-estradiol-3,17-sulfate (βE2–3S-17S) were quantified in the analyzed milk samples. To the best of our knowledge, this work was the first that allows the simultaneous analysis of free and conjugated estrogens with magnetic g-C_3N_4 enrichment in milk samples.

2. Materials and methods

2.1. Chemicals and reagents

All reagents used for sample preparation, HPLC grade solvents, and pure standards of all selected compounds, i.e., E1, βE2, αE2, E3, E1–3S, βE2–3S, βE2–3G, βE2–3S-17S, 17-β-estradiol-3-β-D-glucuronide-17-sulfate (βE2–3G-17S) and synthetic EE2 were purchased from Merck Life Science (Darmstadt, Germany). Melamine was purchased by Fluka. Stock solutions of each analyte were prepared in methanol at 1 mg mL^{-1} level. βE2–3G-17S was prepared in a MeOH/water solution (50:50, v/v) due to its limited solubility in methanol. The stock solutions were diluted in methanol to prepare appropriate working solutions., A working mix solution was prepared by combining the working solutions and diluting them with methanol to obtain final concentrations of 450 ng mL^{-1}. The working solutions were prepared weekly to prevent degradation, stored at −20 °C, and brought to room temperature before use. The quality control standards were prepared from working solutions of the desired analytes concentration in water/MeOH solution (80:20, v/v). 10 mmol L^{-1} ammonium formate.

2.2. Magnetic carbon nitride composites g-C_3N_4/Fe_3O_4 synthesis

g-C_3N_4 was prepared as described by Yang and his coworkers [25] with some modifications. Briefly, the material was prepared by heating 4 g of melamine in a muffle oven, increasing the temperature from room temperature (RT) to 550 °C in 2 h, and keeping it constant for the next 4 h. The g-C_3N_4/Fe_3O_4 were obtained by treating 0.3 g of the carbon nitride g-C_3N_4 with 1.62 g of $FeCl_3 6H_2O$, 0.3 g of trisodium citrate, 7.20 g of sodium acetate, and 2 g poly(ethylene glycol)−10k in 80 mL of ethylene glycol solution. The mixture was sonicated for 3 h and then sealed in an autoclave for 10 h at 200 °C. The autoclave was returned to RT; the final product (g-C_3N_4/Fe_3O_4) was recovered, washed with water and ethanol, and dried at 80 °C for 3 h. After cooling, the material was stored in a glass flask at RT in a desiccator until use.

Table 1
List of the 17 experiments following the BBD with three variables (time, pH elution, dichloromethane percentage).

Exp	pH elution	time	% DCM
1	7	40	40
2	4	40	80
3	4	60	40
4	7	20	0
5	7	60	80
6	7	40	40
7	7	20	80
8	7	40	40
9	7	40	40
10	4	40	0
11	10	60	40
12	10	40	0
13	10	20	40
14	4	20	40
15	10	40	80
16	7	60	0
17	7	40	40

2.3. Sample collection and preparation

The milk samples were collected from the Rome (Italy) local market. The samples were analyzed on the same day the product was opened to prevent alteration of the samples. All samples were treated to eliminate casein, the most abundant protein, and other possible interferent substances. Briefly, 2 mL of milk was reached at pH 4.6 with HCl 5 mol L^{-1}, then the samples were placed at 4 °C for 30 min to favor protein precipitation and centrifuged at 9000 x g for 15 min at 4 °C. The supernatants were recovered and added to 100 mg of g-C_3N_4/Fe_3O_4. The sample was incubated in a sonicator for 60 min (determined by studying the dynamic binding); after the incubation time with magnetic decantation, the solution was eliminated, and the material was ready to be eluted. The elution condition, such as time of contact, the elution pH, and the percentage of dichloromethane in the eluent mixture, were optimized by the Box-Behnken design of the experiment (BBD). The commercial software Design-Expert 13 (StatEase, Minneapolis, USA) was used for the optimization. The parameters evaluated were the elution time considering the range of 20–60 min, the elution pH in the range of 4–10, and the percentage of dichloromethane in the range of 0–80%. The design provided 17 experiments with five replicates at the midpoint to estimate the pure error, as summarized in Table 1. Based on the results of the BBD analysis, the experimental conditions of experiment 12 were used. The results of all the experiments are reported in Supplementary Material Table S1-S2. Elution occurred for 40 min, the elution pH was basic (pH 10), and the percentage of dichloromethane in methanol was 0%. After eluting the analytes from the enrichment material, according to the optimized procedure, the extracts were evaporated to a small volume (<50 µL) in a water bath at 37 °C under a gentle stream of nitrogen. The residues were dissolved in 200 µL of water/methanol (50:50, *v/v*).

The g-C_3N_4/Fe_3O_4 material and related intermediates were analyzed by Fourier Transform Infrared Analysis (FT-IR) using a Nicolet iS50 coupled IR spectrophotometer with a Nicolet Continuum FT-IR microscope (Thermo Scientific), the spectra were obtained between 400 and 4000 cm^{-1} at a scan rate of 20 scans min^{-1} using KBr pellets. The 570 cm^{-1} peak was assigned for binding, the typical s-triazine or tri-s-triazine absorption peak was recorded in the range 1200–1650 cm^{-1}, and finally,

Table 2
Chemical formulas, retention times, precursor and product ions, and MS parameters of the analyzed estroge.

Compound	Chemical formula	Retention time	Precursor ion	Product ions m/z (Collision Energy) (CE)	SLens Voltage (Hz)	Ionic ratio Average[1]
E1	$C_{18}H_{22}O_2$	8.8	$[M-H]^-$ 269.1	**196.8**(41) 240.8(40)	118	16
βE2	$C_{18}H_{24}O_2$	8.3	$[M-H]^-$ 271.1	**183.1**(43) 145(40)	111	80
αE2	$C_{18}H_{24}O_2$	8.8	$[M-H]^-$ 271.1	**183.1**(43) 145(40)	111	85
E3	$C_{18}H_{24}O_3$	6.5	$[M-H]^-$ 287.1	**171.1**(40) 145.1(45)	116	84
EE2	$C_{20}H_{24}O_2$	10.5	$[M-H]^-$ 295.1	**143.0**(45) 145.1(40)	130	82
E1–3S	$C_{18}H_{22}O_5S$	8.8	$[M-H]^-$ 349.0	**269.1**(33) 145.1(57)	151	17
βE2–3S	$C_{18}H_{24}O_5S$	8.8	$[M-H]^-$ 351.0	**271.1**(37) 183.0(61)	109	6
βE2–3S-17S	$C_{18}H_{24}O_8S_2$	7.5	$[M-H]^-$ 351.1	**96.8**(41) 177.0(30)	130	64
βE2–3G	$C_{24}H_{32}O_8$	8.3	$[M-H]^-$ 447.2	**271.1**(45) 113.1(22)	113	58
βE2–3G-17S	$C_{18}H_{24}O_8S_2$	6.3	$[M-H]^-$ 527.1	**351.0**(37) 97(72)	152	34

[1] The relative intensities between the qualifier and the quantifier MRM transitions are reported as percentage.

the adsorption peaks at 3100–3300 cm^{-1} were associated with residual stretching of NH or OH (data not show). The values were in accordance with previous works [15,26].

2.4. UHPLC/ESI-MS/MS analysis

The LC-MS / MS instrumentation used was equipped with a UHPLC system Ultimate 3000 binary pump (Thermo Fisher Scientific, Bremen, Germany) and a triple quadrupole mass spectrometer (TSQ Vantage EMR, Thermo Fisher Scientific, Bremen, Germany) coupled by a heated electrospray (ESI) source. XcaliburTM v.2.2 software (Thermo Fisher Scientific, Bremen, Germany) was used to manage, acquire, and process LC-MS data. The separation was performed on a Hypersil Gold C8 (100 × 2.1 mm, 1.9 μm particle size, Thermo Scientific) equipped with a security guard C8 Hypersil Gold column (2.1 mm i.d. x 4 mm, 5 μm), maintained at 40 °C and operating at a flow rate of 0.3 mL min^{-1}. Mobile phases were water (A) and ACN (B) containing 5 mmol L^{-1} ammonium formate and 0.1% HCOOH. The gradient profile was as follows (t in min): t_0, B = 30%; t_3, B = 30%; t_8, B = 65%; t_{12}, B = 65%; t_{13}, B = 90%, t_{17}, B = 90%, $t_{17.5}$, B = 30%, t_{20}, B = 30%. Selected ion chromatograms of MRM of the investigated free and conjugated estrogens are reported in Supplementary Material Fig S1. Spray voltages of −2.6 and 3.0 kV were applied to the ESI source for the negative and positive ionization modes. The vaporizer temperature was set at 290 °C, and the capillary (ion transfer tube) temperature was at 280 °C. Sheath gas pressure, ion sweep gas pressure, and auxiliary gas pressure have been set to 40, 0, and 20 (arbitrary units), respectively. Mass calibrations and resolution adjustments on quadrupoles and resolution lenses were performed automatically using the manufacturer's solution once a month. For each compound, a solution at a concentration of 1 ng L^{-1} was infused at a flow rate of 10 L min^{-1}, and at least two selected reaction monitoring (SRM) transitions were monitored To optimize the tuning parameters of all the analyzed analytes (see Table 2).

2.5. Method validation

The HPLC-SRM method for analyzing estrogens in milk samples was validated following the main FDA guidelines using a pooled milk sample. The parameters evaluated were recovery (RE), matrix effect (ME), interday and intraday precision, linear dynamic range, linearity, the limit of detections (LOD_S), and the limit of quantification (LOQ_S).

REs were calculated on three replicates from the pool spiked with the analytes at two different concentration levels (C_a 0.5 ng mL^{-1} and C_b 10 ng mL^{-1}) according to Eqs (1). All aliquots were extracted as described in paragraph 2.3, and the peak was compared to those of another aliquot fortified after extraction with the same standard amount (SIM). Area $C_{a,b}$ and $Area_{SIM}$ were both subtracted by preexisting target compound area ($Area_{C0}$)

$$RE = \frac{Area_{Ca,b} - Area_{C0}}{Area_{SIM} - Area_{C0}} \text{X } 100 \quad (1)$$

MEs were calculated by comparing the $Area_{SIM}$ of each targeted compound (subtracted by the preexisting targeted compound $Area_{C0}$) and the area of the same targeted compound dissolved in the reconstitution solvent without extraction ($Area_{Rif}$).calculated with the following formula Eq. (2)

$$ME = \frac{Area_{SIM} - Area_{C0}}{Area_{Rif}} \times 100 \quad (2)$$

Intraday precision was evaluated by the means recovery's relative standard deviation (RSD) calculated from results generated under repeatability conditions of six replicates for each concentration level in a single day. The interday precision was calculated as the RSD of the recoveries obtained from six replicates performed on six different days.

Matrix-matched calibration curves were prepared in milk by spiking milk samples at suitable concentration levels to obtain the concentrations of the final extracts in the range 0.1–100 ng mL^{-1} paragraph by appropriate dilution of the working solutions. After spiking the milk samples, the extraction procedure described in the 2.3 paragraph was followed; 10 μL aliquots were injected into the LC-MS/MS instrument. All samples were analyzed in triplicate, and the results were averaged. For some compounds for which blank samples could not be found, the experimental curve matched to the matrix was subtracted from the endogenous value.

LODs and LOQs were calculated. The LODs were preliminarily estimated by evaluating the signal-to-noise ratio (S/N) of the lowest intensity SRM transition using the following formulas: LODs = 3 S/N; then, for the verification of the estimated values, fortified samples were prepared at the estimated value. The LOQs were set at the lower limit of the linear dynamic range.

2.6. Theory/calculation

2.6.1. Static adsorption tests

The interaction of the prepared g-C_3N_4/Fe_3O_4 material was evaluated by determining the adsorption mechanism [27–29]. The isotherm was constructed with a fixed amount of material (10 mg) dispersed in 1 mL of βE2 aqueous solution (concentration 2.5–1000 ng mL^{-1}). Subsequently, the samples were sonicated for 30 min to reach equilibrium. The material was magnetically decanted, and the supernatant was analyzed to quantify the residual concentration of βE2. The absorbed quantity was calculated with the following formula, Eqs (3):

$$Q_e = \frac{(C_0 - C_e)V}{m} \quad (3)$$

where Q_e was the amount of βE2 adsorbed on the material, C_0 and C_e were the initial and equilibrium concentration of βE2, respectively, m was the amount of adsorbent, and V was the volume of the solution. The pH of the solution was acidic (pH 2) for all experiments. The equilibrium data were fitted by applying the two-parameter model of Langmuir and Freundlich [14,30]. For the Langmuir fit, Eq. (4) was used:

$$Q_e = \frac{Q_{max} K_L C_e}{1 + K_L C_e} \quad (4)$$

where C_e was the equilibrium supernatant concentration, $Q_{e\ was}$ the amount of β-Estradiol adsorbed per unit mass of adsorbent, K_L was the adsorption free energy constant, and Q_{max} was the maximum adsorption capacity. Q_{max} and K_L were calculated linearly, fitting C_e/Q_e vs. C_e. For the Freundlich fit, the equation below was used, Eqs (5):

$$Q_e = K_F C_e^{\frac{1}{n}} \quad (5)$$

where K_F was the Freundlich constant indicative of the relative adsorption capacity of the adsorbent, and 1/n was the heterogeneity factor. The constants n and K_F were calculated by a linear fit of $\log Q_e$ concerning $\log C_e$. All experiments were performed in triplicate analysis by UHPLC-MS/MS.

2.6.2. Dynamic adsorption tests

The dynamic adsorption experiments were performed as previously described for the static adsorption experiments, a constant βE2 concentration at the saturation level (200 ng mL^{-1}) was used, and the supernatant was analyzed at different time points (1–120 mins). The amount of βE2 linked to time t was calculated according to the following equation:

$$Q_t = \frac{(C_0 - C_t)\ V}{m} \quad (6)$$

where Q_t represents the quantity linked to time t and C_t represents the temporal concentration at time t. The adsorption kinetics was described for pseudo-first and pseudo-second order binding with Eqs. (7) and (8), where t represents the adsorption time, K_1 is the pseudo-first-order adsorption constant, and K_2 is the pseudo-second-order adsorption constant.

$$\log (Q_e - Q_t) = log Q_e - \left(\frac{K_1}{2.303}\right) t \quad (7)$$

$$\frac{1}{Q_t} = \frac{1}{K_2 Q_e} + \frac{t}{Q_e} \quad (8)$$

3. Results and discussion

3.1. UHPLC-MS/MS optimization

Analyte standard solutions at 1 ng L^{-1} in H_2O/ACN (50:50, *v/v*) were infused to optimize ESI-MS/MS parameters. All examined estrogens (free and conjugated) were studied in negative ionization mode, with $[M-H]^-$ precursor, except for βE2–3S-17S, whose precursor was the $[M\text{-}H_2SO_3]^-$radical ion. Using a T-junction, a standard solution of the analyte at 1 ng L^{-1} and H2O/ACN (50:50, v/v) at 0.3 mL min^{-1}, was infused to optimize source parameters. ACN was used as an organic modifier for the chromatographic mobile phase, as reported in a previous study [10]. Furthermore, the addition of 0.1% formic acid showed a good response for the glucuronide metabolites, and 5 mmol L^{-1} ammonium formate allowed a simultaneous good response for the sulfate metabolites.

3.2. Optimization of the magnetic solid phase extraction procedure

For the BBD of the experiments, three factors were considered that can significantly influence the recovery ratio, the elution time (20–60 min), the pH (4–10), and the percentage of dichloromethane (0–80%). The BBD resulted in 17 experiments, with the central dot repeated five times (Table 1). In quantitative analysis, it is necessary to have a high recovery, avoid false positives, and identify trace amounts. Table S1 shows the results of the ANOVA tests, X_A X_B and X_C were significant terms for a quantitative recovery.

The result of the BBD analysis is graphically displayed in Fig. 1. The suggested results were: an elution time of 40 min, with a mobile phase at pH 10, and the absence (0%) of dichloromethane in the mobile elution phase to maximize estrogen recovery.

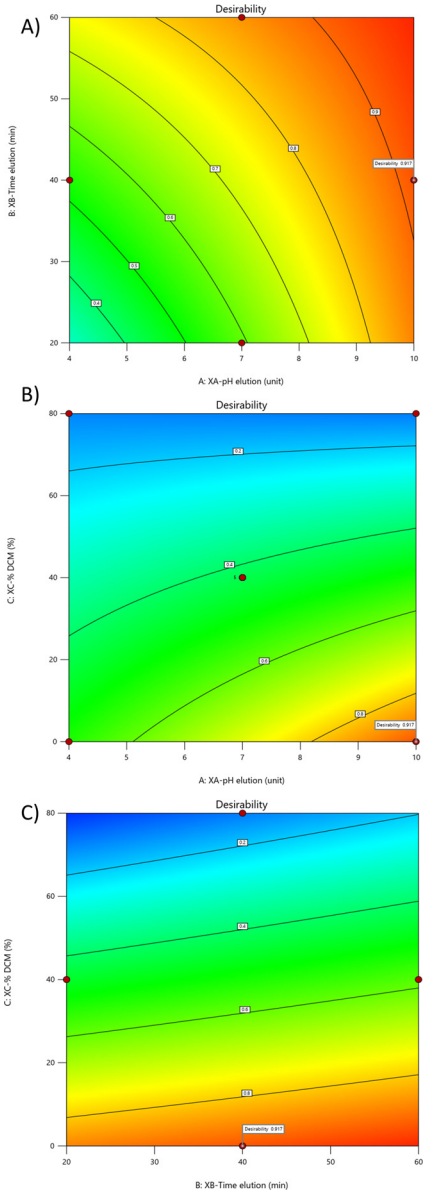

Fig. 1. Desirability function contour plot showing the effects of elution pH (XA), elution time (XB), and% dichloromethane (XC). The surfaces of rest refer to a pair of factors, in A) XAB, with constant XC; in B) XAC, with constant XB; in C) XBC, with constant XA.

3.3. Re-binding characteristics of g-C_3N_4/Fe_3O_4

The loading mixture was chosen based on the proposed application; therefore, an aqueous solution was chosen. The concentration range of β-E2 was 2.5–1000 ng $mL^{-1,}$ determined to reach the saturation level of the material. Two models were chosen to study the thermodynamics of adsorption. The Langmuir isotherm predicts that the analyte was adsorbed uniformly on the no longer adsorbed material beyond a saturation value. The Freundlich isotherm, on the other hand, has multilayered adsorption sites on the surface of the material.

Static adsorption studies of βE2 on g-C_3N_4/Fe_3O_4 showed a better correlation with the Langmuir model, as the experimental data fit the model with an R^2 of 0.97, while the Freundlich model fit less, with the R^2 of 0.82 (Fig. 2).

The calculated values for the Langmuir and Freundlich equation are summarized in Tables 3 and 4.

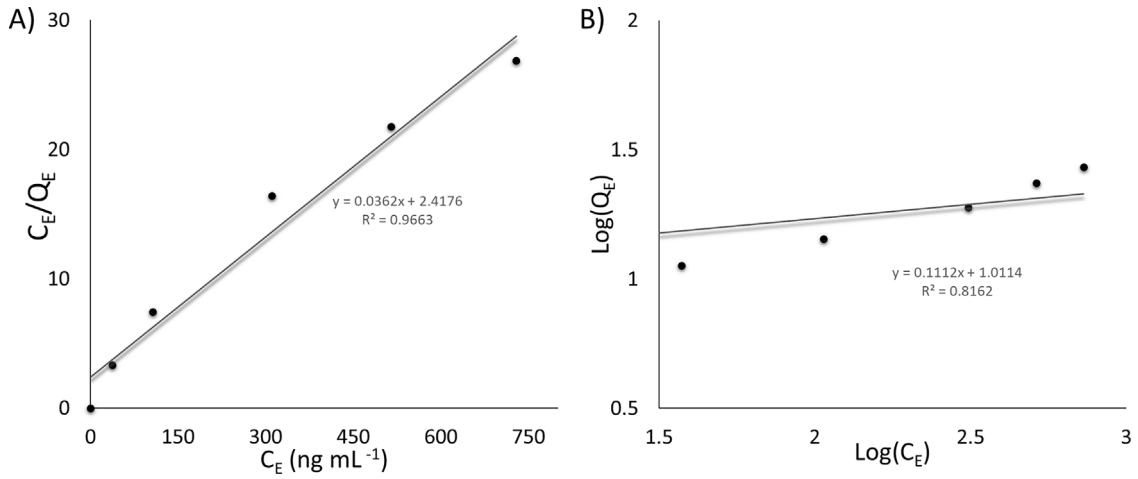

Fig. 2. Static binding for βE2 according to the Langmuir model (A) and Freundlich model (B).

Table 3
Parameters calculated by fitting the isothermal and kinetic models.

Langmuir isotherm	
Q_{Max} (ng mg^{-1})	27.62
K_L (mL mg^{-1})	1.5 E^{-2}
R^2	0.97
Freundlich isotherm	
K_F (mL mg^{-1})	1.29
n	0.99
R^2	0.82
Pseudo-first-order kinetics	
K_1 (min^{-1})	9.2 E^{-5}
R^2	0.53
Pseudo-second-order kinetics	
K_2 (min^{-1})	11.36
R^2	1.00

Table 4
Relative recoveries (n=3 for each level) and ME for the analyzed estrogens analyzed. Data are expressed as mean ± standard deviation.

Compound	RE % (C_a)	RE % (C_b)	ME %
E1	(98 ± 2)	(89 ± 3)	(91 ± 2)
βE2	(98 ± 2)	(94 ± 3)	(92 ± 3)
αE2	(97 ± 1)	(92 ± 1)	(93 ± 2)
E3	(99 ± 1)	(89 ± 2)	(90 ± 2)
EE2	(100 ± 1)	(98 ± 1)	(90 ± 3)
E1–3S	(99 ± 1)	(95 ± 2)	(105 ± 2)
βE2–3S	(100 ± 1)	(98 ± 1)	(110 ± 2)
βE2–3S-17S	(98 ± 2)	(95 ± 2)	(100 ± 2)
βE2–3G	(98 ± 1)	(96 ± 2)	(101± 3)
βE2–3G-17S	(99 ± 3)	(94 ± 1)	(110 ± 1)

Dynamic binding was investigated over a time range of 120 min. The saturation curve showed that complete adsorption occurred within 60 min. The saturation curve indicated that the adsorption of βE2 was rapid and occurred within 30 min (Supplementary Material Figure S2). Two kinetic models were evaluated, and a fit was obtained with second-order kinetics (R^2 1.00, Supplementary Material Figure S3.

The dimensionless equilibrium parameter was also calculated according to the equation below.

$$R_L = \frac{1}{1 + K_L C_0} \quad (9)$$

This parameter describes the type of isotherm as favorable ($0 < R_L < 1$), linear ($R_L = 1$), or unfavorable ($R_L > 1$). In the tested conditions, R_L always indicated a favorable interaction for βE2 on g- C_3N_4/Fe_3O_4.

3.4. Validation results

The analytical method for analyzing analytes in milk samples was performed following the main FDA guidelines using the milk pool sample. The proposed approach was evaluated based on RE, interday and intraday precision, linear regression parameters, LOD, and LOQ. All validation parameters and results are reported in Table S3.

RE values were calculated at two fortification levels (C_a: 0.5 ng mL^{-1}; C_b: 10 ng mL^{-1}) in milk samples, according to Eqs (1). These concentration levels were between the minimum and maximum values of the linear dynamic range.

For the investigated free estrogens, RE values were greater than 89%. In contrast, RE values were greater than 94% for conjugated estrogens, close to the lower and upper values of the considered linear dynamic range, respectively. ME values were calculated as previously described at concentration C_a and ranged from 90 to 110%.

The intraday and interday precision was assessed by performing recovery experiments (n = 6) performed on the same day and six consecutive days and measuring the RSD. Both were less than 15% in accordance with FDA guidelines. The linear dynamic range was evaluated considering the endogenous concentrations of the targeted analytes estimated from the milk pool analysis. Square linear regression coefficients (R^2) were > 0.99 for all analytes. LODs ranged from 0.01 to 0.05 ng mL^{-1} and 0.03–0.04 ng mL^{-1} for free and conjugated estrogens, respectively. LOQs were set at the lower limit of the linear dynamic range (0.1 ng mL^{-1}).

3.5. Estrogens content in different milk samples

The method was applied to analyze six cow's milk samples from different suppliers to demonstrate the applicability of the optimized g-C_3N_4/Fe_3O_4 enrichment method. Milk naturally contains small amounts of various hormones, including estrogens. Most commercial milk in industrialized countries is obtained from pregnant cows, which contains increased estrogen levels compared to non-pregnant cows. Because hormones like estrogen are fat-soluble, hormones are higher in whole milk than in skim milk, so all selected purchased samples consist of whole milk from different brands [31]. The quantitative results are summarized in Table 5.

The results obtained in the sample analysis showed that sample 2 was the richest in estrogen contents, while sample 6 showed no contamination. E3, βE2–3G-17S, and the synthetic estrogen EE2 were not identified in any samples. The absence of EE2 synthetic estrogen in the analyzed milk samples may suggest that the analyzed milk was not sub-

Table 5
Results of the quantitative analysis of estrogens in commercial milk samples. Data are expressed as concentrations in ng mL^{-1} ± RSD.

Compound	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
E1	< LOQ	0.15 ± 0.02	0.11 ± 0.01	< LOQ	0.12 ± 0.01	< LOQ
βE2	< LOD	0.11 ± 0.01	< LOD	< LOD	< LOD	< LOD
αE2	< LOD	0.15 ± 0.01	< LOD	< LOD	< LOD	< LOD
E3	< LOD	< LOQ	< LOD	< LOD	< LOD	< LOD
EE2	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
E1–3S	< LOQ	0.42 ± 0.03	0.25 ± 0.02	0.12 ± 0.01	0.11 ± 0.01	< LOQ
βE2–3S	< LOQ	0.13 ± 0.01	0.15 ± 0.02	< LOQ	< LOQ	< LOD
βE2–3S-17S	< LOQ	0.10 ± 0.01	< LOQ	< LOQ	< LOQ	< LOD
βE2–3G	0.12 ± 0.01	0.54 ± 0.02	< LOQ	0.25 ± 0.01	< LOQ	< LOD
βE2–3G-17S	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOD

Table 6
Comparison of the proposed method with others reported in the literature.

Compound	Pretreatment method	Instrument	LOD	LOQ	RE (%)	Ref
E1, βE2, αE2, E3, E1–3S, E2–3S, E2–17S, E2–3S-17S, E1–3G, E2–3G, E2–17G, E2–3G-17S	SPE-GCB	UHPLC-MS/MS	2–80 (ng L^{-1})	6–110 (ng L^{-1})	85–118	[8]
E1, β E2, αE2, E3	m-μ-Dspe (Fe3O4@pDA m-NPs)	UHPLC-MS/MS	-	0.5–11.8 (μg L^{-1})	72–110	[34]
βE2, αE2, E3, EE2	QuEChERS (d-SPE)	UHPLC-MS/MS	-	0.02–0.60 (μg L^{-1})	99–119	[35]
E1, E2, E3, EE2	Nanoparticles/Polypyrrole MSPE	LC–MS/MS	5.1–66.7 (ng L^{-1})	17.1–222.2 (ng L^{-1})	83.4–108.5%	[18]
E1, βE2, E3, EE2	DMIPMS	HPLC-MS/MS	0.10–0.35 (μg L^{-1})	0.30–0.60 (μg L^{-1})	88.9–102.3	[5]
E1, βE2, E3, EE2, E1–3S, E2–17S, E2–3S, E3–3S, E2–3S-17S, E2–3G-17S, E1–3G, E2–3G, EE2–3G, E2–3G-17G	Oasis™ HLB SPE	HPLC-MS/MS	10–25 (ng L^{-1})	78–105 (ng L^{-1})	23–103	[36]
E1, βE2, αE2, E3, EE2, E1–3S, E2-3S, E2-3S-17S, E2-3G, E2-3G-17S,	g-C_3N_4/Fe_3O_4	UHPLC-MS/MS	0.01–0.05 (ng mL^{-1})	0.1 (ng mL^{-1})	89–110	This work

jected to food fraud. βE2–3S-17S, αE2, and βE2 were identified only in sample 2.

The two major estrogen metabolites identified in milk were E2–3G (0.12–0.54 ng mL^{-1}) and E1–3S (0.11–0.42 ng mL^{-1}). As expected, these metabolites should be much higher in concentration than free estrogens because at least 85% of estrogens in milk are conjugated [32,33]. E1–3S was present in all samples except for 1 and 6, which showed values below the LOQ.

As it has been reported that the value of E1–3S can reach 1 μg L^{-1} in cow milk during pregnancy [33], the result shows the effect of the harmful practice of milking even during the advanced stages of pregnancy (see Table 5)

To the best of our knowledge, g-C3N4/Fe3O4 was applied for the first time to analyze milk samples for the simultaneous determination of free and conjugated estrogens. Recently, Na Li and co-workers reported the application of g-C3N4/Fe3O4 for MSPE of four free polar estrogens (i.e., β-E2, EE, E1, and HEX) in powder milk samples. LOD and LOQ values between the two works were not comparable, being fresh milk, a liquid matrix with a certain water content. Furthermore, compared with our developed method, the extraction was carried out with a non-green mobile phase consisting of n-hexane containing 2% acetonitrile (v/v) [15].

Concerning previous applications of different sorbents for extracting the studied analytes from milk samples, the evaluation of estrogens has been carried out using graphitized carbon black (GCB) [8], magnetic-micro-dispersiveSPE [34], QuEChERS coupled with dispersive-SPE [35], nanoparticles/polypyrrole MSPE [18], dummy molecularly imprinted polymer microspheres (DMIPMS) [5], and Oasis™ HLB- Amino SPE [36] as reported in Table 6. Most of the considered papers reported in the literature focused only on free estrogens, and only two also determined conjugated estrogens. The recovery, LODs, and LOQs achieved in the present work were similar to the previous ones, especially in the cases in which specific MIP sorbents were used, except when GCB or nanoparticles/polypyrrole MSPE was used, for which limits were lower than the ones obtained in our work (see Table 6). However, our developed method was green, avoiding toxic organic solvents. The procedure was more straightforward, mainly if compared to GCB, in which the elution of glucuronide and sulfate metabolites should be carried out in back flushing elution mode.

Some metric evaluation about the greenness of the proposed method, using the analytical greenness metric for sample preparation (AGREEprep), was carried out. AGREEprep is an open-access, intuitive software that produces an easy-to-read pictogram with information on the total performance and structure of the developed methodology [37] Fig. 3 reported the results of the AGREEprep assessment for our optimized procedure.

The procedure was performed *ex-situ,* and consumed 1.1 mL of MeOH. It was assumed the reagent and material were sustainable or renewable. Our material can be used several times for its ability to be used after a regeneration step. Moreover, the sample loading on the sorbent was constituted by acidified milk, which was not considered a waste. According to the fifth principle of green analytical chemistry, our procedure used a small sample size, reducing time, effort, costs, and resources and increasing the potential for automation. The small volume of investigated milk impact also the energy demand and the amount of solvent and reagent necessary for the analysis making our method good from an analytical greenness. The extraction procedure was manual and included the sonication step (~60 min), which was not optimal for criteria 8 of green analytical chemistry. Still it is acknowledged, however, that analysts may choose to perform more extractions simultaneously and, as such, improve the score in this criterion. A ~200 Wh power estimation was considered, and the use of HPLC-MS/MS at the next procedural step. The total score was 0.65, demonstrating that the proposed method possesses moderate analytical greenness.

Fig. 3. The results of AGREEprep assessment of procedure for estrogenic compounds enrichment based on magnetic carbon nitride composites g-C_3N_4/Fe_3O_4.

4. Conclusions

The g-C_3N_4/Fe_3O_4 material was synthesized and used for an MSPE. The experimental design allowed the optimization of the pH, elution time, and percentage of dichloromethane. The best results were obtained with 0% dichloromethane, which allowed this extraction to be green, 40 min elution time at basic pH. The material was studied to elucidate the thermodynamics and kinetics of the bonding process. Langmuir's model better described the adsorption on the material. The kinetic study indicated a rapid absorption occurring within 30 min. Four natural estrogens, five conjugated estrogens and one synthetic estrogen were used to validate the analytical method in cow milk samples. The developed method allowed to identify and quantify simultaneously 3 free and 4 conjugated estrogens in the 6 milk analyzed samples. The proposed method shows good LODs and LOQs, making it usable for other biological and food matrices.

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Declaration of Competing Interests

The authors have no conflicts of interest to declare. All co-authors have seen and agree with the manuscript's contents, and there is no financial interest to report. We certify that the submission is original work and is not under review at any other publication.

Data availability

Data will be made available on request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.greeac.2023.100055.

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