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ANALYSIS OF SEX HORMONES IN GROUNDWATER USING ELECTRON IMPACT IONIZATION

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ABSTRACT

A wide range of estrogenic contaminants has been detected in the aquatic environment, both in natural and synthetic forms. Steroid hormones are endocrine-disrupting compounds, which affect the endocrine system at very low concentrations. This work presents the development of an analytical procedure for the determination of five sexual steroid hormones, 17β-estradiol, estrone, progesterone, and the synthetics contraceptives, 17α-ethynylestradiol and norgestrel in groundwater from São Paulo University campus, specifically at Institute of Energy and Nuclear Research (IPEN). The analytical procedure starting with the sample pre-treatment, where the samples were first filtered and then extracted through solid-phase extraction, using Strata-X cartridges, and ending with detection. The separation method used was gas chromatography (GC), and the detection method was mass spectrometry (MS). The ion source used was electron impact ionization which produced an electron beam generated by an incandescent tungsten/thorium filament, which collide with molecules of gas sample. This interaction between the electrons and molecules, produce ions of the sample. The detection limits 0.06 $\mu g.L^{-1}$ for estrone, 0.13 $\mu g.L^{-1}$ for 17β-estradiol, 0.13 $\mu g.L^{-1}$ for 17α-ethynylestradiol, 0.49 $\mu g.L^{-1}$ for norgestrel and 0.02 $\mu g.L^{-1}$ for progesterone were detected in assays matrix. Validating tests were also used in this work.

1. INTRODUCTION

Water is fundamental to life. Its multiple uses are needed for a broad field of human activities which highlight the industrial and public supply, agricultural irrigation, production of electricity, leisure activities and recreation and preservation of aquatic life. However, the development models adopted by man for agriculture, livestock, industry and urban centers has not taken into account the environment [1]. Therefore, water quality studies are required to improve public health and social needs.

Contamination of groundwater although they are less vulnerable than surface water, may also be affected by contaminants from the surface, among which we can mention the pollution sources: landfills precarious that can provide infiltration of slurry; discharge of sewage from cities without proper treatment in surface bodies - septic tanks, drains, leaking pipes, industrial activities, agricultural activities and direct contamination by wells poorly executed.

Estrogens, androgens and progestogens, both natural and synthetic can be launched daily in water bodies since many organisms excrete them in different amounts depending on age, health status, diet or state of pregnancy woman [2].

Some studies show that the compounds called as endocrine disruptors, which are described by U.S. EPA (U.S. Environmental Programme Agency) as exogenous agents, interfere with the synthesis, secretion, transport connection or elimination of natural hormones [3,1] have been detected in samples from aquatic systems such as domestic sewage, due to the inefficient removal/destruction of these compounds after being treated in STPs (sewage treatment plant). These resistant to the most of the processes employed in the STPs, can also be found in surface waters and even in drinking water and groundwater.

The analysis of trace levels of organic compounds is one of the biggest problems encountered in analytical chemistry due to their low concentration and complexity of environmental matrices wherein these agents are. These factors develop new techniques for pre-processing and analyses of these compounds. Gas chromatography (GC) has been used as a separation technique and mass spectrometry (MS) has been used for more sensitive and selective detection systems.

This article focuses on the determination of the natural and synthetic estrogens estrone (E1), 17β-estradiol (E2), progesterone, 17α-ethinylestradiol (EE2) and norgestrel in groundwater. The main objective was develop a method to evaluate the quality of groundwater from São Paulo University campus, specifically at Institute of Energy and Nuclear Research (IPEN) using GC/MS.

2. EXPERIMENTAL

2.1 Materials and reagents

The standards of estrone, 17β-estradiol, 17α-ethinylestradiol, norgestrel and progesterone were Sigma-Aldrich, methanol and dichloromethane (HPLC-grade), acid chloridric, ultra pure water was obtained with a Milli-Q water puritication system (Millipore). Stock solutions of individual standards were prepared by dissolving each compounds in methanol at a concentration of 100μg/mL. From these stock individual solutions was prepared a mix

solution in the concentration of 10 $\mu g.mL^{-1}$. Working solutions in the concentration of 0.8; 1.2; 1.5; 1.8; 2.0; 2.3 and 2.5 $\mu g.mL^{-1}$ was prepared with a mix solution.

2.2 Sample pretreatment

The groundwater samples were collected from one well (P3) located at 23°33'40.76'' south and 46°44'27.90'' north in the Institute of Energy and Nuclear Research (IPEN) authority of the University of São Paulo. The volume collected consisted of 1 litre of sample packed in ambar glass bottles. After collecting the samples were filtered using a vacuum Satorius device developed in the laboratories of the Center of Chemical and Environment (CQMA/IPEN), with filter of PTFE (Polytetrafluoroethylene) of the 0.4μm of pore to remove suspended solids and stored in the refrigerator for later extraction.

2.3 Solid phase extraction

For solid phase extraction of groundwater samples a "manifold" system and Strata-X cartridges was used. Cartridges were first conditioned with 6mL of methanol and then with a solution of 4 mL of methanol and 2 mL of ultra pure water. Eluted 500 mL of acidified groundwater sample through the cartridge. The end of this step the cartridges were dried by centrifugation at 2500 rpm for 30 minutes and the subsequent elution of the analytes carried out with a solution of 2 mL of the methanol and 4mL of the dichloromethane. The eluate was dryness under nitrogen and then perform the analysis of the solution by CG/MS.

2.4 Instrumentation

The chromatographic instrument was GC-17A coupled mass spectrometer QP-5000 (Shimadzu) being the ion source used, electron impact ionization which produced an electron beam generated by an incandescent tungsten/thorium filament, which collide with molecules of gas sample. This interaction between the electrons and molecules, produce ions of the sample. The chromatographic column was DB-5 (28.8x0.25mm,0.25μm), and the volume injected was 1 μL. The run time was 31.66 min, where the acquisition mode SCAN was used for qualitative analysis while SIM mode was used for quantitative analysis. The mass analyzer used in the study was a type quadrupole analyzer and detector, the electron multiplier detector. The Table 1 show for each compound characteristic fragmentation that were monitored.

Table 1: Ratio mass / charge of the five ions monitored in SIM mode

Compounds	Ratio m/z				
Estrone	146	213	270	271	272
Estradiol	160	213	272	273	274
Ethynylestradiol	213	228	296	297	298
Norgestrel	91	245	297	312	313
Progesterone	124	147	314	315	316

3. RESULTS AND DISCUSSION

The analytical method involved extraction, concentration of the analytes in the sample and their determination by GC/MS, using the OTOMO [3] method. In the method proposed was used the cartridge Strata-X instead of the cartridge of solid phase extraction C18. The choice for this cartridge was due to Strata-X to be filled with polymeric sorbent which has a surface modified with styrene and a pyrrolidone group, whose retention mechanisms are hydrophobic, hydrogen bonds and aromatic, to acids, basic and neutral compounds. In many works the cartridge strata-x has been compared with other sorbents, which is considered as the best sorbent for solid phase extraction of analytes with different physicochemical properties, including hormones. The run time was increased the column temperature from 80ºC to 100ºC to optimize the analyses.

3.1 Validation of the analytical method

3.1.1 Selectivity

Selectivity is the first step in the development and validation of an analytical instrumental method of separation. Some samples suffer degradation, which producing compounds were not observed initially, and may coelute with the substance of interest. To solve this problem applies statistical tests to check the variability of samples. The tests used are the tests of hypotheses, that is, the Snedecor`s test for the ratio of variances and the Student`s test for normal populations with unknown and equal variances [4].

In order to verify the selectivity of the method this work was carried out chromatographic analysis of working solutions with the five compounds of study in solvent and groundwater matrix. It was shown by blank sample (matrix) that there was no interfering matrix at a retention time close to the analytes of interest.

Only analysis is not sufficient to verify that the method is selective or not. This was done using statistical calculations for a better understanding of the results and to check if the matrix effect was observed in the analyzes. The first test was used the Snedecor F to compare the variances of the two populations (with and without matrix) to determine if the populations had the same variance. The F test used was a right-tailed test, which was calculated F_{cal} with a significance level of 5% and n = 6 degrees of freedom, determining that the non-rejection and the rejection region of the null hypothesis, that is, if $F_{cal}>F_{tab}$ the matrix has an effect on the method and if $F_{cal}< F_{tab}$ the matrix does not affect the method. By the Table 2 it can be seen that most of the values obtained were greater than F_{tab} (F_{tab}=4.28), rejecting the null hypothesis concluding that there is a significant effect on the matrix method.

Table 2: Test of selectivity of the compounds studied, the standard addition in the matrix of groundwater (GW) and without matrix.

Estrone-concentration μg.mL^{-1}								
		0.8	1.2	1.5	1.8	2.0	2.3	2.5
Matrix GW	F_{cal}	13.53	16.78	2.78	36.69	0.35	0.73	46.31
	t_{cal}	124.76	249.99	126.47	107.19	43.50	155.10	66.31
Estradiol-concentration μg.mL^{-1}								
		0.8	1.2	1.5	1.8	2.0	2.3	2.5
Matrix	F_{cal}	157.73	3.97	4.73	9.38	2.42	1.11	8.89

GW	t_{cal}	60.72	103.73	80.87	89.63	92.21	86.71	31.72
Ethynylestradiol-concentration $\mu g.mL^{-1}$								
		0.8	1.2	1.5	1.8	2.0	2.3	2.5
Matrix	F_{cal}	50.65	3.85	1.44	12.62	0.20	0.02	0.34
GW	t_{cal}	30.37	80.64	77.28	43.74	46.59	27.01	95.51
Norgestrel-concentration $\mu g.mL^{-1}$								
		0.8	1.2	1.5	1.8	2.0	2.3	2.5
Matrix	F_{cal}	233.41	4.86	2.05	17.54	335.55	0.16	55.63
GW	t_{cal}	12.39	68.65	67.65	35.11	20.57	27.73	12.74
Progesterone-concentration $\mu g.mL^{-1}$								
		0.8	1.2	1.5	1.8	2.0	2.3	2.5
Matrix	F_{cal}	0.06	69.30	66.29	11.20	78.22	4.75	33.46
GW	t_{cal}	34.00	53.90	34.44	69.45	31.14	48.26	25.15

In the case of the t test, this was a right-tailed test, where worked with the averages of the samples. It was calculated the value t_{cal} at a significance level of 5% and n = 12 degrees of freedom in determining that the non-rejection and rejection region of the null hypothesis, and if $t_{cal} > t_{tab}$ matrix has an effect on the method, and if $t_{cal} < t_{tab}$ matrix has no effect on the method. It can be seen the Table 2 that all the values t_{cal} were higher than values t_{tab} (t_{tab}=1.78), it is concluded that the significance of the effect of matrix in the analytical method. These results show that to achieve the quantification of analytes should be used analytical curve prepared at the matrix.

3.1.2 Linearity

Linearity is the ability of an analytical method to produce results which are directly proportional to the concentration of analyte in a given concentration range [5]. It expresses the correlation signal in which the analytical measurement (height or area of the chromatographic peak) called the dependent variable y_i is linearly proportional to the concentration of the substance to be measured, called independent variables x_i. To construct the calibration curve is needed various concentration levels of at least five and the number of replicates at each concentration level should be as close as possible to that employed in routine laboratory [6]. In the present work, the calibration method was based on linear regression, were performed seven replicates injections at concentrations of 0.8; 1.2; 1.5; 1.8; 2.0; 2.3 and 2.5 $\mu g.mL^{-1}$ in methanol and in the matrix, thus obtaining the analytical curves. In the Table 3 are presented the coefficients of determination for test with matrix and without matrix. Second, INMETRO [6], correlation coefficient (r) above 0.90 are acceptable for evaluating the linearity of the analytical method. From the results obtained, the calibration curves were linear and the coefficients of determination were greater than 0.99, concluding that these curves can be used to quantify the samples, and the regression model adopted, linear.

Table 3: Coefficient of determination obtained by adding in the matrix of groundwater and only solvent.

Compounds	Addition in the matrix	Addition in the solvent
	r^2	r^2
Estrone	0.991	0.999

Estradiol	0.994	0.998
Ethynylestradiol	0.993	0.997
Norgestrel	0.995	0.995
Progesterone	0.992	0.993

3.1.3 Detection Limit\Quantification Limit

The detection limit is the lowest amount of analyte which differs significantly from the blank, which is calculated by multiplying the standard deviation of the seven replicates of low concentration calibration curve, the Student's t distribution for (n-1) degrees of freedom and 95% confidence, given by:

$$LD=t_{n-1,1-\alpha}.S \qquad (1)$$

The limit of quantification corresponds to the smallest amount of an analyte which can be measured accurately and faithfully determined. This accuracy is accepted as a variation coefficient of 10% and an accuracy of ±10% [7], given by:

$$LQ=x+10S \qquad (2)$$

where x is the average of seven replicates the concentration of the lower point of the calibration curve and S is the standard deviation. Table 4 and 5 shows the values of the detection and quantification limits of the method for the analysis in groundwater and in solvent.

Table 4: Limit of detection and quantification of the five compounds investigated in groundwater matrix

Compounds	Limit of method - Groundwater	
	LD($\mu g.L^{-1}$)	LQ($\mu g.L^{-1}$)
Estrone	0.062	0.942
Estradiol	0.137	1.158
Ethynylestradiol	0.136	1.160
Norgestrel	0.498	1.033
Progesterone	0.029	1.343

Table 5: Limit of detection and quantification of the five compounds investigated in solvent

Compounds	Limit of method – Solvent	
	LD($\mu g.L^{-1}$)	LQ($\mu g.L^{-1}$)
Estrone	0.030	0.879
Estradiol	0.020	0.833
Ethynylestradiol	0.043	0.916
Norgestrel	0.116	1.081
Progesterone	0.024	1.125

3.1.4 Precision

The precision may be determined in terms of repeatability and reproducibility. In this work, the precision was evaluated by the reproducibility (R), repeatability (r) and coefficient of variation. The samples were injected by the same analyst in the same instrument under the same analysis conditions and the same location. Table 6 presents the values for the coefficient of variation (CV) of the seven replicates in the test with matrix in three concentration levels (low, medium and high), being these values below 20%, which are acceptable by INMETRO [6].

Table 6: Coefficient of variation for the five compounds studied at three levels of concentration in an assay using groundwater matrix

Compound	Coefficient of variation		
	Low	Medium	High
Estrone	3.07	1.22	0.93
Estradiol	2.59	2.10	1.84
Ethynylestradiol	1.48	0.81	0.85
Norgestrel	3.19	2.61	2.30
Progesterone	0.93	2.28	3.28

With respect to repeatability and reproducibility tests were performed by analysis of seven replicates of the working solution in the matrix of groundwater into three concentration levels, and to evaluate reproducibility (R) tests were performed on different days, while the repeatability (r) assays were performed the same day. The values for R and r are described in Table 7 and 8.

Table 7: Values of repeatability limit for the five compounds in three concentration levels in the test with groundwater matrix.

Compounds	Repeatability limit (r)			
		Low	Medium	High
Estrone	$S_{replicates}$	0.025	0.014	0.023
	r	0.071	0.040	0.065
Estradiol	$S_{replicates}$	0.030	0.042	0.043
	r	0.085	0.119	0.121
Ethynylestradiol	$S_{replicates}$	0.030	0.018	0.022
	r	0.084	0.051	0.061
Norgestrel	$S_{replicates}$	0.035	0.034	0.052
	r	0.099	0.095	0.145
Progesterone	$S_{replicates}$	0.012	0.044	0.087
	r	0.033	0.124	0.087

Table 8: Values of reproducibility limit for the five compounds in three concentration levels in the test with groundwater matrix.

Compounds	Reproducibility limit (r)			
		Low	Medium	High
Estrone	$S_{replicates}$	0.009	0.006	0.010
	R	0.026	0.016	0.029

Estradiol	$S_{replicates}$	0.010	0.015	0.020
	R	0.030	0.043	0.056
Ethynylestradiol	$S_{replicates}$	0.013	0.011	0.008
	R	0.035	0.031	0.021
Norgestrel	$S_{replicates}$	0.020	0.014	0.031
	R	0.056	0.041	0.088
Progesterone	$S_{replicates}$	0.004	0.024	0.026
	R	0.012	0.068	0.074

According to Chui et al [8], for the present methodology repeatability and reproducibility, the values R and r must be greater than the difference between the replicates. Therefore the values of Tables 7 and 8 it appears that the proposed method is precise since all values of R and r are greater than their respective standard deviations.

3.1.5 Accuracy

Represents the degree of concordance between the results found in a given individual test and a reference value accepted as true [5]. The accuracy is calculated by the index z score given by:

$$Z=X_{lab}-X_v/S \qquad (3)$$

where X_{lab} is the value obtained by the laboratory, X_v, the value is accepted as true and S is the standard deviation of the test. The assay was performed in the groundwater matrix lower concentration of work, thus obtaining the values of z as shown in Table 9.

Table 9: Result of z score test for the five studied compounds in groundwater matrix and without matrix

Compounds	z Score	
	Matrix GW	Without matrix
Estrone	0.621	1.245
Estradiol	1.407	0.993
Ethynylestradiol	1.486	1.697
Norgestrel	1.629	0.766
Progesterone	1.864	0.473

From the results the method presented values of z less than 2 that are values considered appropriate in both trials, concluding that the analytical method provides accuracy.

3.1.6 Recovery

The recovery of the analyte can be estimated by analyzing samples and spiked with known amounts of the same, at least three different levels: low, medium and high. In most analytical procedures validation, recoveries in the range 70-120% are accepted, unless the desired range is specified with other values [7].

The assay for assessment of recovery was carried out with 500 ml of groundwater fortified with the solution mix of the five compounds in low concentrations. Table 10 shows the results obtained for the recovery test in the test matrix.

Table 10: Recovery rate for the five compounds studied

Compounds	Recovery (%) – Matrix GW
	Low concentration
Estrone	86
Estradiol	94
Ethynylestradiol	105
Norgestrel	105
Progesterone	70

Observing the values contained in the Table 10 verifies-that the percentages recovery ranged 105% being within the recommended range by the literature [5].

4. CONCLUSION

It was proved that there was no presence of any of these compounds (estrone, estradiol, ethynylestradiol, norgestrel and progesterone) in the analysis of the groundwater conditions.

Recovery rates using Strata-X cartridges were between 70-120% for all compounds, being within the range recommended in the literature for environmental matrices.

Optimized the amount of sample used in solid phase extraction, as well as the amount of solvents used in the development work.

The results obtained in the validation of the method were within the standards established in other literature 84. All compounds showed correlation coefficients greater than 0.99, the detection limits ranged from 0.062 to 0.498 $\mu g.mL^{-1}$ and quantification limits were 0.942 to 1.343 $\mu g.mL^{-1}$.

The method proved to be precise with coefficients of variation from 0.93 to 3.28 at concentrations low, medium and high, with all values below 20% and the index z score were all below 2 showing that the method provides accuracy.

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