VALIDATION METHODOLOGY FOR IDENTIFICATION AND MEASUREMENT OF PHENOLIC COMPOUNDS IN OIL REFINERY EFFLUENT BY HPLC

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ABSTRACT

The present work established a method, using High Performance Liquid Chromatography (HPLC) analysis, for the identification and quantification of five phenolic compounds. Chromatographic separation was performed by a reversed phase C18 column with a mobile phase consisting of methanol and aqueous 0.1% phosphoric acid in a ratio (10:90). The calibration curves showed excellent coefficients of determination ($R^2 \geq 0.99$) within the concentrations tested. The recoveries were 84.09% to 99.98% for spiked samples. The method can be adapted for the detection of phenolic compounds, which can be applied to monitor the effluent discharged into the environment.

KEYWORDS

HPLC; validation; phenolic compounds; oil refinery

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1. INTRODUCTION

The effluents of an oil refinery are wastes that originated from industries ~~are~~ involved in the refining of crude oil, manufacturing fuels, lubricants and petrochemical byproducts. These effluents consist of oil and grease, together with other highly toxic organic compounds (Abdelwahab et al., 2009).

Effluents are formed by industrial organic and inorganic components created from raw materials, especially hydrocarbons. These contaminants, in nature, lead to severe environmental impacts due to their high oxygen demand and toxicity of the individual components in wastewater. Thus, restrictive regulations indicate the minimum levels allowed for disposal (Rizzo et al., 2006; Diya'uddeen et al., 2012).

The importance of detecting the presence of toxic substances and assess their impact on the environment is recognized globally. These actions lead to the development of a series of methodologies targeting environmental protection. The occurrence of phenolic compounds in wastewater has become a matter of international concern because of its adverse effects on human health and its dangerousness, persistence, and bioaccumulation in animals and plants (Awomeso et al., 2010; Osibanjo et al., 2011; Achi and Ijeoma, 2011; Wang et al., 2012).

The presence of different concentrations of phenolic compounds has been detected in surface waters, causing a major concern facing the population. Studies have found toxicity in aquatic organisms, being detected in fish and plants. Moreover, polluted water bodies also serve as a source of drinking water for cities (Michalowicz et al., 2008). Several procedures for identification and quantification of phenolic compounds are reported in the literature. Among these techniques, the high performance liquid chromatography (HPLC) stands out (Tiburtius, 2008; Vrsaljko et al., 2012).

HPLC is a separation technique that, in less than thirty years, has become one of the most widely used analytical methods for qualitative and quantitative determinations. The reasons for this growth are related to its adaptability to quantitative analysis with suitable sensitivity. Using HPLC, one is able to perform the separation of non-volatile and thermally unstable analytes, especially

for applications in environmental samples (Collins, 2009). In analytical chemistry, to generate unambiguous data, free of error and uncertainty, is a difficult task. However, knowing the performance characteristics of a particular method, it becomes possible to ensure that the analytical measurements carried out by this method are close to the true unknown value, using the validation of analytical methods (González and Herrador, 2007; Moraci, 2008).

Validation involves procedures that demonstrate the reliability of the method. The results validated provide credibility, precision, and accuracy. Although there is no agreement on which parameters should be included in a validation process, one should consider the following parameters (Lanças, 2009; Rambla-Alegre et al., 2012): linearity, accuracy, repeatability, selectivity, limit of quantification (LOQ), and limit of detection (LOD). Linearity indicates the ability to provide results directly proportional to the concentration of analyte in the sample in a given concentration range (Lanças, 2009; Cassiano et al., 2009).

The correlation between the analytical signal and the concentration of the substance represented by the equation of the regression line is called a calibration curve (Oliveira et al., 2005; Lanças, 2009; Cassiano et al., 2009; INMETRO, 2010; Souza, 2011; Rambla-Alegre et al., 2012).

The equation of the line obtained by the least squares method can be used to estimate the coefficients of the analytical curve and can be described as equation 1.

$$Y = ax + b \quad (1)$$

Where: Y = dependent variable, measured response; x = independent variable, concentration of analyte; a = angular coefficient, expresses the slope of the graph to the axes; and b = linear coefficient, expresses the intersection with the axes of the graph.

The correlation coefficient indicates the percentage of variance explained. Thus, the closer to 1 a correlation is (R^2), the better the fit of the model will be (Otomo, 2010). The regulatory agency recommends a minimum of five concentration levels with a coefficient above 0.90 (INMETRO, 2003). Precision is a parameter evaluated based on the absolute standard

deviation (SD), which uses a significant number of measurements, typically greater than 20. Another expression of precision is given by estimating the relative standard deviation (*RSD*), also known as the coefficient of variation (*CV*), represented by equation 2:

$$RSD (\%) \text{ or } CV (\%) = \frac{s}{\bar{x}} \times 100 \quad (2)$$

Where: *s* = estimate of the standard deviation; $\bar{x}$ = average.

Typically, methods for quantifying compounds in large amounts require a macro *RSD* of 1 to 2%. For trace impurities, analytical methods *RSD* up to 20% are accepted, depending on the complexity of the sample (Huber, 1998).

The precision in validation methods is considered at three different levels: repeatability, intermediate precision, and reproducibility (Oliveira, 2008). Repeatability expresses fidelity obtained under the same operating conditions (same analyst, same equipment, same method) applied in a short time interval (Lanças, 2009). According to the regulatory agency at least seven measurements should be carried out (INMETRO, 2003) in an experiment. The accuracy expresses the agreement between the value found and the value accepted as true, by reference (literature value), by comparison of methods tests, or by standard addition recovery (Oliveira, 2008; Lanças, 2009; Souza, 2011).

The standard addition method is applicable when it is not possible to obtain a sample free of the substance of interest. Based on the method of recovery, which is a ratio of the amount of substance of interest present and added to the sample, it is possible to extract and quantify the analyte (Souza, 2011). In practice, the study of recovery is accomplished by adding known concentrations of the analyte in the sample (Thompson et al., 2002; Ribani et al., 2004). Three levels of concentrations should be chosen for tests of recovery, and they should cover the range of use of the method. For more complex samples, the acceptable recovery ranges from 50% to 120% (Lanças, 2009; Perlatti et al., 2012). The calculation of recovery (*R*) is performed according to equation 3.

$$R (\%) = \left(\frac{C_1 - C_2}{C_3} \right) \times 100 \quad (3)$$

Where: *C*₁ = concentration determined in the sample with addition of the standard; *C*₂ = concentration determined in the sample without addition of the standard; and *C*₃ = concentration of added standard.

The limit of detection (*LOD*) is the smallest amount of an analyte which can be detected. However, it is not necessary to quantify an exact value (Lanças, 2009). The presence of analyte can be calculated in several ways: using the visual method. *LOD* is estimated by multiplying 3 times standard deviation of the response (3 * *s*), divided by the slope or gradient (Ribani et al., 2004; Souza, 2011; Napoleão, 2011).

The Limit of Detection (*LOD*) can be determined by the method based on the analytical curve parameters, using equation 4 (Thompson et al., 2002; Paschoal et al., 2008; Ribani et al., 2004; Napoleão, 2011; Tahmouzi et al., 2011; Perlatti et al., 2012).

$$LOD = 3.3 \times \frac{s}{S} \quad (4)$$

Where: *s* = estimate of the standard deviation and *S* = slope of the analytical curve.

The limit of quantitation (*LOQ*) is the smallest amount of an analyte which can be measured with enough accuracy and precision (Lanças, 2009). The limit of quantification (*LOQ*) can be determined by the relationship between the estimated standard deviation of the response curve and the slope of the analytical curve, as shown in equation 5 (Oliveira, 2008; Ribani et al., 2009; INMETRO, 2010):

$$LOQ = 10 \times \frac{s}{S} \quad (5)$$

This study focuses on validating analytical methodology for identification and quantification of phenolic compounds, with mutagenic and carcinogenic action, from industrial effluents. The validation process has provided objective evidence that the system and method are suitable for the intended use.

Table 1. Specifications of products: phenol, hydroquinone, catechol, resorcinol and p-benzoquinone).

Organic Compound	manufacturer	Lot	Expiration date	Purity
Phenol	Chem Service	463-49A	03/17	99.5±0.5%
Catechol	Chem Service	468-126A	06/15	99.5±0.5%
Hydroquinone	Chem Service	456-90B	12/14	99.5±0.5%
Resorcinol	Chem Service	407-123B	09/17	99.5±0.5%
p-benzoquinone	Chem Service	463-151B	04/13	99.5±0.5%

2. MATERIALS AND METHODS

2.1 Preparation of standard solutions

All the standards (phenol, hydroquinone, catechol, resorcinol, and p-benzoquinone) were weighed separately in analytical balance, accurate to 4 decimal places, and had values equivalent to 2000mg. Table 1 shows the description of the standards of each organic compound (date of manufacture, batch, manufacturer and purity). The aliquots were diluted in deionized water in a volumetric flask to 1000mL measured in order to obtain a concentration of the stock solution of each organic compound 2000mg.L⁻¹.

From a standard solution (stock) prepared at 2000mg.L⁻¹, 15 different dilutions were made (2, 4, 6, 8, 10, 20, 30, 50, 70, 80, 100, 120, 140, 160, and 200mg.L⁻¹) for phenol, hydroquinone, catechol, resorcinol, and p-benzoquinone together. Then, immediately following the dilution, the samples were analyzed by High Performance Liquid Chromatography (HPLC) (Shimadzu – Prominence).

2.2 Determination of phenol using HPLC

The experiment used a HPLC C18 reversed phase column (5µm, 4.6 x 250 mm) and an UV detection (SPD-20A), for wavelengths between 254 and 270 nm for the determination of phenol and aromatic intermediates. The mobile phase consisted of a water solution acidified with phosphoric acid and methanol at a ratio of (90:10) with a flow of 0.75mL min⁻¹ in isocratic mode.

2.2.1 Validation procedure

The validation procedure was performed as

described in the introduction. The recovery was performed by adding known concentrations of the analyte in the sample. Various concentrations of standard (6, 10, and 30mg.L⁻¹) were added to a stock solution of 2mg.L⁻¹ of each compound for the linear range comprised between 2 and 50mg.L⁻¹. Concentrations of 70, 100, and 140mg.L⁻¹ were used for the linear range between 50 and 200mg.L⁻¹ with final concentrations in the respective ranges of linearity of the method. Concentrations used in this work for linear range, were based on literature in wastewater from oil refinery concentrations, ranging between 2 to 200 mg. L⁻¹.

3. RESULTS AND DISCUSSION

3.1 Validation of analytical parameters: analysis by HPLC

Seven analytical curves for phenol and its key intermediates (hydroquinone, resorcinol, catechol and p-benzoquinone) were prepared with eight different concentrations each. To identify each compound analyzed in the matrix by HPLC, the retention time was measured. This study, found that phenol had a retention time of 24.5min – hydroquinone; 7.5min – catechol; 14.5min - p-benzoquinone; 12.0min; and resorcinol, 11.8min. The compounds phenol, hydroquinone, resorcinol, and catechol were detected at a wavelength of 270nm and p-benzoquinone at 254nm. Figures 1 and 2 show the chromatograms obtained for the respective standards of phenol and aromatic intermediates. The peaks present in the chromatograms showed a similar behavior when compared with experiments performed by other authors (Zazo et al., 2005; Timbani, 2011).

Figure 1. Chromatograms obtained for the standards of aromatic hydroquinone, resorcinol, catechol and phenol observed at a wavelength of 270nm.

Figure 2. Chromatogram obtained for standard aromatic compound p-benzoquinone observed at a wavelength of 270nm.

From the obtained peak areas it was possible to calculate the mean and standard deviation between the areas. After obtaining these results, verified the existence of outliers by the method, through Grubbs' test. This test, note anomalous outliers appear larger or smaller than the group measures. Equations 6 and 7 are shown below:

$$G_{<} = \frac{\bar{x} - x_{i<}}{s} \quad (6)$$

$$G_{>} = \frac{x_{i>} - \bar{x}}{s} \quad (7)$$

Where: $G_{<}$ = Grubbs' test to the lowest measured value; $G_{>}$ = Grubbs' test for the greatest measured value; $\bar{x}$ = Average; $x_{i<}$ = minor extent; $x_{i>}$ = superior extent; s = estimated standard deviation.

The mean area of each concentration of the compounds was carried out for 7 replicates, minimum required by regulatory agencies. Tables 2 and 3 show the results for the mean area, the standard deviation and Grubbs' test. The values obtained for the Grubbs' test had a value less than or equal to 2.020, which confirmed a confidence level of 95% when considering the 7 replicates areas (Barros Neto et al., 2002).

3.2 Analysis of linearity

The linearity of the method was assessed by analytical curves of each compound, considering the correlation coefficient (R^2) obtained by linear regression. Table 4 shows the linear ranges for each of the studied compounds, as well as equations of the lines and correlation coefficients obtained.

Table 2. Mean peak area, standard deviation and Grubb's test for phenolic compounds in the range of 2 to 50mg.L⁻¹.

Concentration (mg.L ⁻¹)	Hydroquinone			Resorcinol			Catechol			Phenol			p-Benzoquinone			
	Areas	SD	G<	G>	Areas	SD	G<	G>	Areas	SD	G<	G>	Areas	SD	G<	G>
2	51922.70	2573.10	1.49	1.44	32922.57	636.47	1.32	0.97	75018.86	1451.26	1.46	0.84	43564.14	780.5354	1.89	1.04
4	68481.60	1324.33	1.95	0.88	45034.00	457.79	1.39	1.48	98880.29	644.76	1.58	1.06	62930.10	1193.05	1.09	1.53
6	12150.00	6986.97	1.12	1.85	77884.71	1042.89	1.85	1.02	187384.70	3973.43	1.65	0.75	113200.00	2368.92	1.06	1.13
8	138349.30	6606.99	1.83	1.31	86451.57	1012.81	1.04	1.62	208752.70	1804.84	1.33	0.86	134572.00	3769.14	1.02	1.42
10	151738.40	11371.37	0.93	1.65	94391.29	1591.30	0.23	1.39	215918.60	3664.83	1.12	1.87	147163.00	1850.70	1.43	0.97
20	302558.00	26264.87	0.95	1.91	185109.90	1871.21	1.55	1.03	483867.10	7162.18	2.02	0.78	287439.00	3727.93	0.88	1.99
30	487621.00	26758.01	1.34	1.18	273835.90	4102.80	1.33	1.19	775278.40	2186.44	0.89	1.83	499257.00	8881.28	0.77	1.57
50	809446.00	31562.95	1.73	1.64	435963.00	4116.91	1.80	1.36	1242800.00	22369.07	1.92	0.69	794661.00	15918.10	1.86	0.96
SD = Standard Deviation; G <= Grubb's lowest; G> = Grubb's largest																

Table 3. Average peak areas, standard deviation and Grubb's test for phenolic compounds in the range of 50 to 200 mg L⁻¹.

Concentration (mg.L ⁻¹)	Hydroquinone			Resorcinol			Catechol			Phenol			p-Benzoquinone							
	Areas	SD	G<	G>	Areas	SD	G<	G>	Areas	SD	G<	G>	Areas	SD	G<	G>				
50	812129.70	24150.23	1.86	0.71	430572.10	8709.22	1.38	1.53	1256027.00	2613.05	1.54	1.53	850261.30	13198.83	0.10	0.81	2138699.00	98716.50	1.23	0.95
70	1136284.00	116744.8	1.46	1.42	580506.90	5223.42	0.86	1.43	1755962.00	3668.99	1.07	1.84	1189557.00	5189.10	1.44	1.42	3287104.00	445259.00	0.97	1.50
80	1225962.00	78528.67	1.25	1.27	657794.00	8793.88	1.40	1.56	2004574.00	7154.21	0.87	1.06	1340024.00	15859.80	1.96	0.58	3946943.00	577753.00	0.91	1.10
100	1538504.00	72118.44	1.69	0.96	893812.60	3288.41	2.01	0.50	2541124.00	8007.27	1.02	1.47	1677506.00	28119.60	1.83	1.15	5110530.00	632556.00	1.06	1.28
120	1914175.00	120595.4	1.61	1.41	969972.90	5898.16	1.71	1.59	3069925.00	5565.60	1.31	1.31	1959483.00	37085.20	1.07	1.66	6246580.43	47903.95	1.09	1.38
140	2137177.00	108667.4	1.71	1.03	1126298.00	13799.33	1.91	0.92	3549823.00	69809.40	1.23	0.81	2254431.00	32703.60	0.98	1.14	7414542.00	1070399.00	0.97	1.24
160	2538715.00	212034.7	1.66	1.36	1238906.00	25319.09	1.99	1.02	4012009.00	28697.30	1.85	1.04	2599719.00	41620.70	0.99	1.28	8484984.00	1103733.00	1.00	1.13
200	2936582.00	343499.7	1.43	0.91	1524643.00	16892.22	1.53	1.30	4959328.00	63933.30	1.15	1.76	3223542.00	57027.10	0.89	1.21	10608940.00	1189981.00	0.98	1.13
SD=Standard Deviation; G<= Grubb's lowest; G> = Grubb's largest																				

Table 4. Working range for each compound, straight-line equation, and determination coefficient (R^2).

Compound	Linear range (mg.L ⁻¹)	Equation of the line	R^2
Hydroquinone	2 to 50	$y=15855x+8801.3$	0.9973
	50 to 200	$y=14599x+10104$	0.9936
Resorcinol	2 to 50	$y=8414.6x+17212$	0.9984
	50 to 200	$y=7262.1x+92673$	0.9916
Catechol	2 to 50	$y=24716x+21550$	0.9967
	50 to 200	$y=24912x+30473$	0.9994
p-benzoquinone	2 to 50	$y=42379x+14216$	0.9965
	50 to 200	$y=56466x-579872$	0.9994
Phenol	2 to 50	$y=15822x+3240.3$	0.9958
	50 to 200	$y=15698x+81536$	0.9996

Table 4 shows that the values of the respective compounds correlations were above 0.99, being in accordance with the standards required by the regulatory agencies (ANVISA, 2003; INMETRO,

2010). The graphic representations of calibration curves for each of the compounds can be visualized in Figures 3 and 4.

Figure 3. Graphical representation of linearity for the five compounds studied in the working range between 2-50 mg.L⁻¹.

Figure 4. Graph of linearity for the five compounds investigated in the working range between 50-200 mg.L⁻¹.

3.3 Analysis of precision

The analysis was performed with precision based on the measurement of coefficient of variation (CV). Calculations were performed

according to equation 2. The CV values obtained for each concentration used in the construction of the curve are described in Tables 5 and 6. The results for all compounds included in the work range between 2 to 50mg.L⁻¹ and 50 to 200mg.L⁻¹.

Table 5. Values obtained for the linear CV in the range 2-50 mg.L⁻¹.

Concentration (mg.L ⁻¹)	Coefficient of Variance (CV)				
	Hydroquinone	Resorcinol	Catechol	p-benzoquinone	Phenol
2	4.956	1.933	1.791	10.310	1.792
4	1.934	1.017	0.604	10.240	1.896
6	5.751	1.339	1.963	1.290	2.092
8	4.776	1.172	0.800	3.41	2.801
10	7.494	1.686	1.571	8.980	1.258
20	8.648	1.011	1.714	19.630	1.297
30	5.487	1.498	0.261	5.660	1.779
50	3.899	0.944	1.719	13.660	2.003

Table 6. Values obtained for the linear CV in the range 50-200 mg.L⁻¹.

Concentration (mg.L ⁻¹)	Coefficient of Variance (CV)				
	Hydroquinone	Resorcinol	Catechol	p-benzoquinon	Phenol
50	2.974	2.023	0.229	11.35	1.552
70	10.274	0.900	0.230	13.96	0.436
80	6.405	1.337	0.393	14.73	1.184
100	4.688	0.368	0.315	12.30	1.676
120	6.300	0.608	1.991	0.77	1.893
140	5.085	1.225	2.163	14.53	1.451
160	8.352	2.044	0.787	13.13	1.601
200	11.697	1.108	1.418	11.34	1.769

Table 7. - Limits of quantification of the analytical curve relating to organic compounds.

(mg.L ⁻¹)	Phenol	Limit of quantification (LOQ)			
		Hydroquinone	Catechol	Resorcinol	p-benzoquinone
2-50	0.493	1.623	0.587	0.760	2.354
50-200	8.408	16.540	1.050	11.993	1.107

Table 8. Detection limits of the analytical curve relating to organic compounds.

(mg.L ⁻¹)	Phenol	Limit of Detection (LOD)			
		Hydroquinone	Catechol	Resorcinol	p-benzoquinone
2-50	0.163	0.536	0.194	0.250	1.107
50-200	2.775	5.460	0.350	3.958	14.456

Table 9. Detection limits of the analytical curve relating to organic compounds.

Concentration (mg.L ⁻¹)	Phenol	Hydroquinone	Catechol	Resorcinol	p-benzoquinone
2-6 (recovery)	84.82%	89.38%	85.25%	84.09%	86.85%
2-10 (recovery)	98.26%	88.20%	99.44%	90.35%	94.84%
2-30 (recovery)	95.86%	97.46%	94.75%	99.58%	97.73%
50-70 (recovery)	94.09%	92.51%	98.98%	92.51%	97.73%
50-100 (recovery)	99.15%	90.60%	99.27%	91.38%	94.51%
50-140 (recovery)	98.41%	99.89%	98.83%	99.32%	91.90%

Based on these results, it was found that all determined coefficients of variance (CV) in the two linear ranges of each compound were acceptable for trace analysis or impurities (Ribani et al., 2004; Souza, 2011).

3.4 Limit of Detection and Quantification

Tables 7 and 8 show the results of LOQ and LOD, respectively, for each of the organic compounds examined.

3.5 Analysis of accuracy

The experiments were performed in triplicate. The calculations for recovery were obtained according to equation 2. The results of the analysis (Table 9) show that, for all organic compounds studied, the average percentage of recoveries obtained were equal or higher than 84%. These results demonstrate that the values were within the range recommended in the literature of 50 to 120% for complex samples (Lanças, 2009; Ribani et al., 2004; Souza, 2011).

4. CONCLUSIONS

The analytical methodology used a reverse phase HPLC that allowed a rapid detection and quantification of the phenolic compounds. The method comprised a mobile phase consisting of water acidified with phosphoric acid, methanol pH 2.2 (90:10), and UV detection at 270nm for the compounds phenol, resorcinol, hydroquinone, and catechol, and 254nm for p-benzoquinone. The technique was sensitive enough to identify and quantify all compounds proposed in this paper.

The proposed analytical method for the detection and quantification of five compounds were satisfactory in terms of linearity, precision, accuracy and stability in the range of interest.

The issues addressed in this work highlight the importance of validating the analytical methodology applied in chromatography. In the experimental part, the results show that all main parameters for validation of analytical methods required were within the range established by regulators.

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