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Validation of an Analytical Method Using HPLC for Identification and Quantification of Aliphatic Acids Present in Effluents

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ABSTRACT: There is a great interest in developing rapid analytical methods and provide appropriate parameters for quantitative analysis of compounds derived from refractory pollutants. Such methods are important for routine quality control analysis of an effluent before being discharged into the environment. Therefore, a rapid methodology for identification and quantification of some aliphatic acids is proposed. A method using High Performance Liquid Chromatography (HPLC) analysis was established for the identification and quantification of six aliphatic acids including: maleic, acetic, fumaric, malonic, succinic, and oxalic acid. 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)) with a flow of 0.75 mL min^{-1} in isocratic mode.The calibration curves showed excellent coefficients of determination (R^2 e" 0.99) at the concentrations tested. The recoveries were 54.72 to 99.70% for spiked samples. The method is appropriate for the detection of aliphatic acids, which can be applied to monitor the effluent.

Key words: HPLC, Validation, Aliphatic acids, Wastewater, Effluents

INTRODUCTION

Disposal of organic contaminants in the aquatic environment from sewage effluents have been well addressed. Excessive use of pesticides, pharmaceuticals and personal care products in modern society also brings many types of organic pollutants in the aquatic environment (Sirtori *et al*., 2009; Broseus *et al*., 2009; Magureanu *et al*., 2010; Alvarez *et al*., 2012; Lang and Kahidai, 2012). Due to industrial development, various effluents containing organic pollutants are produced; among them, some of the organic pollutants present in industrial effluents are refractory, and can not be eliminated by traditional treatment (Martinez-Huitle and Brillas, 2009; Cui *et al*., 2009; Yang *et al*., 2010; Goméz *et al*., 2012; Lewis, 2012; Wanga *et al*., 2012; Li *et al*., 2012; Yang *et al*., 2012). Whatever the source of organic pollutants, their presence in water can affect aquatic organisms and may cause a potential risk to human health through drinking water or food (irrigated by polluted water) consumption. During the oxidation of many refractory compounds the formation of some intermediates, present among the aliphatic acids (maleic, fumaric, malonic, succinic, acetic and oxalic), is often observed. It is, therefore, necessary to develop procedures for new and effective treatments of organic pollutants in water and to apply analytical methods able to identify and quantify these compounds before being discharged to the environment. Unreliable analytical data can lead to take wrong decisions, devastating financial losses as well as irreparable and irreversible environmental damage. To ensure that a new analytical method generates reliable and interpretable information about the sample, it must undergo an evaluation called validation. The validation of a method is a continuous process that begins in the planning of analytical strategy and continues throughout their development and transfer (Silva *et al*., 2012; Gouveia and Castilho, 2102; Ribani *et al*., 2012; Rambla-Alegre *et al*., 2102; Gomes and Garcia, 2012). The separation techniques such as high performance liquid chromatography (HPLC) have gained prominence in analytical chemistry for the capability to perform qualitative and quantitative analysis in environmental samples. The parameters followed for the validation process are usually: linearity, accuracy, precision, limit of quantification, limit of detection and selectivity. The linearity can be established by evaluation of a signal (response)

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diagram as a function of concentration or content analyzed. Having a linear relationship, the analysis results should be assessed based on suitable statistical methods, for example, the least squares method which can be used to estimate the coefficients of the analytical curve. The equation of the straight line obtained is depicted in the equation y = ax + †b, where "y" is the dependent variable (measured response), "x" is the independent variable (analyte concentration), "a" is the angular coefficient (slope of the calibration curve) and "b" is the linear coefficient (intersection of the curve on the y axis). The number of points required to perform an analytical curve is variable depending on the reference consulted, as well as the number of replicates. Some regulatory agencies recommend at least five levels of concentrations, and a number of duplicate at each level (Thompson *et al*., 2002; INMETRO, 2003; Ribani *et al*., 2004; Lanças, 2004; Mowafy *et al*., 2012; Shewiyo *et al*., 2012). The correlation coefficient (R) is a parameter that expresses the relationship between x and y, and that allows to assess the quality of the calibration curve. The square of the correlation coefficient, called coefficient of determination (R^2) can be used to estimate the quality of the obtained analytical curve. R^2 can be between zero and one, the closer to 1, the less dispersion curve values †from the values †expected for linear behavior, then the better the fit of the model (Otomo, 2010; Souza, 2011; Gomes and Garcia , 2012; Camino-Sánchez *et al*., 2012). Through the precision of an analytical method it is possible to evaluate the dispersion of results between independent assays, repeated on the same sample or standards under similar preconditions. The precision can be evaluated in terms of repeatability, reproducibility and intermediate precision. As the repeatability of the most used one, in which expressed the fidelity obtained in the same operating conditions (same analyst, same equipment, same method) applied in a short time interval (Lanças, 2004; Ribani, 2004; Souza, 2011). The precision is expressed in terms of standard deviation (SD) and relative standard deviation (RSD), also known as the coefficient of variation (CV), as shown in equation 1. The acceptable value of the coefficient of variation depends on the concentration level analyzed and the complexity of the matrix considered. Some authors mention a RSD acceptable up to 20% in trace impurities analyzes (Thompson *et al*., 2002; Peruga *et al*., 2012; De Baere *et al*., 2012; Gasson *et al*., 2013).

$$CV(\%) = \frac{SD}{\bar{x}} \times 100 \qquad (1)$$

Being:
SD= Standard deviation of measurements
X= arithmetic mean of determinations

The Limit of Detection (LOD) is the lowest amount of analyte which can be detected, but not necessarily quantified using a particular method. This parameter is important for the use of threshold tests, since it defines the level below which the method can not work. There are several ways to calculate the LOD for HPLC methods. The most common approach is to determine the amount of sample that provides a signal-to-noise ratio of 2:1 or 3:1. An alternative approach to signal to noise is to estimate LOD based on the standard deviation of response. For this calculation, LD = 3.3 (SD / a), where SD is the standard deviation of the response and "a" is the slope of the calibration curve. The limit of quantification (LOQ) is the lowest level that an analyte can be quantified with any degree of certainty. The determination of LOQ is similar to that used for approaches LOD, although it should be consistent, taking into account that these figures of merit are calculated within a given method. Since the calculation of LOD and LOQ are similar and can be inferred, it is common to find LOD and LOQ reported together even if such a method may not need both values. The LOQ can be determined by a signal-to-noise ratio of 10:1, or approximated by multiplying LOD by 3.3. Likewise, LOQ can be estimated by the equation LOQ = 10 (SD / a) (Gimeno *et al*., 2012; Kong *et al*., 2012; Klein *et al*., 2012). To demonstrate the accuracy of a method certified reference materials can be used, as well as comparison of methods, tests or standard addition recovery (Ribani *et al*., 2004). The method of standard addition is carried out by adding known concentrations of the analyte in the sample (Thompson *et al*., 2002; Ribani *et al*., 2004; Otomo, 2010; Souza, 2011). Some regulatory agencies establish that three concentration levels should be chosen for tests of recovery, and they should cover the range of use of the method. Some researchers claim that, in the analysis of residues, acceptable recovery interval ranges are 70 - 120%, or 50 - 120% for complex samples (Thompson *et al*., 2002; INMETRO, 2003; Ribani *et al*., 2004; Simonelli *et al*., 2007). The calculation of recovery is performed according to equation 2.

$$R(\%) = \left(\frac{C_1 - C_2}{C_3} \right) x\,100 \qquad (2)$$

being:
C_1 = concentration determined in the sample with addition of the standard;
C_2 = concentration determined in the sample without addition of the standard;
C_3 = concentration of added standard.
Several concentrations of standard (6, 10 and 30 mg/L) were added to a stock solution of 2 mg/L of each compound for the linear range comprised between 2 and 50 mg/L and concentrations (70, 100 and 140 mg/

L) for the linear range between 50 and 200 mg/L with final concentrations in the respective ranges of linearity of the method (Thompson *et al*., 2002; Otomo, 2010; Souza, 2011).

MATERIALS & METHODS

Each standard of aliphatic acids (maleic, fumaric, malonic, succinic, and oxalic) was separately weighed (2000 mg), in analytical balance. The standards were purchased from Chem Service manufacturer with purity of 99.5 ± 0.5%. The portions weighed were dissolved with deionized water in a volumetric flask to 1000 mL in order to obtain a concentration of the stock solution of each organic compound of 2000 mg/L. From standard stock solution were prepared 15 dilutions: 2, 4, 6, 8, 10, 20, 30, 50, 70, 80, 100, 120, 140, 160, 200 mg/L of each acid, and then immediately analyzed by the High Performance Liquid Chromatography (HPLC) of Shimadzu instrument.

For the determination of aliphatic acids, a C18 reverse phase column (5µm, 4.6 x 250 mm) and UV detector (SPD-20A) for wavelength 254 nm, were used. The mobile phase consisted of a water solution acidified with 0.1% phosphoric acid and methanol at a ratio of (90:10) with a flow of 0.75 mL min^{-1} in isocratic mode.

RESULTS & DISCUSSION

To achieve the validation of the HPLC method for the seven acids, seven analytical curves (with eight different concentrations each) were prepared. The HPLC retention time analysis of each compound, in order to make their identification in the matrix, was measured. These are: oxalic acid (1.5 min), acetic acid (5.3 min), malonic acid (5.4 min), succinic acid (6.4 min), maleic acid (6.5 min) and fumaric acid (7 min). All acids were detected at a wavelength of 254 nm. Fig. 1 shows the chromatograms obtained for the respective standards of aliphatic acids. From the areas obtained from each concentration, the calculations of mean and standard deviation were made. Tables 1 and 2 show all results of this part of the study.

The linearity of the method was assessed by analytical curves of each compound, considering the determination coefficient (R^2) obtained by linear regression. Table 3 shows the linear ranges for each of the studied compounds, as well as equations of the lines and determination coefficients (R^2) obtained. As can be seen, all the values †of the respective compounds correlations were above 0.99, according to the standards required by regulatory agencies.

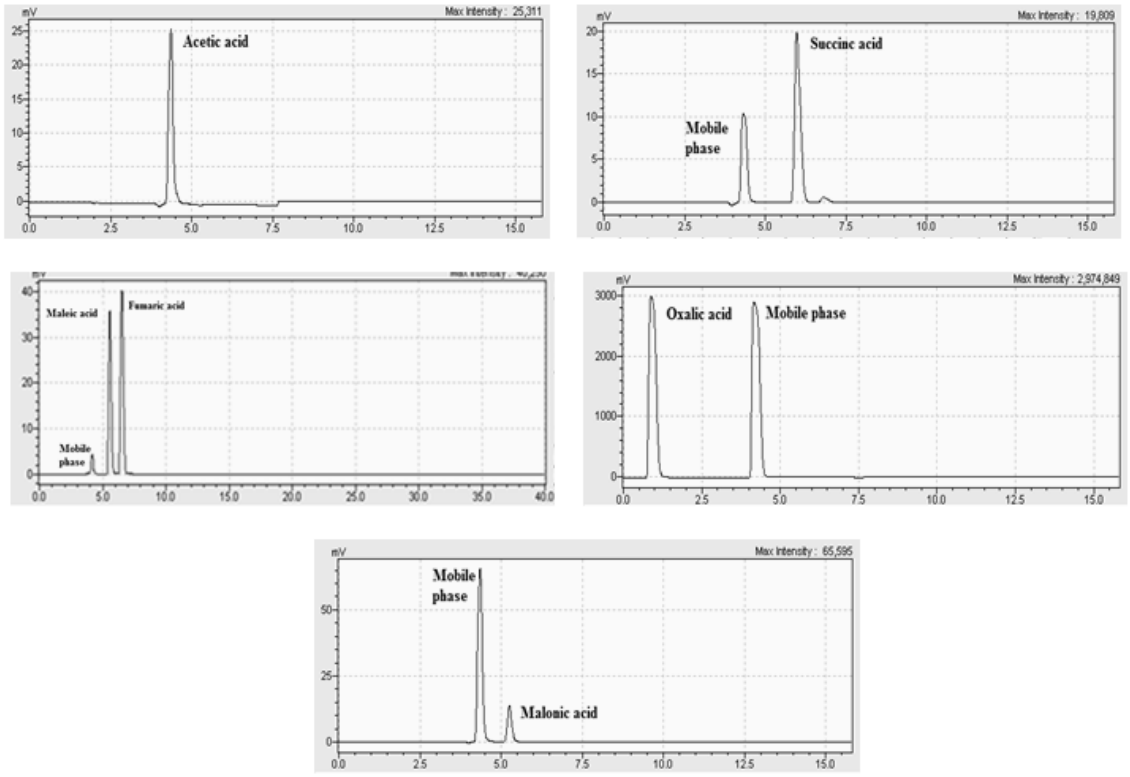

Fig. 1. Chromatograms obtained for aliphatic acids analytical standards at wavelength of 254nm

Table 1. Mean peak area and standard deviation for acids in the range of 2 to 50 mg/L

Concentration (mg/L)	Oxalic		Acetic		Malonic		Succinic		Maleic		Fumaric	
	Areas	SD	Areas	SD	Areas	SD	Areas	SD	Areas	SD	Areas	SD
2	235182.43	2768.01	2721.14	71.57	2016.43	297.01	2127.26	192.35	347217.90	15107.09	428925.98	12335.40
4	262974.53	20265.61	4527.57	391.57	6029.57	1079.21	4307.50	339.59	658658.50	10688.65	817603.66	9865.53
6	386626.20	8251.04	7123.90	660.84	8610.43	471.87	6466.10	588.66	1008887.85	16600.29	1260773.04	15512.77
8	407068.33	14634.44	9646.19	1493.79	11836.55	799.23	9083.52	1037.70	1330260.32	29341.60	1663472.31	35624.50
10	447534.57	15245.43	11929.12	1961.37	15445.07	413.23	11231.76	986.59	1660440.49	88619.19	2094850.22	78470.15
20	613616.33	29803.90	24874.14	2293.15	34086.29	1434.33	22216.24	1979.46	3403351.25	110874.36	4307114.53	74009.61
30	793383.17	47344.49	41114.38	2523.83	50980.90	694.30	33464.12	3590.85	4998293.11	44834.36	6277540.83	52671.89
50	1173716.71	32970.82	67266.29	67266.29	84904.29	2283.77	61137.93	806.38	8097816.86	96037.14	10201158.07	114031.80

SD = Standard Deviation

Table 2 . Mean peak area and standard deviation for acids in the range: 50 to 200 mg/L

Concentration (mg/L)	Oxalic		Acetic		Malonic		Succinic		Maleic		Fumaric	
	Areas	SD	Areas	SD	Areas	SD	Areas	SD	Areas	SD	Areas	SD
50	1171692.88	33876.11	67127.76	6147.18	85592.42	1948.06	61070.93	785.280	8171150.76	56834,.04	10211326.90	104411.30
70	1549280.00	44728.14	94706.86	9128.86	118042.95	2185.03	82555.36	6497.04	11429679.83	94637.01	14355636.95	160405.40
80	1807353.00	142912.66	106438.00	6692.97	137677.31	1622.48	92786.81	9962.44	12750312.76	231516.60	16096195.79	143770.20
100	204028.93	61874.48	133275.81	5872.99	172981.24	1298.05	120106.55	6017.44	16050504.14	68090.11	20123469.38	190887.40
120	2330210.36	25542.98	159243.00	9515.32	204785.10	784.11	145498.95	2648.36	19547967.95	447319.80	24258019.52	194513.70
140	2873060.00	95684.14	184772.50	10631.00	248653.57	1519.35	167922.38	6444.44	22159923.90	137563.30	27300731.52	1222039.00
160	3072636.95	46340.01	208787.38	12412.06	277197.62	55177.56	193894.83	5907.38	25329118.52	1108889.00	31577264.29	304553.70
200	3843192.98	94447.74	258206.93	16114.99	347400.38	1829.14	242449.81	3461.73	31523866.33	514551.10	39690874.90	694877.20

SD = Standard Deviation

The analysis was performed based on the measurement of coefficient of variation (CV). Calculations were performed in accordance with equation 1. The CV values obtained for each concentration used in the construction of the curve are described in Table 4. The results for all compounds were included in the work ranges between 2 and 50 mg/L and from 50 to 200 m/L. Based on these results, it was found that all coefficients of variation (CV) in the two linear ranges of each compound were acceptable ($\leq$20%).

Tables 5 and 6 show the results of LOD and LOQ for each of the organic compounds examined.The experiments were performed in triplicate to obtain accurate data on responses of experimental analysis. The calculations for recovery were obtained according to equation 2. The results of the analysis (see Table 7), showed that for all aliphatic acids studied a percentage recovery average of over 50%, were obtained, demonstrating that the compounds recovery values †are within the range recommended in the literature, 50-120 % for complex samples (Ribani *et al*., 2004; Lanças, 2004; Souza, 2011).

Table 3. Working range for each compound, straight-line equation and determination coefficient (R^2) obtained

Acid	Linear range (mg/L)	Equation of the line	R^2
Oxalic	2 a 50	y=18971x+231215	0.9906
	50 a 200	y=17552x+315495	0.9942
Acetic	2 a 50	y=1366.9x+1062.5	0.9986
	50 a 200	y=1273.2x+5147.5	0.9996
Malonic	2 a 50	y=1734x-1420.4	0.9996
	50 a 200	y=1761.2x-3609	0.9991
Succinic	2 a 50	y=1212.2x-944.63	0.9972
	50 a 200	y=1220.6x-2201.7	0.9994
Maleic	2 a 50	y=162259x+46539	0.9995
	50 a 200	y=156291x+416523	0.9996
Fumaric	2 a 50	y=204923x+51439	0.9994
	50 a 200	y=194116x+608581	0.9992

Table 4. Values obtained for the linear CV in the range 2-50 mg/L

	Coefficient of Variance (CV)					
Concentration(mg/L)	Oxalic	Acetic	Malonic	Succinic	Maleic	Fumaric
lower range						
2	1.18	2.63	14.73	9.04	4.35	2.87
4	7.71	8.65	17.90	7.88	1.62	1.20
6	2.13	9.28	5.48	9.10	1.65	1.23
8	3.60	15.49	6.75	11.42	2.21	2.14
10	3.41	16.44	2.68	8.78	5.34	3.75
20	4.86	9.22	4.21	8.91	3.26	1.72
30	5.97	6.14	1.36	10.73	0.90	0.84
50	2.81	6.81	2.69	1.32	1.19	1.12
greater range						
50	2.89	9.16	2.28	1.29	0.70	1.02
70	2.89	7.41	1.85	7.87	0.83	1.12
80	7.91	6.29	1.18	10.74	1.82	0.89
100	3.06	4.41	0.75	5.01	0.42	0.95
120	1.10	5.98	0.38	1.82	2.29	0.80
140	3.33	5.75	0.61	3.84	0.62	4.48
160	1.51	5.94	19.91	3.05	4.38	0.96
200	2.46	6.24	0.53	1.43	1.63	1.75

Table 5. Limits of quantification of the analytical curve relating to organic compounds

Limit of Quantitation (LOQ)						
Range	Oxalic	Acetic	Malonic	Succinic	Maleic	Fumaric
Less	1.459	0.524	1.713	1.587	0.929	0.602
More	19.30	48.281	11.061	6.434	3.636	5.379

Table 6. Detection limits of the analytical curve relating to organic compounds

Limit of Detection (LOD)						
Range	Oxalic	Acetic	Malonic	Succinic	Maleic	Fumaric
Less	0.481	0.173	0.565	0.524	0.307	0.199
More	6.369	15.932	3.650	1.123	1.200	1.775

Table 7. Results of mean recoveries for phenol, hydroquinone, catechol and p-benzoquinone in the working range between 2 to 50mg/L

Concentration (mg/L)	Oxalic	Acetic	Malonic	Succinic	Maleic	Fumaric
2-6	88.49	90.87	97.39	87.31	96.93	94.70
2-10	96.41	88.99	59.04	98.01	95.18	95.37
2-30	67.04	85.55	52.91	52.07	99.28	95.96
50-70	56.81	95.21	92.55	99.61	98.70	96.97
50-100	54.72	94.72	82.54	97.67	98.10	95.53
50-140	60,50	95,67	81,65	97,33	99,70	98,82

CONCLUSION

The aspects discussed in this work, showed the importance of validating analytical HPLC methods, since the validation process of analytical data supplied statistical reliability, confirmed sensitivity, precision and accuracy of the method for all compounds in the matrices evaluated. The measured areas are found to be directly proportional to the concentrations of the analytes within a statistically acceptable variation. In the experimental part, it was found that the main validation parameters of the analytical HPLC method were within the acceptable ranges.

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REFERENCES

Alvarez, D. A., Rosen, M. R., Perkins, S. D., Cranor, W. M. , Schroeder, V. L. and Jones-Lepp, T. L. (2012). Bottom sediment as a source of organic contaminants in Lake Mead, Nevada, USA, Chemosphere, **88 (5),** 605–611.

Angela S., Pascale B., Nadia M., Loredana C., Rossell G., Antonio A. and Nicola S. (2007). Analytical method validation for the evaluation of cutaneous occupational exposure to different chemical classes of pesticides. Journal of Chromatography B, **860 (1)**, 26–33.

Broseus, R., Vincent, S., Aboulfadl, K., Daneshvar, A., Sauve, S., Barbeau, B. and Prevost, M. (2009). Ozone oxidation of pharmaceuticals, endocrine disruptors and pesticides during drinking water treatment, Water Research, **43 (18)**, 4707–4717.

Camino-Sánchez, F. J., Zafra-Gómez, A., Oliver-Rodríguez, B., Ruiz-Naranjo, I., Ruiz-García, J. and Vílchez, J. L. (2012). Validation of a method for the determination of tributyltin in seawater by stir bar sorptive extraction–liquid chromatography tandem mass spectrometry. Journal of Chromatography A, **1263**, 14– 20.

Charlotte G., Michelle L. and Bridget E. B. (2013). The development and validation of an immunoassay for the measurement of anti-thymidine phosphorylase antibodies in mouse and dog sera. Journal of Pharmaceutical and Biomedical Analysis, **72**, 16–24.

Cui, Y. H., Li, X. Y. and Chen, G. H. (2009). Electrochemical degradation of bisphenol A on different anodes, Water Research, **43 (7)**, 1968–1976.

De Baere, S., Osselaere, A., Devreese, M., Vanhaecke, L., De Backer, P. and Croubels, S. (2012). Development of a liquid–chromatography tandem mass spectrometry and ultra-high-performance liquid chromatography high-resolution mass spectrometry method for the quantitative determination of zearalenone and its major metabolites in chicken and pig plasma. Analytica Chimica Acta, **756**, 37–48.

Gimeno, P., Maggio, A., Bousquet, C., Quoirez, A., Civade, C. and Bonnet P. (2012). Analytical method for the identiûcation and assay of 12 phthalates in cosmetic products: Application of the ISO 12787 international standard "Cosmetics–Analytical methods–Validation criteria for analytical results using chromatographic techniques". Journal of Chromatography A, **1253** , 144–153.

Gomes, F. P. and Garcia, O. L. (2012). Development and validation of a simple and sensitive high performance liquid chromatographic method for the simultaneous determination of anastrozole, bicalutamide, tamoxifen, and their synthetic impurities. Talanta, **101**, 495–503.

Gómez, M. J., Herrera, S., Solé,D., García-Calvo, E. and Fernández-Alba, A. R. (2012). Spatio temporal evaluation of organic contaminants and their transformation products along a river basin affected by urban, agricultural and industrial pollution. Science of the Total Environment, **420**, 134–145.

Gouveia, S. C. and Castilho, P. C. (2012). Validation of a HPLC-DAD–ESI/MSn method for caffeoylquinic acids separation, quantification and identification in medicinal Helichrysum species from Macaronesia. Food Research International, **45 (1)**, 362–368.

INMETRO, (2003). Instituto Nacional de Metrologia, Normalização e Qualidade Industrial, Orientações sobre Validação de Métodos de Ensaios Químicos – Revisão: 01 de março de.

Klein, T., Longhini, R. and Mello, J. C. P. (2012). Development of an analytical method using reversed-phase HPLC-PDA for a semipuriûed extract of Paullinia cupana var. sorbilis (guaraná). Talanta, **88**, 502–506.

Kong, W., Zhang, X., Shen, H., Ou-Yang, Z. and Yang, M. (2012). Validation of a gas chromatography-electron capture detection of T-2 and HT-2 toxins in Chinese herbal medicines and related products after immunoafûnity column clean-up and pre-column derivatization. Food Chemistry, **132 (1)**, 574–581.

Lanças. F. M. (2004). Validação de métodos cromatográficos de análises. São Carlos: RiMa.

Lang, J. and Kohidai, L. (2012). Effects of the aquatic contaminant human pharmaceuticals and their mixtures on the proliferation and migratory responses of the bioindicator freshwater ciliate Tetrahymena, Chemosphere, **89 (5)**, 592–601.

Lewis, C. and Ford, A. T. (2012). Infertility in male aquatic invertebrates: A review. Aquatic Toxicology, **120–121**, 79 – 89.

Li, A. Z., Zhao, X., Hou, Y. N., Liu, H. J., Wu, L. Y and Qu, J. H. (2012). The electrocatalytic dechlorination of chloroacetic acids at electrodeposited Pd/Fe-modified carbon paper electrode, Applied Catalysis B: Environmental, **111–112**, 628–635.

Magureanu, M., Piroi, D., Mandache, N. B., David, V., Medvedovici, A. and Parvulescu, V. I. (2010). Degradation of pharmaceutical compound pentoxifylline in water by non-thermal plasma treatment, Water Research, **44 (11)**, 3445–3453.

Martinez-Huitle, C. A. and Brillas, E. (2009). Decontamination of wastewaters containing synthetic organic dyes by electrochemical methods: a general review, Applied Catalysis B: Environmental, **87 (3-4)**, 105–145.

Mowafy, H. A., Alanazi, F. K. and El Maghraby, G. M. (2012). Development and validation of an HPLC–UV method for the quantification of carbamazepine in rabbit plasma. Saudi Pharmaceutical Journal, **(1) 20**, 29–34.

Otomo, J. I. (2010). Desenvolvimento e validação de metodologia analítica para determinação de hormônios, considerados disruptores endócrinos, nas águas destinadas ao abastecimento público na região do rio Paraíba do Sul, SP. Dissertação de mestrado, Instituto de Pesquisas Energéticas e Nucleares – IPEN/USP, São Paulo.

Peruga, A., Grimalt, S., López, F. J., Sancho, J. V. and Hernández, F. (2012). Optimisation and validation of a speciûc analytical method for the determination of thiram residues in fruits and vegetables by LC–MS/MS. Food Chemistry **135 (1),** 186.

Rambla-Alegre, M., Esteve-Romero, J. and Carda-Broch, S. (2012). Is it really necessary to validate an analytical method or not? That is the question. Journal of Chromatography A, **1232**, 101– 109.

Ribani, M., Bottoli, C. B. G., Collins, C. H., Jardim, I. C. F. S. and Melo, L. F. C. (2004). Validação em métodos cromatográficos e eletroforéticos. Química Nova, **27**, 771-780.

Shewiyo, D. H., Kaale, E., Risha, P. G., Dejaegher, B., Smeyers-Verbeke, J. and Heyden Y. V. (2012). HPTLC methods to assay active ingredients in pharmaceutical formulations: A review of the method development and validation steps. Journal of Pharmaceutical and Biomedical Analysis, **66**, 11– 23.

Silva, M. A. M., Ferreira, M. H., Braga, J. W. B. and Sena, M. M. (2012). Development and analytical validation of a multivariate calibration method for determination of amoxicillin in suspension formulations by near infrared Spectroscopy. Talanta, **89**, 342– 351.

Sirtori, C., Zapata, A., Oller, I., Gernjak, W., Aguera, A. and Malato, S. (2009). Solar photo-Fenton as finishing step for biological treatment of a pharmaceutical wastewater, Environmental Science & Technology, **43 (4)**, 1185–1191.

Souza, R. R. (2011). Desenvolvimento e validação de metodologia analítica para determinação de disruptores endócrinos resultantes de atividades antrópicas nas águas da região do Rio Paraíba do Sul. Dissertação de Mestrado, São Paulo.

Thompson, M., Ellison, S. L. R. and Wood, R. (2002). Harmonized guidelines for single laboratory validation of method of analysis. IUPAC Technical Report. Pure Applied Chemistry, **72**, 835-855.

Wanga, C., Feng, Y., Zhao, S. and Li, B. (2012). A dynamic contaminant fate model of organic compound: A case study of Nitrobenzene pollution in Songhua River, China. Chemosphere, **88 (1)**, 69–76.

Yang, S. X., Liu, Z. Q., Huang, X. H., and Zhang, B. P. (2010). Wet air oxidation of epoxy acrylate monomer industrial wastewater, Journal Hazardous Materials, **178 (1-3)**, 786–791.

Yang, S., Yan, Z., Xu, F., Wang, S. and Wu, F. (2012). Development of freshwater aquatic life criteria for Tetrabromobisphenol A in China. Environmental Pollution, **169**, 59-63.