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Article

Optimization of Extraction and Determination of Chloramphenicol in Livestock Meat Samples using Aqueous Two-Phase System of *n*-propanol and Potassium Citrate Coupled with HPLC

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Um sistema de duas fases aquosas (ATPS) com base em álcool de cadeia curta e sal foi o método para a pré-concentração, separação, e análise de cloranfenicol (CAM), associado à cromatografia líquida de alta performance com detector de ultravioleta-visível (HPLC-UV). A fim de selecionar o ATPS adequado para a extração de CAM, diferentes ATPSs foram testados, e o ATPS *n*-propanol/citrato de potássio foi escolhido para a separação e concentração de CAM nos experimentos subsequentes. As influências do pH, da concentração de sal, do volume de *n*-propanol na eficiência de extração e do coeficiente de partição da CAM foram examinadas. A metodologia de superfície de resposta foi utilizada para aperfeiçoar as condições experimentais. Em condições ótimas, este método tem sido aplicado para a determinação quantitativa de CAM em amostras de carne com um limite de detecção de 0,48 ng g^{-1} e um limite de quantificação de 1,6 ng g^{-1}, com recuperação no intervalo de 92,39-104,12 %. Esse ATPS usou solventes orgânicos de custo baixo e forneceu um ambiente moderado e biocompatível, que é adequado a biomoléculas.

An aqueous two-phase system (ATPS) based on short chain alcohol and salt was the method for preconcentration, separation and analysis of chloramphenicol (CAM), coupled with high performance liquid chromatography with ultraviolet-visible detector (HPLC-UV). In order to select the suitable ATPS for CAM extraction, different ATPSs were tested and *n*-propanol/potassium citrate ATPS was chosen for separating and concentrating CAM in the subsequent experiments. The influences of the salt concentration, pH and the volume of *n*-propanol on the extraction efficiency and partition coefficient of CAM were examined. Response surface methodology was employed to optimize the experimental conditions. Under the optimal conditions, this method has been applied to quantitative determination of CAM in livestock meat samples with limit of detection of 0.48 ng g^{-1} and limit of quantification of 1.6 ng g^{-1} with a recovery range of 92.39-104.12%. This ATPS used low cost of organic solvents and supplied a moderate and biocompatible environment, which is suitable for biomolecules.

Keywords: HPLC, aqueous two-phase system, extraction, chloramphenicol, livestock meat, ATPS

Introduction

Aqueous two-phase systems (ATPS) can be obtained when solutions of two differently hydrophilic polymers or solutions of a polymer and a salt above certain concentrations are employed. ATPS have been applied for extraction and purification of proteins,[1-4] antibiotics[5] and metal ions.[6,7] ATPS consisting of a short chain alcohol and a salt solution may be economically advantageous as the alcohol can be recycled by distillation and it has attracted much attention in several fields.[8,9]

The chloramphenicol (CAM), although prohibited for raising animals for meat production in the EU and USA,[10] is still used in some economically less developed areas because of its low cost.[11] Due to the complexity and low concentration of CAM residues in food, the sample

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pretreatment methods are recurrent problem, mainly solid-phase extraction (SPE)[12] and liquid-liquid extraction (LLE)[13] nowadays. Both methods demand volatile and toxic organic solvents, SPE requires a solvent desorption step which is time-consuming and complicated. ATPS, a new LLE technique, has advantages of quick phase separation, a moderate and biocompatible environment containing large amounts of water in each phase which is suitable for biomolecules. Our group[14] have reported that an ionic liquid ATPS was used to extract CAM in feed water, milk and honey samples. Ionic liquids used as "green solvent"[15] are still expensive and it is necessary to develop a simple, rapid and inexpensive method for sample pretreatment.

To better understand the functional relationship between experimental factors and responses, and to identify the optimal conditions, the optimal design of the experiment is an extremely crucial aspect. Box-Behnken design (BBD)[16,17] which has been widely applied in analytical chemistry[18] is one of the response surface methodologies.[19] In this study, the factors influencing the partitions of CAM were investigated. Under the optimal conditions, the alcohol-based ATPS coupled with high-performance liquid chromatography (HPLC) was successfully applied to the separation and determination of trace CAM in livestock meat.

Experimental

Chemicals and materials

The standard drug sample of CAM was procured from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol of HPLC grade, the alcohols and the organic salts of analytical grade were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). They were all used without further purification.

The stock solution of CAM which should be replaced every two months was prepared by dissolving it in methanol at a concentration of 200 μg mL^{-1} and storing at 4 °C in a refrigerator. The working solutions of CAM were prepared by appropriately diluting the stock solution with deionized water.

Apparatus

A BS124S electron balance (Beijing Sartorius instrument Co., Ltd., China) was used for weighting. A digital pH meter (Shanghai LIDA Instrument Factory, China) was applied to determine the pH of solutions. A thermostatic waterbath (Gongyi City Yuhua instrument Co., Ltd., China) was used to control temperature. The analysis of variance was calculated using the Design-Expert.V.8.0.5.b. A high performance liquid chromatography Agilent 1200 HPLC (Agilent, USA) equipped with a quaternary pump and an ultraviolet-visible detector (UV) was used for the analysis of extracted products. The instrument control and data processing were actualized by using Agilent ChemStation software.

Preparation of real samples

The meat samples purchased from local marketplace was stored at −10 °C in a refrigerator. Before being used, they were thawed for several hours at ambient temperature. The trichloroacetic acid solutions (10 mL, 15% in water) containing different concentrations of CAM (0-128 ng mL^{-1}) were mixed with 1.5 g of meat, and then were thoroughly grinded. The solutions were centrifuged at 357 × g for 30 min and finally filtered through 0.45 μm microfiltration membrane made of nitrocellulose to remove the denatured proteins. The homogenous sample was stored at 4 °C for future use.

General procedure

In a 10.0 mL centrifuge tube, 8.5 mL of $K_3C_6H_5O_7$ solution (0.8 g mL^{-1}) containing 1 μg mL^{-1} of CAM was added, and then was added 1.5 mL of *n*-propanol. The mixture was gently shaken for 5 min at room temperature, centrifuged at 357 × g for 30 min, and then placed into the waterbath at 25 ± 0.05 °C for 2 h to equilibrate and allow for phase separation. The top phase was primarily comprised of *n*-propanol and CAM, while the bottom phase was mainly of salt solution. The volume of the top and bottom phases was recorded precisely. The desired pH was adjusted by putting hydrochloric acid or ammonia water into salt solutions if necessary.

CAM in the top phase was determined by HPLC after extraction without any treatment. An analytical reversed phase column was used for chromatographic separations at the column temperature of 25 °C. The ratio of mobile phase methanol and water was 43:57 at the flow rate of 1.0 mL min^{-1}. The injected volume was 20 μL and the column effluent was monitored at a wavelength of 278 nm.

Determination of the partition parameters of CAM

The partition coefficient (K) and extraction efficiency (E, %) of CAM can be calculated by,

$$K = C_t / C_b \quad (1)$$

$$E = C_t V_t / m_s \times 100 \quad (2)$$

where C_t and C_b represented the equilibrium concentrations of CAM in the top phase and bottom phase, respectively; V_t was the volume of the top phase and m_s was the mass of CAM initially added.

Results and Discussion

Selection of ATPS

In order to choose the suitable phase forming salt, the partitions of CAM were carried out in different ATPSs listed in Table 1. The results indicated that these organic salts could form ATPSs with the three alcohols, except for the combination of ethanol and $(NH_4)_2C_4H_4O_6$. When the ethanol volume was 1 mL, the separation phase couldn't occur, even if the concentration of $(NH_4)_2C_4H_4O_6$ reached the maximum. When the ethanol volume was 2 mL, $(NH_4)_2C_4H_4O_6$ was easy to precipitate from the solution. In Table 1, the extraction efficiency and partition coefficient of CAM were effectively influenced by the type of salts following the order: $K_3C_6H_5O_7$ > $(NH_4)_3C_6H_5O_7$ > $K_2C_4H_4O_6$ > $(NH_4)_2C_4H_4O_6$. Thus, $K_3C_6H_5O_7$ was selected as phase forming salt.

In ATPSs of $K_3C_6H_5O_7$ with *n*-propanol, isopropanol or ethanol, the highest extraction efficiency and partition coefficient of CAM were in *n*-propanol/$K_3C_6H_5O_7$ ATPS. Apparently, it was the best choice to use *n*-propanol as extraction solvent. In this work, it was chosen *n*-propanol/$K_3C_6H_5O_7$ ATPS to extract CAM.

Table 1. Extraction efficiency and partition coefficient of CAM in different ATPSs

Salt	Organic solvent	Extraction efficiency / %	Partition coefficient
$(NH_4)_3C_6H_5O_7$	*n*-propanol	91.34	73.83
	isopropanol	88.01	53.23
	ethanol	78.74	56.80
$K_3C_6H_5O_7$	*n*-propanol	96.07	154.04
	isopropanol	93.53	87.26
	ethanol	91.52	72.27
$(NH_4)_2C_4H_4O_6$	*n*-propanol	70.62	21.63
	isopropanol	69.40	38.76
	ethanol	– [a]	–
$K_2C_4H_4O_6$	*n*-propanol	88.01	53.23
	isopropanol	84.09	37.44
	ethanol	82.54	36.95

[a]ATPS couldn't form or salting-out.

Effect of the concentration of $K_3C_6H_5O_7$

In *n*-propanol/$K_3C_6H_5O_7$ ATPS, the concentration of $K_3C_6H_5O_7$ was evaluated in the range of 0.3-0.85 g mL^{-1}, with the increase of salt concentration, the extraction efficiency and partition coefficient of CAM first increased, and then kept invariable. The reason was that the salting-out effect of $K_3C_6H_5O_7$ has reached to the maximum degree, and both of them were not able to be improved.

Effect of pH

According to a previous study,[14] the appropriate pH suitable for CAM was in the range of 6.0-10.0. In strong acid or alkaline condition, CAM could not exist at the molecular state, and it was decomposed. The pH used in this experiment was 7.0-10.0, because some salt was dissolved out in pH 6.0. From the results, the extraction efficiency of CAM was higher than 95%, meanwhile, the partition coefficient increased in pH 7.0-9.0 and then decreased. At the pH of 9.0, they both reached the maximum values.

Effect of the volume of *n*-propanol

When the mixture of *n*-propanol and salt solution including CAM was gently shaken at room temperature, CAM was surrounded by *n*-propanol and water molecules. In the structure of CAM, there are hydrophobic groups as $-NO_2$, $-Cl$, and hydrophilic groups such as $-OH$. The hydrophobic groups were embedded in *n*-propanol molecules, while the hydrophilic groups were embedded in water molecules. Because of space steric effect, the effect of hydrophobic groups was dominant. Meanwhile, due to the salting-out effect of $K_3C_6H_5O_7$, CAM was transferred to the top phase.

With the increase of the volume of *n*-propanol, the amount of CAM extracted to the top phase increased and the extraction efficiency increased continuously. In this case, the concentration of CAM in the two phases was changed. Based on the combined influences, the partition coefficient of CAM first increased and then decreased.

Experimental design

A three-factorial and three-level of BBD was chosen for optimizing the process parameters affecting CAM extraction. Simultaneously considering the effect of the discussed factors on the extraction efficiency and partition coefficient, the three factors which were selected to optimize the parameters by BBD were the concentration of $K_3C_6H_5O_7$ (*A*, 0.50-0.80 g mL^{-1}), pH (*B*, 8-10) and the

volume of *n*-propanol (*C*, 1.0-2.0 mL). The factor levels were coded as −1 (low), 0 (central point), 1 (high).

According to the experimental design, the results from the experimental research were analyzed and tabulated in Table 2. The second-order polynomial equations in terms of coded factors were established as follows,

$$Y_E = 95.49 + 4.14\times A + 0.34\times B + 1.93\times C + 0.40\times A\times B + 0.83\times A\times C + 1.07\times B\times C - 2.66\times A^2 - 1.38\times B^2 - 2.15\times C^2 \quad (3)$$

$$Y_K = 128.94 + 41.88\times A + 8.79\times B - 11.84\times C + 10.38\times A\times B + 16.96\times A\times C + 14.71\times B\times C - 13.40\times A^2 - 18.96\times B^2 - 22.27\times C^2 \quad (4)$$

The coefficients of the equation were procured by regression analysis of the experimental data, where Y_E and Y_K stood for the response, from the extraction efficiency and partition coefficient, respectively.

Table 2. Design matrix and responses for *n*-propanol/$K_3C_6H_5O_7$ ATPS

Run	Concentration of $K_3C_6H_5O_7$	pH	Volume of *n*-propanol	Extraction efficiency / %	Partition coefficient
1	1	0	1	96.83	132.14
2	1	1	0	96.66	167.66
3	0	0	0	95.97	134.79
4	0	0	0	94.83	116.78
5	1	−1	0	95.42	127.10
6	0	1	1	95.72	97.49
7	−1	−1	0	87.06	46.28
8	−1	0	−1	86.19	88.33
9	0	0	0	96.23	148.65
10	0	−1	1	92.65	52.71
11	0	0	0	95.00	116.66
12	0	−1	−1	90.33	107.37
13	0	1	−1	89.13	93.29
14	0	0	0	95.42	127.85
15	−1	1	0	86.68	45.31
16	−1	0	1	87.77	32.29
17	1	0	−1	91.91	120.34

From the analysis of variance (ANOVA) about the response of extraction efficiency, the *F*-value of 47.86 implied that the model was significant. There was only a 0.01% chance that the model could occur due to noise. Values of "Prob > *F*" less than 0.05 indicate that the model terms are significant while values greater than 0.10 indicate that the model terms are not significant. In this case, A, C, BC, A^2, B^2, C^2 were significant model terms, and A, C, A^2, C^2 were highly significant model terms ($p < 0.001$). The non-significant lack of fit ($p > 0.05$) showed that the model was significant for the response. The determination coefficient (R^2) of 0.9840 and the adjusted R-squared (R^2_{adj}) of 0.9635 demonstrated a good degree of correlation between the experimental and the predicted data of the response.[20] Adequate precision measures the signal to noise ratio, and a ratio greater than 4 is desirable. So a ratio of 21.232 indicated an adequate signal, and this model could be used to navigate the design space.

From the ANOVA, about the response of partition coefficient, the model *F*-value of 13.06 implied that the model was significant. There was only a 0.13% chance that the model could occur due to noise. The R^2 of 0.9438 predicted that the model represented good relationships between the factors chosen. A, C, AC, B^2, C^2 were significant model terms, and A was a highly significant model term. A ratio of 12.572 indicated an adequate signal, and this model was fit to navigate the design space.

From the results of BBD, the optimal conditions were obtained when the concentration of $K_3C_6H_5O_7$, pH and the volume of *n*-propanol were 0.80 g mL^{-1}, 9.17, 1.45 mL, respectively. Simultaneously the extraction efficiency and partition coefficient of CAM could reach 97.78% and 164.30, respectively.

Method validation

The analytical curve was performed by adding standard CAM in the range of 8-160 ng mL^{-1} to ATPS. After phase separation, the top phase was determined by HPLC-UV method. The analytical curve for CAM was area = 0.15645495 × c − 0.5129424 with R^2 = 0.9995, where c represented the concentration of CAM (ng mL^{-1}), R^2 was the correlation coefficient. Successive eight-time extraction and analysis of a 10 ng mL^{-1} standard solution of CAM were performed to check the repeatability of this method. The relative standard deviation (RSD) was 1.16%.

The limit of detection (LOD) was a signal value of three times the noise and the limit of quantification (LOQ) was a signal value of ten times the noise. The LOD obtained was 0.48 ng g^{-1} and the LOQ was 1.6 ng g^{-1}. The LOQ for CAM of 1.6 ng g^{-1} of HPLC-UV detection system used in this study is lower than that of the matrix solid-phase dispersion/HPLC[21] and that of HPLC-mass spectrometry.[22] Nevertheless, the minimum required performance limit of 0.3 ng g^{-1} cannot be reached by these systems. Concerning the complexity of the sample matrix, this efficient pretreatment method combined with a more sensitive detector can be applied, such as the combination of ATPS with LC-mass spectrometry or other electrochemistry methods.

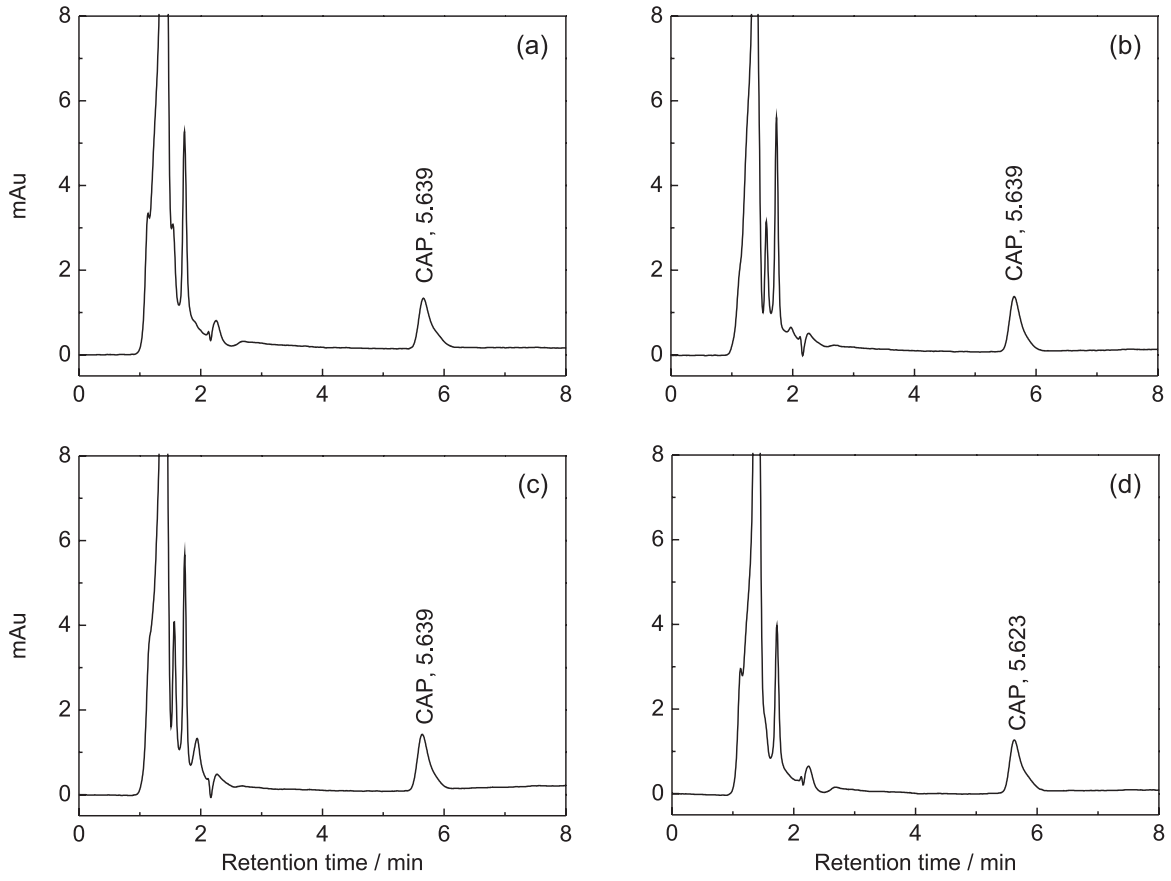

Figure 1. HPLC chromatograms with UV detection after ATPS extraction, a sample of pork (a), beef (b), chicken(c) and fish(d) added with 128 ng mL^{-1} CAM.

Table 3. Analysis results (n = 3) for CAM in livestock meat samples

Samples	Concentration added / (ng mL^{-1})	Concentration determined / (ng mL^{-1})	Recovery / %	RSD / %
pork	0	ND[a]	–	–
	32	29.49	92.16	1.23
	80	75.95	94.94	0.97
	128	123.77	96.70	1.67
beef	0	ND	–	–
	32	31.96	99.88	1.14
	80	77.87	97.33	1.43
	128	124.41	97.20	0.52
chicken	0	ND	–	–
	32	33.24	103.86	2.19
	80	78.50	98.13	2.86
	128	128.87	100.68	1.26
fish	0	ND	–	–
	32	31.96	99.88	2.37
	80	79.14	98.93	1.25
	128	124.41	97.20	1.84

[a]None found.

Sample analysis

Under the optimum conditions, four kinds of livestock meat were analyzed to demonstrate the applicability of the proposed extraction technique. At detectable levels, no contamination of CAM residues was found in meat before CAM was added. The recoveries of CAM were studied by adding known concentration of CAM standard solution into real samples. After phase separation, CAM in real samples was extracted to the top phase and determined by HPLC-UV (Figure 1 and Table 3). As shown in Table 3, the recoveries of CAM were in the range of 92.39-104.12% when the real samples were spiked with 32-128 ng mL^{-1} CAM. The results showed that the reproducibility and recovery of the method were satisfactory for CAM determination and the method can be gratifyingly applied to quantitative analysis of CAM.

Conclusions

The factors influencing the partitions of CAM were studied and three factors containing the concentration of $K_3C_6H_5O_7$, pH and the volume of *n*-propanol were

chosen for further evaluating the experimental conditions and optimizing the process parameters by BBD. ATPS is a green, simple and efficient technique for only simultaneously separating and concentrating the target in the top or bottom phase but also purifying the target. The advantages of alcohol-based ATPS are low interfacial tension and viscosity, little emulsion formation, quick phase separation, high extraction efficiency, low cost of organic solvents and a friendly biocompatible environment suitable for biomolecules. The determination of CAM by HPLC is a quick, sensitive and useful method. As a viable pretreatment technique, this separation method coupled with HPLC has been successfully applied to the extraction and determination of trace CAM in livestock meat samples.

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References

1. Naganagouda, K.; Mulimani, V. H.; *Process Biochem.* **2008**, *43*, 1293.
2. Mistry, S. L.; Kaul, A.; Merchuk, J. C.; Asenjo, J. A.; *J. Chromatogr., A* **1996**, *741*, 151.
3. Ross, K. C.; Zhang, C. M.; *Biochem. Eng. J.* **2010**, *49*, 343.
4. Rosa, P. A. J.; Azevedo, A. M.; Ferreira, I. F.; Sommerfeld, S.; Bäcker, W.; Aires-Barros, M. R.; *J. Chromatogr., A* **2009**, *1216*, 8741.
5. Jiang, Y. Y.; Xia, H. S.; Yu, J.; Guo, C.; Liu, H. Z.; *Chem. Eng. J.* **2009**, *147*, 22.
6. Bulgariu, L.; Bulgariu, D.; *J. Serb. Chem. Soc.* **2008**, *73*, 341.
7. Bulgariu, L.; Bulgariu, D.; *Sep. Purif. Technol.* **2011**, *80*, 620.
8. Li, Z. G.; Teng, H.; Xiu, Z. L.; *Process Biochem.* **2010**, *45*, 731.
9. Liu, H. L.; He, C. Y.; Wen, D. W.; Liu, H. W.; Liu, F.; Li, K. A.; *Anal. Chim. Acta* **2006**, *557*, 329.
10. European Commission; *Off. J. Eur. Communities* **2002**, L71.
11. Farombi, E. O.; *Tohoku J. Exp. Med.* **2001**, *194*, 91.
12. Schirmer, C.; Meisel, H.; *J. Chromatogr., A* **2006**, *1132*, 325.
13. Chen, H. X.; Chen, H.; Ying, J.; Huang, J. L.; Liao, L.; *Anal. Chim. Acta* **2009**, *632*, 80.
14. Han, J.; Wang, Y.; Yu, C. L.; Yan, Y. S.; Xie, X. Q.; *Anal. Bioanal. Chem.* **2011**, *399*, 1295.
15. Han, X. X.; Armstrong, D. W.; *Acc. Chem. Res.* **2007**, *40*, 1079.
16. Box, G. E. P.; Behnken, D. W.; *Technometrics* **1960**, *2*, 455.
17. Francis, F.; Sabu, A.; Nampoothiri, K. M.; Ramachandran, S.; Ghosh, S.; Szakac, G.; Pandey, A.; *Biochem. Eng. J.* **2003**, *15*, 107.
18. Ferreira, S. L. C.; Bruns, R. E.; Ferreira, H. S.; Matos, G. D.; David, J. M.; Brandão, G. C.; da Silva, E. G. P.; Portugal, L. A.; dos Reis, P. S.; Souza, A. S.; dos Santos, W. N. L.; *Anal. Chim. Acta* **2007**, *597*, 179.
19. Bezerra, M. A.; Santelli, R. E.; Oliveira, E. P.; Villar, L. S.; Escaleira, L. A.; *Talanta* **2008**, *76*, 965.
20. Su, S. N.; Nie, H. L.; Zhu, L. M.; Chen, T. X.; *Bioresour. Technol.* **2009**, *100*, 2336.
21. Guo, L. Y.; Guan, M.; Zhao, C. D.; Zhang, H. X.; *Anal. Bioanal. Chem.* **2008**, *392*, 1431.
22. Fernandez-Torres, R.; Bello Lopez, M. A.; Consentino, M. O.; Mochon, M. C.; Payan, M. R.; *J. Pharmaceut. Biomed. Anal.* **2011**, *54*, 1146.

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