Talanta 89 (2012) 526–530

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Short communication

Automatic flow-batch system for the sample treatment and determination of hydroxyproline in sausages

J.A. Belloni, M.S. Di Nezio, M.F. Pistonesi, M.E. Centurión*

Department of Chemistry, Universidad Nacional del Sur, INQUISUR (UNS-CONICET), Av. Alem 1253 (B8000CPB), Bahía Blanca, Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 18 October 2011
Received in revised form 2 December 2011
Accepted 6 December 2011
Available online 20 December 2011

Keywords:
Flow-batch
Automatization
Meat products
Sausages
Hydroxyproline

ABSTRACT

In this study an automatic method for sample treatment and spectrophotometric determination of hydroxyproline in commercial sausages was developed. A flow-batch system that includes the steps of sample hydrolysis and determination of the analyte was designed. The method presents a linear range between 0.60 and 3.60 μg mL^{-1} of hydroxyproline. A relative standard deviation of 1.68% was obtained from hydroxyproline standard solution (n = 6, 1.20 μg mL^{-1}) and the detection limit was 0.12 μg mL^{-1}. The sample throughput was 1 sample h^{-1} while the reference method (AOAC) was carried out in about 17 h. This method employs 16 h of hydrolysis while in the proposed method the hydrolysis time was 15 min. For this purpose a pressure hydrolysis chamber with a low cost halogen lamp was used. The flow-batch system is simple and allows the use of both chambers (hydrolysis and detection) simultaneously. The obtained results with the flow-batch method are in good agreement with that obtained with the reference method. Moreover, it is good alternative to the quality control of meat products.

1. Introduction

Collagen, the major component of muscle connective tissue, is important in regard to meat quality, because a high content affects the tenderness as well as the biological value of meat protein [1,2]. It is present in skin, tendon, vascular system and other waste materials and by its thermal denaturation, gelatine is obtained. From a nutritional point of view, collagen and gelatine are proteins very unbalanced in their amino acid composition. Collagen is deficient in tryptophan and gelatine devoid of it. On the other hand, collagen contains high amounts of glycine, proline and hydroxyproline [3]. The presence of hydroxyproline is often used as analytical approach to assess the amount of collagen present in meat products.

Nowadays, there are little information in the literature related to the employment of different analytical techniques to determine the content of hydroxyproline in meat and meat products. A novel liquid chromatography–mass spectrometry (LC–MS/MS) analytical method for the quantification of hydroxyproline was described by Colgrave and co-workers [4]. Micellar electrokinetic chromatography with laser-induced fluorescence detection has also been reported for internal standard based quantification of 4-hydroxyproline in muscle hydrolysates [5]. In both studies, the authors propose a laborious sample preparation that includes numerous steps that consume quite a lot of hours [4,5]. Recently, the determination of hydroxyproline content in meat products was developed by Mazorra-Manzano and co-workers. An acidic sample hydrolysis and amino acid derivatization was carried out before capillary electrophoresis detection [6]. On the other hand, a high performance anion exchange chromatography and pulsed amperometric detection (HPAEC–PAD) was developed to quantify 4-hydroxyproline in meat products. In this paper the sample hydrolysis time is reduced from 24 h to 20 min by using microwave procedure [7]. The main disadvantage of these studies is that they use high cost instrumentation and some of them employed a laborious and complex sample preparation. González-Martín et al. [8] determined hydroxyproline in cured pork sausages and dry cured beef by using near infrared spectroscopy (NIRS) technology with a remote reflectance fibre optic. However, the sample treatment was carried out using the AOAC method [9]. The determination of hydroxyproline levels by this method is based in hydroxyproline oxidation using chloramine T and subsequent formation of a red compound with p-dimethylaminobenzaldehyde (Ehrlich's reagent). The spectrophotometric determination was carried out at 560 nm. In order to obtain hydroxyproline from meat products, this method includes sample treatments that involve several previous operations: grinding, weighted, dissolving and heating in acidic medium during approximately 16 h. This treatment has the disadvantage of being laborious, employed large amounts of reagents and time consuming procedure. Besides, numerous mistakes can be discussed during this stage as well as contamination of the sample. To overcome these drawbacks, sample treatment was improved by Centurión et al. [10]. In this paper in a first step, sample

* Corresponding author. Tel.: +54 291 4595100; fax: +54 291 4595160.
E-mail address: mecentur@criba.edu.ar (M.E. Centurión).

0039-9140/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2011.12.019

solubilisation was carried out with sodium dodecyl sulphate and sodium hydroxide at 80 °C for 40 min with magnetic stirring. Then, this slurry is filtered and made up to water. Finally, an acid hydrolysis of the solubilised sample was performed at 100 °C for 1 h. This treatment was applied to different beef muscles.

Moreover, an automated method using flow injection analysis has been developed. However, this method employs the official manual methodology to carry out the sample treatment [11]. The development of automatic methods, that include sample preparation since it is a critical step, represents an important advantage for the quality control of meat products. Among the automatic methods, flow-batch methodology (FB) is a good option since it combines the intrinsic favourable features of the flow and batch techniques. These systems are very flexible and versatile (multi-task characteristic) and are characterised by the use of a mixing/reaction chamber and three-way solenoids valves or a multi-port selecting valve fully computer-controlled. One of the advantages of the FB is that is considered as a universal purpose accessory tool easily attached to any conventional equipment for instrumental analysis. Using FB systems, conventional methods can be updated to improve analytical properties. Furthermore, they present characteristics such as low sample and reagent consumption, low cost and high sample throughput. Another advantage is the measures of the analytical signal can be performed directly inside the chamber [12–18]. Flow-batch systems have been used in several analytical procedures such as: nephelometric [12] and chemiluminescence determinations [15], chemometric-assisted method [13], extraction procedures [19], titrations [20,21], preparation of calibration solutions [22], on-line matching of pH [23], screening analysis [24].

In this paper, an automatic method to determine hydroxyproline in various commercial sausages was proposed. For this purpose a flow-batch system was designed that includes two laboratory-made polytetrafluoroethylene (PTFE) connected chambers. One of them is a pressure hydrolysis chamber, used for the sample treatment with the experimental conditions proposed by Centurión et al. [10]. The inclusion of this chamber in the FB system represents a novelty and shows FB systems versatility. Within our knowledge, in the literature, there is no information about FB systems with a chamber that allows carrying out sample hydrolysis by heating and pressure. The hydrolysis temperature is achieved by using a la low-cost halogen lamp. The other chamber is used for spectrophotometric detection of hydroxyproline in the sample at 560 nm. This chamber was placed instead of the cell holder of the spectrophotometer. Therefore, this methodology requires minimal intervention by the operator because sample treatment and detection step are coupled. By other hand, is much faster than the reference methodology. The proposed method was validated by comparing the obtained results with those generated by the reference method (AOAC) when both were applied to real samples.

2. Experimental

2.1. Reagents and solutions

All reagents were of analytical grade and ultra pure water (18 MΩ) was used. Hydroxyproline stock solution was prepared by dissolving an appropriate amount of hydroxyproline (Merck) in water. Standard solutions were prepared by appropriate dilution of the stock solution. Solubilising solution was prepared by mixing 20 mL of sodium dodecyl sulphate 10% (w/v) (Merck), 50 mL 2 mol L^{-1} sodium hydroxide (Anedra) and made up to 100 mL with water. Chloride acid 6.0 mol L^{-1} was prepared with a suitable volume of hydrochloric acid (Anedra) in water. Buffer solution pH 6.0 was prepared by dissolving 5.0 g of citric acid monohydrate (Cicarelli), 2.63 g sodium hydroxide (Anedra) and 14.61 g sodium acetate trihydrate (Anedra) and made up to 100 mL with water. Chloramine T solution was prepared by dissolving 1.41 g of N-chloro-p-toluenesulphonamide (Merck) in 10 mL of distilled water, add 10 mL of propanol (Anedra) and 80 mL of buffer solution. Ehrlich's reagent was prepared by dissolving 10 g of p-dimethylaminobenzaldehyde (Aldrich) in 35 mL of perchloric acid (Cicarelli) (69%, w/v), add 65 mL of propanol (Anedra).

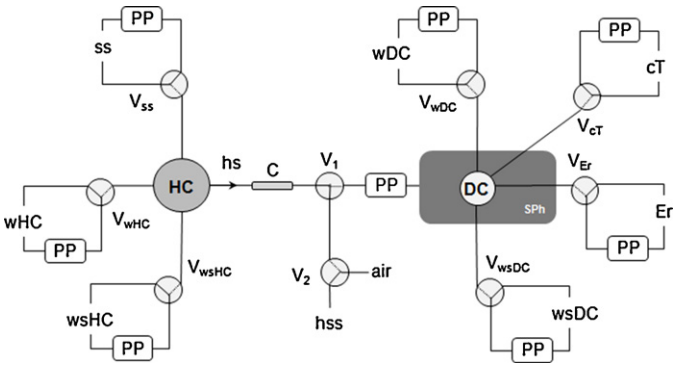

Fig. 1. Hydrolysis chamber (HC), detection chamber (DC) and solenoid valve (V).

2.2. Flow-batch assembly

The spectrophotometric measurements were carried out in an UV–vis Agilent 8453 spectrophotometer with diode array detector at 560 nm. Solutions were propelled by an eight channel Gilson Minipuls-3 M312 peristaltic pump. The system is composed of two connected laboratory-made PTFE chambers, one of hydrolysis (HC) and the other one of detection (DC) (Fig. 1). Furthermore, nine three-way solenoid valves (model 137 161T031, Nresearch) were used allowing the admission and removal of fluids used in the chambers: V_{ss} solubilising solution, V_{cT} chloramine T solution, V_{Er} Ehrlich's reagent, V_{wDC} and V_{wHC} water, V_{wsHC} and V_{wsDC} waste, V_1 hydrolysed sample/hydroxyproline stock solution/air and V_2 hydroxyproline stock solution/air. PTFE pumping tubes of different inner diameters were employed. Then, a glass cartridge C (5 cm length and 2 mm internal diameter) filled with filter paper was placed between both chambers. Hanna Instruments magnetic stirrers (model HI 190 M), were placed underneath the HC and DC.

The pressure hydrolysis chamber was built with PTFE, hydrophobic material resistant to high temperatures (up to about 300 °C) and to most acids, bases and organic solvents. This chamber offers the following features: 30 mL internal volume (3.0 cm internal diameter and 4.3 cm height), screw cap that allowing a hermetic seal and contains two holes for connecting a thermocouple and a 24 V halogen lamp (L1). The lamp voltage is controlled with a voltmeter to obtain the hydrolysis temperature. For monitoring the pressure inside the chamber hydrolysis, the system has a pressure gauge (located on the top of the camera). In this way the hydrolysis step is achieved in less time than in conventional heating.

The detection chamber also was built in PTFE and has an inner volume of 5 mL and two quartz windows for spectrophotometric measurements. In order to reach a temperature of 60 °C, a second lamp (L2) (12 V) was used.

A PC microcomputer with an interface (USB6009, National Instruments) is employed to control the flow-batch system. The solenoid valves, the peristaltic pumps, magnetic stirrer and lamps are controlled through a computer program developed in Lab View 8.0 graphic language (National Instruments, Austin, TX, USA).

Table 1
Optimum values for flow batch parameters.

q_{ss}	q_{hs}	q_{cT}	q_{Er}	q_{wDC}	q_{wHC}	q_{hss}	q_{wsHC}	q_{wsDC}
Flow rate (mL min^{-1})								
5.70	0.47	0.52	0.47	0.55	6.60	0.47	8.00	5.80

	V_{ss}	V_1	V_{cT}	V_{Er}	V_{wDC}	V_{wHC}	V_1–V_2	V_{wsHC}	V_{wsDC}
Valve switching time intervals (s)									
Hydroxyproline stock solution	–	–	2.2	3.5	0.9–2.2	–	0.4–1.2	7.0	4.2
Samples	120.0	8.0	2.2	3.5	1.6	110.0	–	7.0	4.2

ss: solubilising solution, hs: hydrolysed sample, cT: chloramine T solution, Er: Ehrlich's reagent, hss: hydroxyproline stock solution, w: water, ws: waste, q: flow rate, V: solenoid valve, HC: hydrolysis chamber, DC: detection chamber.

2.3. Sample preparation

A total of 4 different commercial samples of Vienna sausages purchased in local supermarkets, were analysed. Samples were taken from the gondolas of a supermarket and kept at 4 °C for 48 h until analysis. The samples were processed using a blender until a smooth and homogenized paste was obtained. Taking into account the quantity of hydroxyproline presents in the samples, suitable amounts were weighed and placed in the hydrolysis chamber.

2.4. Procedure

A schematic diagram of the proposed FB system is shown in Fig. 2. Before starting the analysis, the valves were activated for 2 s to load the channels with the respective solutions.

The sample was placed in the hydrolysis chamber and the solubilising solution valve (V_{ss}) was turned on during 120 s. Then, the chamber was sealed with the PTFE screw cap to start hydrolysis. To carry out the sample treatment, the lamp L1 was switched on by applying 6 V through a power supply to achieve the optimum values of temperature (115 °C) and pressure (0.6 bar). This step was carried out during 15 min with magnetic stirring.

After the hydrolysis step, valve V_1 was switched on during 8.0 s and a certain volume of the hydrolysed sample (hs) passes through the cartridge C and entering the detection chamber and V_2 was switched off. Afterwards, valve V_{cT} was switched on for 2.2 s promoting the aspiration of chloramine T towards the detection chamber. This oxidation step was allowed to proceed for 20 min at room temperature. Finally, valve V_{Er} was switched on (3.5 s) and the colour reagent was added. The desired reaction product was obtained after 20 min at 60 °C. This temperature was reached by using lamp L2. Absorbance was read at 560 nm. The magnetic stirrer was always activated during the insertion of the fluids aliquots into the DC in order to assure a good homogenization of the solutions. Two cleaning steps may be carried out with water between each recorder. For this purpose valves V_{wHC} and V_{wDC} were switched on during 41 s. In order to empty both chambers, V_{wsHC} and V_{wsDC} were turned on.

The standard solutions were prepared by using the same procedure in the DC. However, time interval of valves V_2 and V_{wDC} increased and decreased respectively while V_1 was switched off. Standard solution and water valves are activated sequentially during t_2 and t_{wDC}. Time intervals of valves V_{cT} and V_{Er} remain fixed.

This procedure allows that sample hydrolysis and standard solutions can be prepared simultaneously and the wash cycles are carried out independently.

3. Results and discussion

3.1. Optimization of the sample treatment and analysis

The flow-batch system was optimized and the optimum values were selected as a compromise between sensitivity and reproducibility of the analytical signals. Table 1 shows the optimum values for the different flow rates and the switching time intervals of the solenoid valves.

To optimize sample hydrolysis, the concentration of solubilising solution (sodium dodecyl sulphate and sodium hydroxide) was fixed as Centurión and co-workers [10]. In order to corroborate that sample treatment was effective, a sample of sausage was used. This sample has been analysed by the reference method and its concentration of hydroxyproline was 0.257 g/100 g. This sample was used in the FB system to establish the optimum conditions.

The temperature and time of hydrolysis were optimized by a full factorial design with two factors and four levels. In this case, the design requires sixteen experiments. Temperature and time of hydrolysis were studied between 95 and 125 °C at intervals of 10 °C and between 5 and 20 min with increments of 5 min respectively. The temperature was established by varying the voltage applied to the lamp. The temperature and pressure measurements were done with a thermocouple (accuracy of ±1 °C) and a manometer respectively.

The statistical analysis (STATGRAPHICS Plus, version 5.1 STSC, Rockville, MD, USA) of the results was performed considering all possible interactions between the variables. The obtained results indicate that both variables (temperature and time) can be considered significant and the interactions were found to be statistically negligible (95% probability). The optimum values of temperature and time of hydrolysis were 115 ± 1 °C and 15 min respectively. The pressure inside the chamber at 115 °C is 0.6 bar.

3.2. Analytical parameters

The calibration curve was linear for hydroxyproline, in the concentration range from 0.60 to 3.60 μg mL^{-1}. The calibration curve was $y = (0.151 \pm 0.005)x + (0.007 \pm 0.007)$, where y is the absorbance and x is the concentration of hydroxyproline μg mL^{-1}. The precision was expressed as percentage of the relative standard deviation of replicate measurements and it was calculated by using standard solutions. The obtained value was 1.68% ($n = 6$, 1.20 μg mL^{-1}). The detection limit estimated as 3 times $S_{y/x}$/slope [25], was 0.12 μg mL^{-1}. The sample throughput was 1 h^{-1}.

3.3. Application to real samples

The proposed method was applied to the determination of hydroxyproline in commercial sausages samples, using the optimum experimental conditions. In order to validate the flow-batch proposed method, the AOAC method was used. The results obtained with both methods are shown in Table 2. A paired t-test was used to compare the methods. The t calculated value (0.479) is less than t critical value (3.18 for $n = 4$, $\alpha = 0.05$), therefore there is no significant difference between the results obtained by both methods.

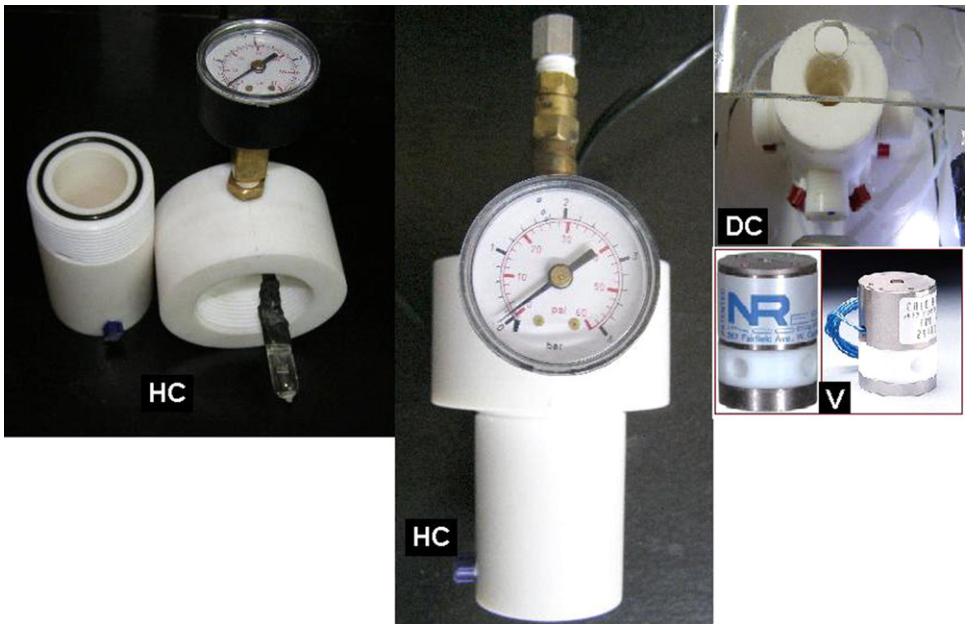

Fig. 2. Flow-batch system to determine hydroxyproline concentration in sausages. HC: hydrolysis chamber, DC: detection chamber, SPh: spectrophotometer, PP: peristaltic pump, V: solenoid valve, C: glass cartridge, ss: solubilising solution, hs: hydrolysed sample, cT: chloramine T solution, Er: Ehrlich's reagent, hss: hydroxyproline stock solution, w: water, ws: waste.

Table 2
Analysis of real samples.

Samples	Hydroxyproline (g/100 g)		Relative error (%)
	Proposed method[a]	AOAC method[a]	
M1	0.166 ± 0.009	0.177 ± 0.006	−6.21
M2	0.248 ± 0.007	0.257 ± 0.009	−3.50
M3	0.212 ± 0.005	0.205 ± 0.003	+3.41
M4	0.485 ± 0.010	0.453 ± 0.008	+7.06

[a] The samples were analysed by triplicate.

4. Conclusions

A flow-batch system for the determination of hydroxyproline in Vienna sausages was developed. In this system the sample treatment is performed in a PTFE chamber coupled to a detection chamber placed instead of the spectrophotometer cell holder.

The advantage of the designed flow-batch system is that has two independent chambers that allow the sample treatment and the standard solution preparation at the same time. Furthermore, the inclusion of the hydrolysis chamber for the sample treatment at a given temperature and pressure, represents a novelty and shows the versatility of the FB systems. For the sample treatment a low cost halogen lamp was placed into the sealed pressure hydrolysis chamber to achieve the appropriate temperature and pressure. Thus, the digestion takes place in 15 min, unlike the reference method that requires 16 h of hydrolysis. In this way the analysis time was reduced to 1 h per sample.

Four trademarks Vienna sausages were analysed and the obtained results were validated using the reference method, showing good agreement between them.

This methodology regarding the AOAC method is simple, low cost, low consumption of reagents, requires minimal human intervention and provides results in considerably less time. Besides, the proposed method allowed the automatic sample treatment and the hydroxyproline detection in sausages and contributes to quality control of meat products.

Acknowledgements

J.A. Belloni acknowledge CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas) for support. M. Pistonesi is also grateful to CIC (Comisión de Investigaciones Científicas de la Provincia de Buenos Aires). The authors acknowledge financial support from Universidad Nacional, and Proyecto Grupo de Investigación (PGI) granted for Secretaría General de Ciencia y Tecnología (Argentina).

The authors thank Dr. Beatriz S. Fernández Band (Universidad Nacional del Sur, Argentina) for her support and assistance during this paper.

References

[1] A. Bosselmann, C. Moller, H. Steinhardt, M. Kirchgessner, F.J. Schwarz, J. Food Sci. 60 (1995) 953–958.
[2] Y.-K. Lin, C.-Y. Kuan, Food Chem. 119 (2010) 1271–1277.
[3] S. Damodaran, O.R. Fennema, K.L. Parkin, Química de los Alimentos, third ed., Acriba, Zaragoza, 2010.
[4] M.L. Colgrave, P.G. Allinghama, A. Jones, J. Chromatogr. A 1212 (2008) 150–153.
[5] M.E.R. Dugan, R.D. Thacker, J.L. Aalhus, L.E. Jeremiah, K.A. Lien, J. Chromatogr. B 744 (2000) 195–199.
[6] M.A. Mazorra-Manzano, M.J. Torres-Llanez, A.F. González-Córdova, B. Vallejo-Cordoba, Food Analytical Methods, doi:10.1007/s12161-011-9271-8, http://www.springerlink.com/content/0282553t73j43463/, 2011.
[7] M.C. Messia, T. Di Falco, G. Panfili, E. Marconi, Meat Sci. 80 (2008) 401–409.
[8] Ma.I. González-Martín, C. Fernández Bermejo, J.M. Hernández Hierro, C.I. Sánchez González, Food Control 20 (2009) 752–755.
[9] AOAC International, in: W. Horwitz (Ed.), Official Methods of Analysis of AOAC International. AOAC Official Method 990.26, seventeen ed., AOAC International, Gaithersburg, MD, USA, 2000.
[10] M.E. Centurión, E. Agulló, An. Asoc. Quím. Argent. 82 (61) (1994) 71–77.
[11] J.Möller, A. Sjödin, Fresenius Z. Anal. Chem. 329 (1988) 732–734.
[12] C.C. Acebal, M. Insausti, M.F. Pistonesi, A.G. Lista, B.S. Fernández Band, Talanta 81 (2010) 116–119.
[13] M. Grünhut, M.E. Centurión, W.D. Fragoso, L.F. Almeida, M.C.U. de Araújo, B.S. Fernández Band, Talanta 75 (4) (2008) 950–958.
[14] E.L. Nascimento, M.C.U. Araújo, R.H. Galvão, J. Braz. Chem. Soc. 22 (2011) 1061–1067.
[15] M. Grunhut, V. Martins, M. Centurion, M.C.U. Araújo, B. Fernandez Band, Anal. Lett. 44 (2011) 67–81.
[16] M.C. Souza, V.L. Martins, L.F. Almeida, O.D. Pessoa Neto, E.N. Gaião, M.C.U. Araujo, Talanta 82 (2010) 1027–1032.
[17] V.B. Nascimento, T. Selva, E.C.S. Coelho, F.P. Santos, J.L.S. Antônio, J.R. Silva, E.N. Gaião, M.C.U. Araujo, Talanta 81 (2010) 609–613.

[18] L.F. Almeida, V.L. Martins, E.C. Silva, P.N.T. Moreira, M.C.U. Araújo, Anal. Chim. Acta 486 (2003) 143–148.
[19] M.J. Silva, E.V. Anjos, R.S. Honorato, M.F. Pimentel, A.P.S. Paim, Anal. Chim. Acta 629 (2008) 98–103.
[20] E.P. Medeiros, E.C.L. Nascimento, A.C.D. Medeiros, J.G. Veras Neto, E.C. Silva, M.C.U. Araújo, Anal. Chim. Acta 511 (2004) 113–118.
[21] C. Pasquini, E.V. Aquino, M.V. Rebouças, F.B. Gonzaga, Anal. Chim. Acta 600 (2007) 84–89.
[22] L.F. Almeida, M.G.R. Vale, M.B. Dessuy, M.M. Silva, R.S. Lima, V.B. Santos, P.H.D. Diniz, M.C.U. Araújo, Talanta 73 (2007) 906–912.
[23] R.S. Honorato, J.M.T. Carneiro, E.A.G. Zagatto, Anal. Chim. Acta 441 (2001) 309–315.
[24] R.A.C. Lima, S.R.B. Santos, R.S. Costa, G.P.S. Marcone, R.S. Honorato, V.B. Nascimento, M.C.U. Araujo, Anal. Chim. Acta 518 (2004) 25–30.
[25] J.C. Miller, J.N. Miller, Estadística Para Química Analítica, segunda ed., Addison-Wesley Iberoamericana, S.A., Wilmington, Delaware, E.U.A., 1993.