Analytical Methods

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A SIMPLE AND NEW REVERSE LIQUID-LIQUID MICROEXTRACTION FOR THE AUTOMATED SPECTROMETRIC DETERMINATION OF DOXYCYCLINE IN CHICKEN FAT

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Abstract

This work presents a new, simple and inexpensive reverse liquid-liquid microextraction of doxycycline (DOC) from chicken fat. In this just 13 min extraction methodology, acidulated water, as extraction solvent (400 µL), was used. A monochannel flow injection system was designed for the spectrometric determination of the analyte (ʎ = 344 nm). The extracted solution containing DOC was loaded into the injection valve of the continuous flow manifold. A lineal range between 100 and 700 µg DOC kg^{-1} sample was obtained. The LOD and LOQ were 33 µg kg^{-1} and 100 µg kg^{-1} respectively. The relative standard deviation was 4.87% and the sample throughput for the entire process was 4.5 h^{-1}. As recovery values when the method was applied to real samples showed variability, the expanded uncertainties were calculated. Their values indicated that the new method is independent of the concentration of the analyte and the origin of the sample.

Keywords: Doxycycline; Reverse liquid-liquid microextraction; Chicken fat samples; Flow injection spectrometric determination.

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1. Introduction

Tetracyclines (TCs) are broad-spectrum antibiotics used in animal therapy for the prevention, control and treatment of bacterial infections (Samanidou, Nikolaidou, & Papadoyannis, 2007). Due to their low cost and absence of major adverse effects, they are widely used to fight gram positive and gram negative bacteria, anaerobes, mycoplasmas, and as feeding additives to promote growth and weight gain. Unfortunately, TCs have been widely and improperly used causing serious problems. Since TCs residues may be present in animal tissue, they can generate the evolution of microorganisms promoting resistance to antibiotics and generating toxic and harmful effects for human health (García, Sarabia, & Ortiz, 2004). One member of this group of antibiotics is doxycycline (DOC) which is more lipid soluble than other TCs. For this reason it is more persistent in the body of animals and above all in animal fat where the concentration can reach the maximum residue limit (MRL) (Santos et al., 1996). These MLRs are established by the European Union, for the different TCs in different kinds of pigs, poultry and bovine tissues (muscle, liver and kidney). For fat and skin, only DOC is regulated for pigs and poultry, and this concentration is 300 μg kg^{-1} (The European Commission, 2010). There are several methods to determine TCs residues in different matrixes using, capillary electrophoresis (García-Ruiz, Crego, Lavandera, & Marina, 2001) and HPLC (Cinquina, Longo, Anastasi, Giannetti, & Cozzani, 2003), but in food of animal origin there are just a few, probably due to the complexity of the sample (Anderson, Rupp, & Wu, 2005). Referring to the determination of DOC in chicken fat only one paper has been published until now, which using UV liquid chromatography and liquid chromatography-tandem mass spectrometry detection (Gajda, Posyniak, Zmudzki, & Tomczyk, 2013). One of the most important difficulties to determine TCs is the treatment of biological samples. This procedure includes an extraction step with a suitable solvent, a clean-up and a preconcentration stage. Usually, the extraction methodologies use an acidic buffer at pH 3-5, a homogenization step and then a centrifugation one. In some cases, ultrasonication is used to improve the efficiency of the extraction (Shalaby, Salama, Abou-Raya, Emam, & Mehaya, 2011). Sometimes, in order to isolate TCs from

complex matrix interferences, a solid phase extraction and/or liquid-liquid extraction is required (Suárez, Santos, Simonet, Cárdenas, & Valcárcel, 2007; Ibarra, Rodriguez, Miranda, Vega, & Barrado, 2011).

According to literature, there are many analytical procedures to determine DOC in animal tissues(Shalaby et al., 2011), pork fat (Cherlet, Schelkens, Croubels & De Backer, 2003), eggs (Song et al., 2014) and plasma (Santos et al., 1996), and as it is mentioned above, only one work for this determination in chicken fat and skin has been made (Gajda et al., 2013) .In that paper, the sample preparation was tedious: the extraction was done using an oxalic acid buffer (pH = 4.00), ethyl acetate and 20% trichloroacetic acidic solutions. The mixture was stirred and centrifuged and the extraction was repeated twice with ethyl acetate. Then, the SPE procedure was carried out.

On the other hand, the liquid-liquid extraction is a traditional methodology used for many years for separation and preconcentration of analytes. It is based in a distribution of a solute between two immiscible liquid phases. However, this methodology is time-consuming, often requiring large amounts of hazardous organic solvents and, in some cases, multistep procedures. So, the miniaturizing, automating and cleaning procedures were developed to replace the conventional sample treatment step. In this way, the liquid-liquid microextraction methodology is being widely used. It is a simple and fast procedure and it is known as an environmentally friendly treatment technique because of its low consumption of organic solvents. There are different categories of this methodology: single drop microextraction (SDME) (Kokosa, 2015), dispersive liquid–liquid microextraction (DLLME)(Saraji & Boroujeni, 2014), reversed-phase dispersive liquid–liquid microextraction (RP-DLLME) (Hashemi, Raeisi, Ghiasvand, & Rahimi, 2010) and a new way, reverse liquid-liquid microextraction. The last one is a simple procedure, non dispersive, based on the use of water as extraction solvent, and the analytes go from the organic phase (usually, oil samples) to the aqueous phase. So, the methodology becomes more eco-friendly because organic solvents are not necessary and the cost of the procedure is lower.

In this work, a new reverse liquid-liquid microextraction/preconcentration method for DOC from chicken fat samples was developed. The procedure was simple and fast and it was a very good option for the treatment of this complex matrix. In order to automatize the determination of the analyte, a simple monochannel flow injection system, with spectrometric detection, was designed and optimized. A volume of extracted solution containing DOC was cleaned-up on-line and loaded in a loop of the injection valve and by using acidulated water as a carrier it was transported to the flow cell. The spectrophotometric measurement was done at 344nm.

2. Experimental

2.1 Reagents and solutions

All solutions were prepared from analytical grade chemicals and using deionized water (18.0 MΩ) provided by Milli-Q system (Millipore, Bedford, USA).

A stock solution of DOC (Sigma, Buenos Aires, Argentine) was prepared dissolving a suitable amount of doxycycline chlorhydrate in methanol in order to obtain a concentration of 1 mg L^{-1}. This solution was stored under refrigeration at -18ºC until its use and it was stable for at least 90 days. Standard working solutions of DOC were daily prepared diluting the above mentioned stock solution with acidulated water (pH: 1.24).

A solution of HCl 0.0100 M was used as extractant solution and as carrier in the flow injection system.

2.2 Instrumentation

Spectrophotometric measurements were performed on a Lambda 2S Perkin Elmer UV-visible spectrophotometer coupled to an FIAS 300 Perkin Elmer manifold, with a 178-712 QS Hellma flow cell (inner volume = 8 µL, optical path = 10 mm).

A Rheodyne 5041 injection valve was used (sample loop of 150 µL). The reaction coils and sampling loop were made with i.d. 0.5 mm PTFE tubing.

The flow injection system contained a Tygon minicolumn (length 1.5 cm, i.d 0.3 cm) filled with cotton, and then a nylon syringe filter of 0.45 µm of pore size (CAMEO 25).

A Rolco 2036 centrifuge, Orion 710A pH-meter, Vortex Velp Zx3 and a thermostatic bath Cole Parmer BT-15 were also used.

2.3 Samples

Three chicken fat samples were used. The samples came from chickens of different origin: one was purchased at the retail market (A) and other at a supermarket and it was a frozen chicken (C). The last one was bought from an ecologic farm to ensure the absence of DOC (B). All samples were from Bahia Blanca, Buenos Aires, Argentine and they have been acquired during 2016.

2.3.1 Sample preparation and on-line clean-up procedure

The solid chicken fat was heated at 75ºC ± 1ºC. The liquid sample was filtered under vacuum through an 8µm paper filter, and then 5 g was weighed and placed into a 15 mL polypropylene centrifuge tube. 400 µL of extractant (acidulated water, pH=1.24) were added and the mixture was put in a vortex for 10 minutes in order to favour the contact between the two phases. Finally, the mixture was centrifuged during 3 min at 1200 rpm and then the lower phase (aqueous phase) was introduced into the flow injection system shown in Fig. 1, where it was on-line cleaned up using a cotton minicolumn followed by a nylon syringe filter.

At first, a pure extract of the sample and an extract of the spiked sample were measured at 344 nm to verify the presence of DOC in the sample. The spectra of both solutions indicated the absence of DOC in the pure extract, because at this wavelength only the spiked extract presented the corresponding absorption band (Fig.2). So, the samples were spiked with different concentration of analyte before the weighing and then, they were mixed in a vortex during 5 min to achieve the equilibrium.

3. Results and discussions

3.1 Optimization of reverse liquid-liquid microextraction variables

The reverse liquid-liquid microextraction procedure is a relatively new methodology to extract different kinds of analytes. This methodology is very simple and ecologic because the extraction solvent usually used is water. The variables involved in this step were optimized and are summarized in Table 1, in which the study ranges and the optimum values for each variable are shown. These values were selected taking into account the best sensitivity and reproducibility of the signal.

3.1.1 Selection the working wavelength

The spectra of DOC in different extractant solvents were analyzed scanning from 200 to 500 nm on a spectrophotometer. According to the literature (Anderson et al., 2005 ; Remon, Schelkens, Haesebrouck, & Backerh, 1996) two absorption maxima were found for doxycycline (260 and 344 nm) working under acidic solution. However, at 260 nm interferences of matrix substances of the blank were observed (Fig. 2). Hence the wavelength of 344 nm was chosen.

3.1.2 Selection of the extractant solvent

The selection of a suitable extractant agent is the most important parameter for the RP-LLME procedure. The study was carried out using different extractant solutions and the spectra of the different pure extracts of the sample and spicked extracts were recorded. The work was started using citrate buffer at pH:4.00 because this extractant solution was previously used for the extraction of TCs from different biological samples (Papadoyannis, 2007; Lv, Zhao, He, Yang, & Sun, 2013). However, in this case, the spectrum of the pure extract of the sample showed interference at the selected wavelength, probably due to the co-extraction of other

species from the matrix. In addition, there were not any considerable differences in the various concentrations used for the fortification of the sample indicating poor sensitivity. For these reasons, the study continued using water as extractant. Although the extraction of the analyte improved, once again the blank presented interferences. So, it was decided to use acidulated water at pH 1.2, 2.0, 3.0. The results showed that the lower the pH the better the analyte extraction and the lower the interferences of the matrix. This is possible because the analyte was protonated ($pH < pK_{a1}$) and under this chemical form it had much more affinity with the aqueous phase (Anderson et al., 2005). Therefore, acidulated water at pH 1.24 was selected as extractant solution because the best analyte extraction and the lowest matrix interferences were achieved.

3.1.3 Selection of mode and time extraction

In order to improve the extraction procedure, different modes of extraction such as ultrasonic bath, vortex and magnetic stirrer were studied. While it is true that the extraction modes tested here enhance the contact between the two immiscible liquids, a problem was observed using the ultrasonic bath. This could be related to the lack of uniformity in the transmission ultrasound energy (Yiantzi, Psillakis, Tyrovola, & Kalogerakis, 2010), causing variability in the results. Similar results were obtained using magnetic stirrer. The best recoveries were obtained with the vortex. This can be attributed to the way of vortex mixing, that generated a more efficient and faster analyte transfer to the extractant solution. Usually, the extraction of analyte increases with longer times. So, vortex time was tested between 5 to 15 min, observing that any longer extraction times that 10 min did not considerably affect the extraction efficiency. Therefore, 10 min of vortex agitation was chosen as optimum value.

3.1.4 Selection of volume of extractant solvent

Besides the extraction, the preconcentration of the analyte is necessary. So, volumes in the range 200 - 600 µL were tested. The best results were obtained with 400 µL because using 200 µL the extraction was incomplete. Volumes between 400 and 600 µL showed similar results. Thus, it was decided to use the smaller volume to obtain the best preconcentration.

3.2 Optimization of FIA variables

A FIA system was designed in order to automatize the spectrometric determination of DOC. Several configurations were tested and the best results were obtained with the monochannel manifold shown in Fig.1. The variable optimization was done using the univariant mode and taking into account the best sensitivity and reproducibility of the spectrometric signal.

The volume of the sample loop was tested between 50 and 300 µL and the optimal value was 150 µL. Keeping fixed this variable, the flow rate of the carrier was proved in the range 3.82 – 5.73 mL min^{-1}. Working at 5.73 mL min^{-1} lower dispersion and higher sample throughput were obtained, but a reactor prior the flow cell was necessary to obtain a signal without double peak. The length and type of it were optimized and the best results were obtained with a 200 mm knocked reactor.

This study was performed using the extraction solution (acidulated water pH: 1.24) as a carrier. Although, Milli-Q water was tested to check the best carrier solution, worse signals were obtained.

In order to clean-up the samples, a minicolumn filled with cotton followed by a nylon syringe filter were introduced into the channel that was used to load the sample at 4.62 mL min^{-1}. With a Tygon minicolumn of 1.5 cm of length and 0.3 cm of i.d it was possible to carry out the clean-up of the sample on-line.

3.3 Analytical parameters

Using the proposed method with all optimized variables, a calibration curve for the DOC determination, using the standard working solution, in a range between 100 and 700 µg kg^{-1} was obtained. The regression equation was Y= (0.0002 ± $4.4x10^{-6}$) X – (0.0047 ± 0.0018), where X was expressed as [µg DOC kg^{-1} sample] and the determination coefficient was R^2 = 0.998. This linear range contains the MLR (300 µg kg^{-1}) established by the European Union. The limit of detection (LOD) was 33.0 µg kg^{-1} and the limit of quantification (LOQ) was 100 µg kg^{-1}, both calculated from the calibration curve (Miller, Miller, 1993). The relative standard deviation, calculated from 8 replicates of spiked sample with 300 µg kg^{-1} was 4.87%. The sample throughput, taking into account the treatment step, was 4.5 h^{-1}.

Usually, TCs are not fat soluble compounds, only the DOC has a significative $K_{o/w}$ (-0.02), so only DOC is regulated in this kind of samples. In this way, the selectivity of the method is assured, because only DOC will be present in chicken fat samples.

3.4 Analysis of real samples

Different poultry fat samples, which are described in Section 2.3.1, were analyzed to check the applicability of the proposed method to determine DOC. Preliminary analysis of the samples showed that they were free from DOC (Fig.2). So, a recovery study was carried out at three concentration levels. Table 2 shows the obtained results when the proposed method was applied to real samples. As can be seen, the recoveries presented certain variability. Thus, it was decided to calculate the combined and relative uncertainties (µ and µ%) for each concentration level and for samples of different origins (Maroto, Boqué, Riu, & Rius, 2001). These values include the contributions of the intermediate precision of the method and the verification of the trueness. Besides, the expanded uncertainty (U) was evaluate with a coverage factor k = 2, at a confidence level of 95%. The obtained values were much lower than those recommended for CAC / GL 59 – 2006 (CAC/GL 59 –2006, 2011) for each concentration level ($U_{expanded\ \%}$ = 16 to 53 % for 1 µg kg^{-1} to 1000 µg kg^{-1}). In addition, these relative expanded uncertainties

showed statistically similar values ($p>0.2$), indicating that the new method is independent of the analyte concentration levels and the origin of the samples. For these reasons, the usefulness of the new method was demonstrated, taking into account the effective, simple and eco-friendly extraction step.

On the other hand, only one work presents results for the determination of DOC in chicken fat using the LC-UV-V technique (Gajda et al., 2013).This paper presents recovery values in agreement with the values obtained with the proposed method, although the concentrations are not exactly the same, but they are very close.

4. Conclusions

The automated FIA spectrometric method presented in this work is a very good alternative to determine DOC in chicken fat. It is a simple, fast and low cost technology because only an easy extraction step, a spectrophotometer and a simple flow injection system are used.

The main advantage of the method is the extraction/preconcentration step. A reverse liquid-liquid microextraction methodology, using only 400 µL of acidulated water as extraction solvent, has been developed. The procedure is effortless, clean, eco-friendly and the extracted solution is very easy to load in the injection valve of the FIA system.

The obtained results show good recovery values, a good reproducibility and sample throughput, taking into account the extraction/preconcentration step. It is worth mentioning that it is not necessary to use the matrix matched calibration, in spite of the complexity of biological samples. Here, the calibration curve is constructed with acidulated water standards and it contains the MLRs for DOC in fat. The obtained values of $U_{expanded\%}$ demonstrated that the method is useful to determine DOC in samples of different origin and at any concentration level. This selective method is environmentally-friendly and only uses a spectrophotometric determination without the need for sophisticated instruments.

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447 **Table 1:** Studied ranges and optimal values of extraction variables.

Variable	Studied range	Optimal value
Extractant	Citrate pH:4.0 Water Acidulated water	Acidulated water
pH extractant	1.2-3.0	1.2
Vol. extractant (µL)	200-600	400
Time of extraction mode (min)	5-15	10

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Table 2: Analysis of spiked real samples using the proposed method

Sample	Added(μg kg^{-1})	Recovery*(R)	SD_R	μ (combined)	μ (relative %)	U (expanded %)
A	200	0.825	0.019	5.04	2.52	5.04
	300	0.787	0.013	5.39	1.80	3.59
	400	0.906	0.016	7.67	1.92	3.83
B	200	0.785	0.017	4.82	2.41	4.82
	300	0.846	0.012	4.50	1.50	3.00
	400	0.933	0.014	6.62	1.65	3.31
C	200	0.650	0.016	5.54	2.77	5.54
	300	0.683	0.018	8.71	2.90	5.81
	400	0.611	0.012	8.68	2.17	4.34

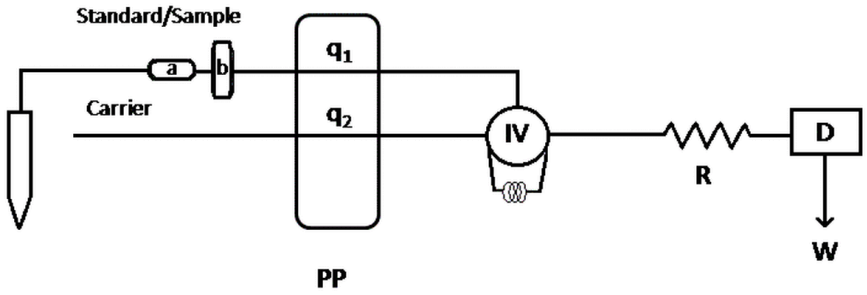
* Average of five replicates

SD_R: standard deviaton of the recoveries

Figure captions

Figure 1: Flow injection system to determine DOC in chicken fat samples. PP: peristaltic pump, q_1: load flow rate, q_2: carrier flow rate, a: cotton minicolumn, b: nylon syringe filter, IV: injection valve, R: knocked reactor, D: detector (ʎ= 344 nm), W: waste.

Figure 2: UV-Vis Spectra of fat spicked sample with (A) 1600 µg kg^{-1} ; (B) 1200 µg kg^{-1} ; (C) 800 µg kg^{-1} ; (D) blank sample.



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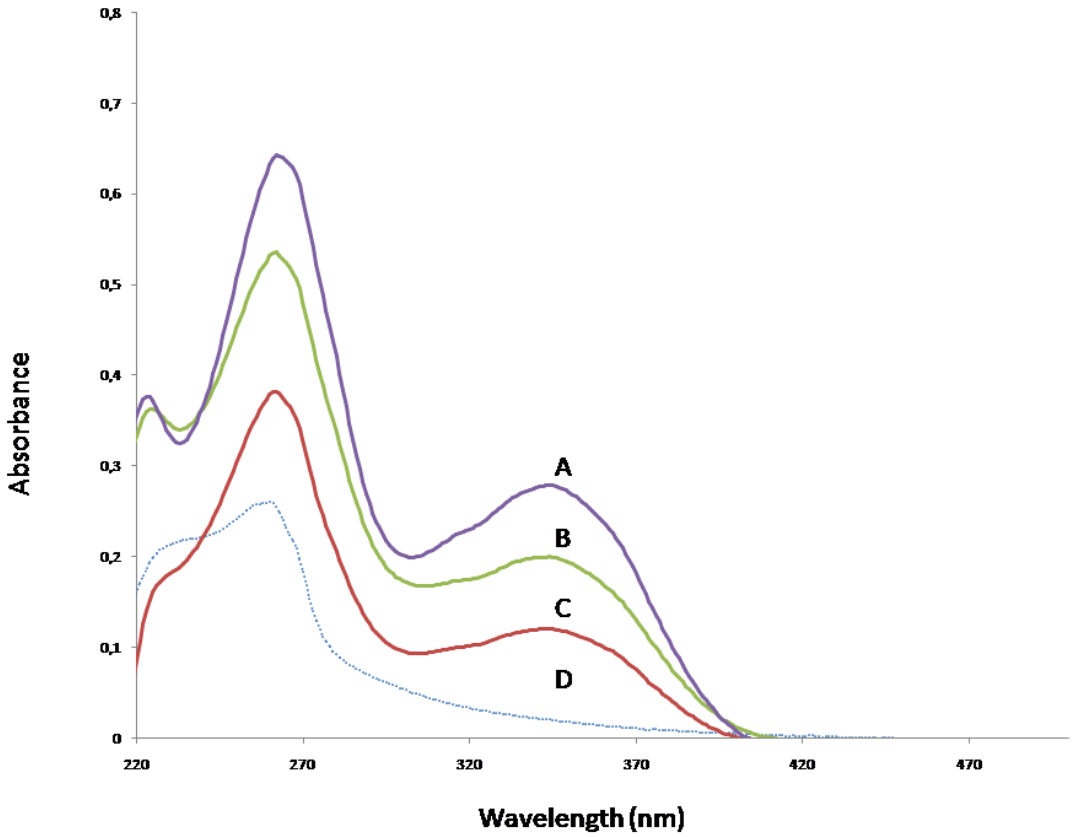

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Highlights

- A new reverse liquid-liquid microextraction was developed for determining doxycycline (DOC).
- 400 µL of acidulated water was used as extractant.
- The new methodology is free of toxic organic solvents and eco-friendly.
- A simple and cheap monochannel spectrometric flow injection system was used.
- The method was applied for the determination of DOC in chicken fat samples.