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High-performance liquid chromatography using pressurized liquid extraction for the determination of seven tetracyclines in egg, fish and shrimp

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ABSTRACT

A simple and especially rapid method, pressurized liquid extraction, has been developed and applied to the quantitative determination of oxytetracycline, tetracycline, chlortetracycline, minocycline, methacycline, demeclocycline and doxycycline in egg, fish and shrimp. The procedure consisted of a trichloracetic acid/methanol extraction conducted at elevated temperature (60 °C) and pressure (65 bar), without further clean-up, the extraction solution was concentrated and finally for high-performance liquid chromatography analysis. The limits of detection were 5.0–10.0 μg/kg and the limits of quantification were 10.0–15.0 μg/kg for tetracyclines in egg, fish and shrimp using UV detection. The analytical limits CCα and CCβ were also calculated. The recoveries of tetracyclines spiked at levels of 15–300 μg/kg, averaged 75.6–103.5% with the relative standard deviation values less than 11%. The optimized procedure has been successfully applied to real samples in our laboratories. It demonstrated that the new method was robust and useful for monitoring and quantification of 7 tetracycline residues in food of animal origin.

1. Introduction

Oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC), minocycline (MINO), methacycline (MTC), demeclocycline (DEMC) and doxycycline (DOX) are representative members of tetracyclines (TCs). Because of the easy availability and efficiency for the treatment of bacterial infected disease, they were widely used in animal husbandry. Due to the misuse, the antibiotic residues in products of animal origin brought a concern to consumers. The residue of this kind of drugs can be directly toxic or else cause allergic reactions in some hypersensitive individuals [1]. In addition, low-level doses of antibiotic in foodstuffs consumed for long periods can lead to the spread of drug-resistant micro-organisms [2]. In China, the maximum residue limits (MRLs) of TC, CTC, OTC were set at 100 μg/kg in fish and shrimp and 200 μg/kg in eggs, respectively, while DOX was not permitted to residue in milk and eggs and no MRLs values were set for MINO, MTC and DEMC [3], this is the same as EU has established [4]. To ensure confidence in the animal production and to avoid the residues of TCs, determination of these drugs in egg, fish and shrimp is of considerable importance.

Analytical methods such as microbiological, immuno-assays and thin-layer chromatography had been described for monitoring TCs in biological matrices or pharmaceuticals [5,6], however, immuno-assays are usually used as screening methods, some of them were either time consuming or precision variable and even can not identify certain TCs. Therefore, a precise chromatographic analysis method for the TCs has been required.

Several instrumental methods such as HPLC, LC–MS/MS, SPME-LC/MS and UPLC/MS had been used for TCs residues determination [7–15]. However, sample pretreatment of some HPLC methods was time consuming and laborious [8–10], LC/MS/MS and UPLC/MS need expensive instruments which were not suitable for the routine monitoring, while SPME resulted in poor reproducibility caused by instability. Additionally, prior studies mostly focused on the improvement of detection, only a few papers described new and rapid sample pretreatment for the further analysis of TCs in egg, fish or shrimp [11,12,16], and most of the reported HPLC-based methods in the previous literatures only allow the determination of three or four analytes [17].

As is known, TCs are very unstable and decomposed rapidly under the influence of light and atmospheric oxygen, forming more than fourteen different degradation products, such as the epi- and anhydro-compounds [18], long time of pre-condition in the conventional extraction process always resulted in low recovery, further affected the sensitivity. The critical challenge for a quantitative determination of trace-level TCs in complicated matrices is linked with the extraction process. Pressurized liquid extraction (PLE) is a recent advance in sample pretreatment for trace analyte and this technique uses conventional solvents at elevated pressures and temperatures to extract solid samples quickly [19–21]. It has been used in environmental sample pretreatment [22–25] and other antibiotics in foodstuffs [26–29]. However, in the previous

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work, no paper has described applicability of PLE for the extraction of TCs in foods of animal origin, such as chicken eggs, fish and shrimp.

The present study is to develop a rapid, sensitive and reliable method for simultaneous determination of 7 TCs in chicken eggs, muscle of fish and shrimp by PLE combined with HPLC-UV. After the optimization of PLE conditions such as extraction solvents, temperature, pressure and extraction cycles, high extraction efficiency has been obtained and this method could be applied to real sample analysis.

2. Experimental

2.1. Materials and materials

Acetonitrile and methanol of HPLC grade were purchased from Fisher Chemical Company (New Jersey, USA). Oxalic acid, disodium ethylene–diamine tetraacetate (Na_2EDTA), trichloracetic acid (TCA) and sodium hydroxide (NaOH) of analytical-reagent grade were obtained from Beijing Chemicals Company (Beijing, China). Deionized water (Milli-Q; Millipore, Bedford, MA, USA) was used through the study. Standards of OTC, TC, CTC, DOX, MINO, MTC and DEMC were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Standard solutions

OTC, TC, CTC, DOX, MINO, MTC and DEMC stock standard solutions (1.0 mg/mL) were prepared every 2 months by dissolving each in methanol and stored at −20 °C. Working combined mixed standard solution (100 μg/mL) was diluted to volume with mobile phase and the working standard was stable for at least 1 week when stored in amber tube at 4 °C.

2.3. Blank sample

Eggs were from laying hens fledged by our own lab, fish and shrimp muscle was purchased from supermarket, after being homogenized in a high-speed food blender and samples were stored below −20 °C in a freezer until the time of analysis.

2.4. PLE conditions for sample pretreatment

The TCs were extracted with a Dionex accelerated solvent extractor 200 (Dionex, Sunnyvale, CA, USA). This method implies the use of solvents at temperature up to 80 °C and pressure to 85 bar, the solvents selected were methanol and 1 mmol/L TCA adjusted to pH 4.0. Approximately 5 g of the blank/spiked sample material mixed with 5 g of Na_2EDTA-washed sand was packed in a 22 mL stainless steel extraction cell. Each cell was locked with stainless steel screw caps equipped with teflon O-ring sealings, and circular glass microfiber filters of 1.98 cm diameter (Dionex) were placed above and below the packing. Conditions used in the extraction can be seen in Table 1.

Table 1
Pressurized liquid extraction conditions.

	PLE
Solvent	TCA/methanol (v/v = 1/3)
Pressure	65 bar
Temperature	60 °C
Heat time	3 min
Flush volume	80%
Purge time	60 s
Static cycles	2
Final extraction volume	25 mL

Finally, the resulting extracts were diluted to 25 mL and 5 mL was evaporated to dryness at 40 °C under a nitrogen flow. The residue was dissolved in 1 mL of mobile phase and votexed and filtered through a 0.22 μm nylon Millipore chromatographic filter for HPLC analysis.

2.5. HPLC analysis

The HPLC system consists of a Waters 2695 separations module and 2487 dual λ absorbance detector (Waters, USA). A ZORBAX SB-C18 (150 mm × 4.6 mm I.D., 5 μm) (Agilent Technology, USA) HPLC column was used for separating TCs. Temperature of the column was set at 35 °C. The mobile phase used was methanol/acetonitrile/0.01 M oxalic acid solution, the injection volume was 50 μL, a gradient elution was selected and the mobile phase composition of methanol/acetonitrile/0.01 M oxalic acid solution (pH 3.0) was 7:8:85 at 0 min, switching to 10:20:70 after 15 min and maintaining at 10:20:70 for 3 min, the flow rate was 1.0 mL/min. The wavelength of UV detector was set at 355 nm. For each drug, a series of six concentration points (10, 50, 100, 200, 400, 800 μg/L) were prepared and each solution was injected three times, The calibration curves were obtained by plotting concentration (μg/kg) against peak area, identification was performed by matching the retention time and their spectral characteristics examined by the UV detector against those of standards.

2.6. Validation study

The method was validated with reference to the implemented validation procedure for residues in food animal products as described in the EU Commission Decision 2002/657/EC under Council Directive 96/23/EC [30]. The validation of specificity, linearity, decision limits (CCα) and detection capability (CCβ), recovery and precision for the method were determined. The blank samples were spiked with the TCs at each of six concentrations from 10 μg/kg to 800 μg/kg and the linearity of these matrices spiked curves was established.

2.6.1. CCα and CCβ

The analytical limits CCα and CCβ were determined as required by Commission Decision 2002/657/EC.

2.6.2. Recovery

The recovery was measured in blank samples that were spiked at the levels of 0.5, 1 and 1.5 times of MRL (1, 2, and 4 times of CCβ of DOX). As the MRLs of DEMC, MINO and META have not been set, the spiked levels were the same as OTC, TC and CTC. The spiked samples were analyzed and the recoveries were calculated by comparing the peak area of measured concentration to the peak area of the spiked samples concentration.

2.6.3. Repeatability and reproducibility

Samples that have been spiked with TCs at three concentration levels as described above, each 6 sets, respectively, were analyzed on the same day with the same instrument and operator. The mean concentration and the relative standard deviation (RSD) were calculated as repeatability. Samples that have been spiked with TCs at three concentration levels, respectively, were analyzed for 3 days with the same instrument and operator. The overall mean concentration and RSD were calculated as reproducibility.

2.6.4. Stability

The stability experiment was carried out aimed at testing possible conditions in which the samples might be exposed to. The solutions of seven TCs (1 μg/mL) were stored at −20 °C, 4 °C, and room temperature in the darkness. The solutions were analyzed

every 1 week and were compared with freshly prepared standards (1 μg/mL).

2.7. Application of the method to real samples

The present method was used to analyze the seven TCs in real samples to evaluate its quantitative capability. 30 eggs, 20 fish samples and 20 shrimp samples bought from market were determined using the method described above and seven drugs contents were calculated.

3. Results and discussion

3.1. Optimization of PLE procedure

As mentioned previously in Section 1, sample pretreatment was an important part of our method. The extraction procedure was outlined in Table 1 and the total extracting time was less than 25 min. With the aim to find the most effective conditions, the influence of solvents proportion, temperature, pressure and extraction cycles were investigated.

Pure organic solvents such as methanol and acetonitrile were able to extract these drugs from matrices, the only drawback was that many other soluble organic impurities were also extracted. Water was also used to extract analytes as the prior study [28], when egg, fish muscle and shrimp muscle samples were analyzed, there were strong interference peaks with MINO, OTC and TC, which might due to the different matrices in different samples. The pK_a 1 and pK_a 2 of these 7 TCs are in the range of 7.5–8.0 and 8.9–9.6, respectively [31], and these drugs are unstable under strong acid conditions and apt to form reversible epimers under pH 3.0 [32]. Therefore, aqueous solution mixed with organic solvents was studied. Sczesny et al. used acetonitrile and citrate buffer (1 M, pH 5) with the ratio of 20:1 to extract tetracyclines and their metabolites in eggs [17]. We compared initially an extraction solution consisting of citrate buffer and acetonitrile or TCA and acetonitrile. The results showed that TCA had a more favorable effect on the extraction efficiency. Additionally, when the buffer was pH 4.0 (adjust to pH 4.0 using 1 mol/L NaOH), a more satisfactory HPLC chromatogram with fewer impurities was obtained and an obvious high recovery was found when mixed with methanol in the rate of 1:3 (v/v) (Figs. 1 and 2 and Table 2). To our investigation, methanol or acetonitrile as organic solvent had little or no difference for the extraction, considering the expense, methanol was selected.

The extraction was tested at different temperatures (40–80 °C) for their effectiveness in extracting TCs from egg, fish and shrimp, and recovery values arranged from 69% to 93% (n = 3; RSD = 4–10%). Extraction efficiency showed an optimum at 60 °C. At higher temperatures the recoveries decreased, which is probably due to a destruction of the tetracycline or increased formation of 4-epimers. Another problem is that at high temperature, the extractant was not clear which may be due to the matrix dispersion of the sample. Below 50 °C, low recoveries were obtained, that was most probably attributable to the inefficient desorption and dissolution of the TCs (Fig. 3a).

To determine extraction recoveries, the pressure of the extracting system has also been investigated ranging from 45 to 85 bar. The recovery was obtained between 43% and 95% with RSD of 5–11%. It was found that good recovery could be obtained at the pressure of 65 bar (Fig. 3b) and this pressure is considerably above the minimum pressure to keep the solvent liquid.

The lengthy of exposure to solvents allows the matrix to swell and improve the penetration of the solvent into the sample interstices and the contact of the solvent with the analytes [33]. According to our study, the number of extracting cycle was not the most important factor. After twice of extraction within 4 min of one extraction cycle, the recovery could not increase any more, on the contrary, the impurity raised and this result was consisted to a prior study [34]. Therefore, each sample was extracted twice.

This work proved that using optimized conditions of PLE, without the need for further clean-up or for increasing throughput,

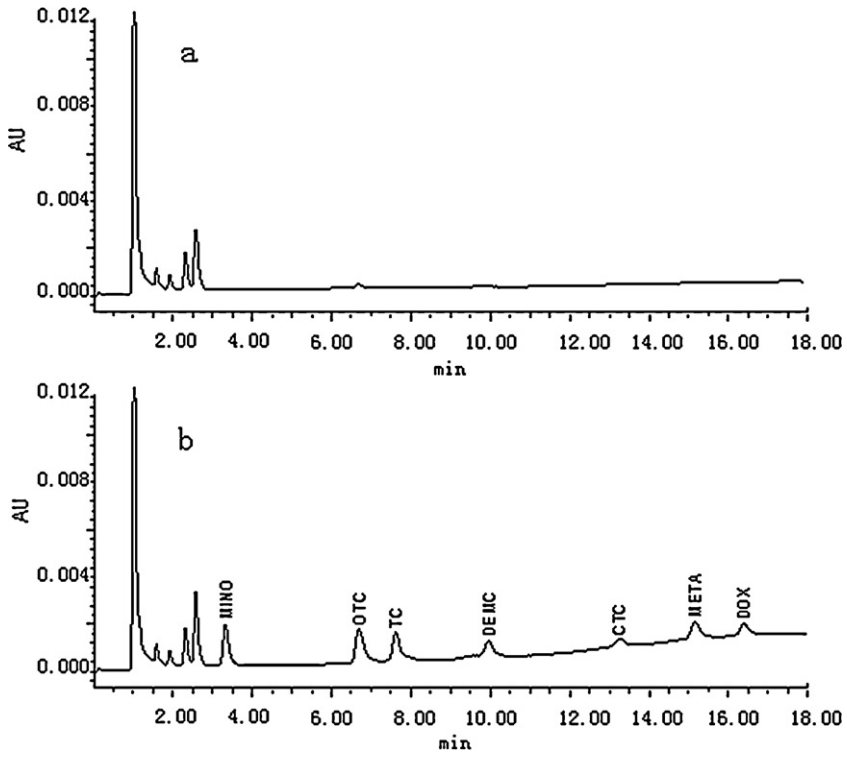

Fig. 1. Chromatograms of blank egg (a), and spiked egg (b) at concentration of 10 μg/kg for each of individual drug.

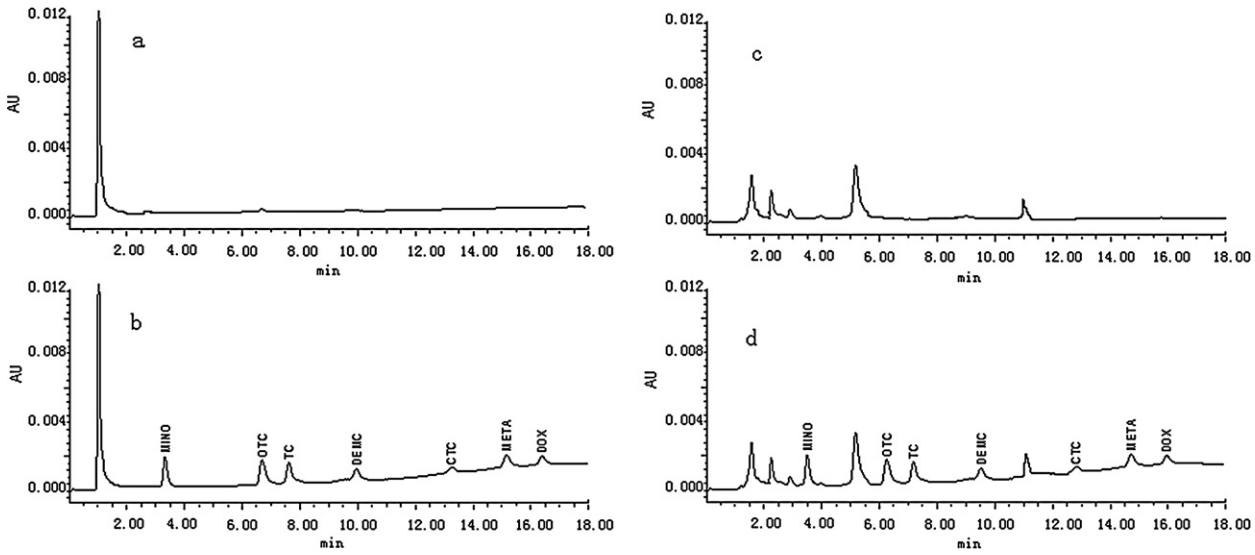

Fig. 2. Chromatograms of blank muscle of fish (a), shrimp (c), and spiked muscle of fish (b), shrimp (d) at concentration of 10 μg/kg for each of individual drug.

did not seem to extract more background material in comparison to conventional techniques [1,35]. Additionally, PLE could reduce analysis time, had good recoveries and the precision was satisfactory.

3.2. HPLC-separation

Column was an important factor for separating 7 TCs, various columns were applied in the previous work. In our research, columns including Xterra C8, Microbondapack C18 and Agilent ZORBAX SB-C18 were tested. When an Xterra C8 was used, good peak shape of OTC, TC and MINO was obtained, however, the peak shape for CTC and DOX was not satisfactory even gradient elution was used. Microbondapack C18 and Agilent ZORBAX SB-C18 performed the same results in the chromatography separation. Considering the routine analysis, Agilent ZORBAX SB-C18 was selected because it was commonly used in the laboratory.

The selected organic phases were methanol and acetonitrile. It must be highlighted that in our study, the acetonitrile content >8% was required to elute analytes with the good peak shapes. It has been reported that TCs can be adsorbed on the silanol group in a reversed phase column which leads to tailing peaks, while the addition of oxalic acid (pH 2.0–3.0) could avoid this [36]. In the present study, the concentration of oxalic acid in the mobile phase is 0.01 M.

Optimization of the mobile phase for HPLC separation of the 7 compounds was accomplished by investigating various volume ratios of 0.01 M oxalic acid solution and organic phase. Because of the different polarity of these 7 drugs, when isocratic elution was used, the retain time of CTC and DOX extended and the peak width was not satisfactory. Good separation was achieved when gradient elution was used. The mobile phase composition of methanol/acetonitrile/0.01 M oxalic acid solution (pH 3.0) was 7:8:85 at 0 min, switching to 10:20:70 after 15 min and maintaining 3 min, so the analysis time was reduced to 18 min. The UV wavelength was selected at 355 nm after scanning all the 7 drugs by the ultraviolet spectrophotometer and the peaks were reasonably sharp.

3.3. Method validation

3.3.1. Linearity and specificity

The commercially supplied internal standard such as substituted could not be obtained from reagent's company. Thus, we employed external reference method for quantification. The linearity and regression study were performed according the description as Section 2 validation. The high correlation coefficients (r = 0.9988–0.9990) indicated good correlations and it also indicated that this method can be used to determine the 7 compounds at a wide contamination level range.

Table 2
Solvent influences on the extraction recovery of studied TCs from egg, fish muscle and shrimp muscle.

Extraction solvent	Sample	Recovery (%RSD)						
		MINO	OTC	TC	DEMC	CTC	META	DOX
Methanol/TCA (1:1)	Egg	61 (14)	60 (17)	58 (4)	41 (9)	56 (9)	46 (11)	70 (13)
	Fish muscle	63 (5)	47 (5)	50 (2)	52 (6)	58 (4)	62 (9)	69 (6)
	Shrimp muscle	72 (6)	55 (3)	61 (3)	60 (7)	52 (5)	67 (6)	60 (10)
Methanol/TCA (2:1)	Egg	75 (4)	60 (5)	58 (9)	41 (6)	58 (12)	46 (4)	70 (4)
	Fish muscle	78 (10)	77 (6)	62 (6)	71 (8)	65 (8)	80 (5)	78 (5)
	Shrimp muscle	70 (5)	63 (8)	53 (10)	47 (5)	58 (10)	49 (5)	71 (6)
Methanol/TCA (3:1)	Egg	89 (5)	88 (4)	82 (6)	85 (3)	80 (5)	91 (3)	90 (2)
	Fish muscle	88 (5)	82 (6)	80 (5)	85 (5)	81 (5)	90 (7)	80 (5)
	Shrimp muscle	83 (4)	87 (7)	89 (2)	80 (6)	85 (6)	84 (6)	84 (6)
Methanol/TCA (4:1)	Egg	68 (15)	65 (13)	46 (6)	51 (17)	44 (11)	65 (10)	73 (15)
	Fish muscle	46 (6)	51 (17)	65 (10)	73 (15)	65 (13)	48 (9)	81 (8)
	Shrimp muscle	52 (4)	57 (13)	61 (13)	80 (9)	60 (9)	57 (7)	82 (6)

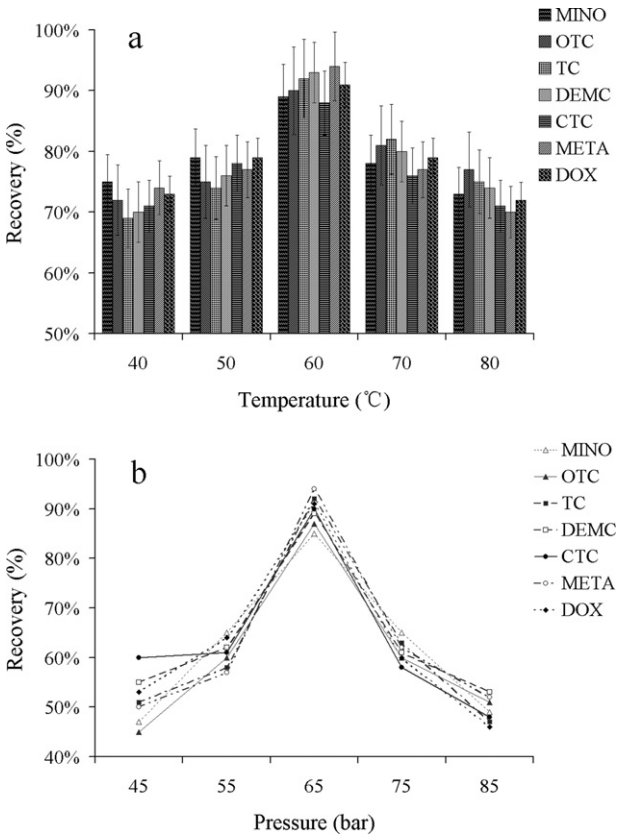

Fig. 3. The influence of different temperature (a) and pressure (b) on PLE.

The specificity of the method was checked by analyzing different types of blank samples. None interfering peaks could be detected at the retention time of the 7 analytes (see Figs. 1 and 2).

3.3.2. LOD, LOQ, CCα and CCβ

For the limits of detection (LOD) and limits of quantification (LOQ) calculation, samples were spiked with a standard mixture of the analytes at serially diluted concentrations. The LOD were estimated by calculating the signal to noise ratio of 3 at matrices. The LOD were 5–10 μg/kg for all compounds and LOQ, considered as being at the lower point of the linear range, were 10–15 μg/kg for most compounds. This showed that the method could be useful for determining TCs residues in contaminated samples.

The CCα and CCβ of the method were determined in chicken eggs, fish muscle and shrimp muscle. Table 3 shows the CCα and CCβ with an error of 5%, considering the experimental standard deviation of within-laboratory reproducibility at the adequate contamination level. For OTC, TC, and CTC, which have established MRLs, CCα and CCβ were calculated by analyzing 20 blank eggs, fish muscle, and shrimp muscle, all fortified with the analytes at the maximum permitted limit according to the EU criteria. However, since the MRLs of META, DEMC and MINO were not established, CCα was calculated by analyzing 20 blanks to be able to calculate the signal to noise ratio at the time window in which the analyte is expected. The signal to noise ratio 3/1 can be used as CCα, values of CCα plus 1.64 times the standard deviation of the within-laboratory reproducibility of the measured content equals CCβ (β = 5%).

Table 4
Accuracy and precision of the method for TCs in spiked eggs at three levels ($n = 6$ at each concentration on three separation days).

Sample	Compound	Spiked level (μg/kg)	Overall recovery (%)	Within-day RSD (%)	Between-day RSD (%)
Egg	OTC	100	75.6	5.1	8.8
		200	78.4	7.5	6.1
		300	79.0	4.9	5.7
	TC	100	80.4	3.8	8.1
		200	82.7	6.1	9.6
		300	85.4	5.9	9.2
	CTC	100	78.8	5.8	9.2
		200	79.2	3.4	6.9
		300	102.9	4.7	8.8
	DOX	15	86.7	6.3	7.1
		30	82.1	6.9	6.1
		60	85.0	5.6	5.9
	DEMC	100	80.5	6.1	9.1
		200	86.7	7.9	7.2
		300	89.1	4.8	8.9
	MINO	100	81.8	8.1	9.9
		200	88.3	6.7	8.1
		300	89.4	5.8	8.0
	MTC	100	80.9	8.4	7.7
		200	82.6	6.1	9.1
		300	87.4	5.9	9.0

3.3.3. Recovery and RSD

The method was further tested by applying the extraction procedure to the analysis of spiked samples. Tables 4 and 5 give the results of the recovery and repeatability of the method over the concentration range (15–300 μg/kg) on three separate days. It was also reflected that the method provided a wide concentration range over which to assess the performance of the developed method. Recoveries from spiked samples were more than 80%, except for CTC (>78%), and between-day RSD were lower than 10%.

3.3.4. Stability

The solutions were analyzed using the developed method every 1 week and were compared with freshly prepared standards (1 μg/mL) in the darkness. The standard solutions were found to be stable for 3 months when refrigerated at −18 °C, but about one-third of OTC, TC and CTC degraded at 4 °C for 1 month and half of OTC, TC, CTC and OTC degraded at room temperature for 1 week.

3.4. Real sample analysis

The developed method was successfully applied to real samples. 30 eggs, 20 fish samples and 20 shrimp samples bought from

Table 3
Decision limit (CCα) and detection capability (CCβ) of the method for egg, fish muscle and shrimp muscle (μg/kg).

	MINO	OTC	TC	DEMC	CTC	META	DOX
Egg							
CCα	5.8	201.4	205.4	9.5	202.4	8.5	11.3
CCβ	7.1	214.9	206.9	11.0	208.9	10.9	14.9
Fish muscle							
CCα	5.3	100.8	102.1	10.1	103.5	9.3	10.8
CCβ	7.0	103.1	103.6	11.7	106.8	11.3	14.8
Shrimp muscle							
CCα	5.5	101.8	102.2	6.5	106.8	8.8	13.0
CCβ	7.8	104.2	104.4	8.6	108.1	10.5	15.3

Table 5
Accuracy and precision of the method for TCs in spiked fish muscle and shrimp muscle at three levels ($n = 6$ at each concentration on three separation days).

Sample	Compound	Spiked level (μg/kg)	Overall recovery (%)	Within-day RSD (%)	Between-day RSD (%)
Fish muscle	OTC	50	81.5	8.1	9.6
		100	82.4	8.5	9.1
		150	85.7	7.9	8.4
	TC	50	85.0	8.0	7.1
		100	87.1	7.3	9.1
		150	82.3	5.1	8.5
	CTC	50	79.6	8.3	9.5
		100	82.4	8.4	8.8
		150	85.7	5.0	5.9
	DOX	50	82.4	8.2	8.8
		100	85.4	6.8	8.1
		150	100.9	4.1	5.9
	DEMC	50	82.1	4.9	8.5
		100	82.5	7.1	9.4
		150	85.4	5.9	5.9
	MINO	50	82.5	8.0	9.6
		100	82.4	7.2	9.0
		150	80.6	6.4	8.7
	MTC	50	80.6	8.0	6.6
		100	81.4	8.1	9.0
		150	103.5	7.6	8.7
Shrimp muscle	OTC	50	80.5	6.9	7.2
		100	86.4	8.2	8.5
		150	101.8	5.1	7.9
	TC	50	81.5	7.6	9.5
		100	82.4	8.0	8.8
		150	85.4	5.2	6.7
	CTC	50	78.7	8.1	8.5
		100	82.4	7.0	10.4
		150	85.7	5.3	7.9
	DOX	50	83.4	8.2	8.1
		100	87.3	8.5	8.6
		150	89.1	8.7	9.9
	DEMC	50	82.1	9.1	9.6
		100	84.0	7.2	9.1
		150	85.3	6.3	8.4
	MINO	50	80.6	5.3	7.3
		100	80.4	7.1	9.5
		150	99.7	5.9	8.4
	MTC	50	81.4	5.8	7.0
		100	84.3	8.0	8.5
		150	86.2	8.0	9.4

market were determined and these seven drugs contents were calculated. The results demonstrated that over 16 samples and 5 eggs contained TCs, in which CTC were at higher levels (mean more than 271.8 μg/kg) and MINO had a moderate level (each sample did not exceed 50 μg/kg). TCs in fish and shrimp samples were not detected. The successful use of the developed method in the quantification of TCs in real eggs, fish, and shrimp samples also demonstrates the accurate quantitative capability of the method.

4. Conclusion

A rapid sample extraction method involving PLE has been developed for determination of seven TCs in eggs, fish and shrimp with HPLC-UV. Solvents proportion of TCA solution and methanol from 1:1 to 1:4 was investigated and it showed that 1:3 was the most effective one. Moreover, temperature (60 °C) and pressure (65 bar) allowed for easier handling of analytes and cleaner extractions. After optimization, PLE reduced the use of solvents and extraction time compared to traditional liquid–liquid extractions, especially; solid phase extraction (SPE) step was not involved. The LOD were 5.0–10.0 μg/kg and the LOQ were 10.0–15.0 μg/kg for TCs in and eggs. The recoveries of TCs spiked at levels of 15–300 μg/kg, averaged 78.8–102.9% with RSD values less than 10%. The sensitivity of this method was good and the accuracy and precision were satisfactory. The results demonstrated that the method was a reliable tool and could be applied to analyze TCs residues in eggs, fish and shrimp for surveillance programs, it generated less hazardous waste and was friendly to the environment.

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