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Experimental Design Assisted HPLC/UV and LC-MS/MS for Simultaneous Determination of Selected Veterinary Antibiotics in Broiler Chicken

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Abstract: Antibiotics are used in the poultry industry to treat and prevent diseases. Their frequent use resulted in the appearance of antibiotic residuals in poultry meat, which is considered a serious public health issue. Among frequently used antibiotics are cefotaxime (CTX), ciprofloxacin (CIP), colistin (CST), doxycycline (DOX), flumequine (FLU), sulfamethoxazole (SMZ), trimethoprim (TMP) and tylosin (TYL). This study aimed to develop an optimized and validated method for concurrent estimation of the eight antibiotics in broiler chicken samples based on an easy extraction method followed by HPLC-UV and LC/MS/MS analysis. An experimental design was used for the optimization of the extraction procedure. Optimal conditions for separation were determined by using a central composite design after studying (1) mobile phase initial concentration, (2) column temperature, and (3) flow rate. The method was validated on the bases of ICH guidelines. The detection limits ranged from 3 to 5 μg kg^{-1} for HPLC- UV and ranged from 0.01 to 0.05 μg kg^{-1} for LC/MS/MS, while quantification limits ranged from 10 to 16 μg kg^{-1} for HPLC- UV and ranged from 0.01 to 0.11 μg kg^{-1} for LC/MS/MS. The chromatographic techniques were utilized for the analysis of spiked broiler chicken samples at a concentration range from 30 to 300 μg kg^{-1}) for HPLC-UV and 0.01–20 μg kg^{-1} for LC/MS/MS. The proposed methods were used for quantification of the residues of the studied antibiotics in real broiler samples obtained from local supermarkets in Ismailia governorate, Egypt. The detected levels of residual antibiotics were within the permissible limits.

Keywords: antibiotics; food analysis; liquid chromatography; sustainability of natural resources

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1. Introduction

Antibacterial medicines are used in poultry for treatment and protection purposes as well as increasing efficiency and growth factors [1]. Several groups of antibacterial agents, including cephalosporins, tetracyclines, beta-lactams, sulfonamides, lincosamides, fluoroquinolones, aminoglycosides, macrolides, and others, are widely used in the treatment of food-producing animals [2]. Macrolides are an important group of antibiotics extensively

used in veterinary fields for treating respiratory infections or as growth-promoting feed additives. Macrolides consist of macrocyclic lactones with twelve up to sixteen fields in which numerous amino groups and/or neutral sugars are attached [3]. After oral administration, macrolides are easily absorbed and widely distributed throughout tissues, especially the lungs, kidneys, and liver. Tylosin (TYL) is a widely used antibiotic in poultry farming [4]. Sulfonamides (SAs) are considered one of the most important classes of antibacterials broadly used for treating both human and animal illnesses. SAs are added to animal feed at a subtherapeutic level to avoid infections, encourage growth, elevate weight, also decrease daily animal feed requirements [5]. It acts similarly to sulfamethoxazole (SMZ). Trimethoprim (TMP) exhibits its action by inhibiting dihydrofolate reductase. It prevents the production of bacterial tetrahydrofolic from dihydrofolic acid and thus inhibits the synthesis of several amino acids, thymidine, purines, and DNA [6]. Tetracyclines (TCAs) are a group of antibiotics with efficacy against Gram-positive and Gram-negative bacteria. They are also used as growth promotors in cattle and poultry [7]. Doxycycline (DOX) is the tetracycline most widely applied to food-producing animals. Quinolones and fluoroquinolones are antibacterials widely prescribed in humans and in the prevention and management of veterinary infections. Quinolones and fluoroquinolones are applied in aqua-culture as well. Ciprofloxacin (CIP) and flumequine (FLU) are common types of this group [8]. Cefotaxime (CTX) is a third-generation cephalosporin. It is effective against many gram-positive and gram-negative bacteria [9]. Colistin (CST), also known as polymyxin E, is a polymyxin antibacterial. CST disrupts the bacterial cytoplasmic membrane by attaching to membrane phospholipids [9]. Colistin is a widely used growth promoter, specifically in the poultry sector [10]. These drugs were determined either individually or with each other using numerous techniques and in the presence of other drugs in broiler chicken samples.

The reported techniques include HPLC using different detection techniques [11–19], LC/MS/MS [4,14,20–28], TLC [14,17,29], enzyme-linked immunosorbent assay (ELISA) [17,30], microbiological methods [31–34] and spectrophotometry [34,35]. By reviewing the literature regarding the separation of the most used antibiotics in Ismailia, Egypt poultry farms, it was found that there is no method for the simultaneous estimation of TMP, CIP, CTX, DOX, TYL, SMZ, FLU, and CST in broiler chicken samples. Moreover, the previous methods suffer from some drawbacks, such as using sophisticated and long extraction procedures with using large volumes of organic solvents [11,12,14,17,20,21,24,27], poor percentage of recovery [17,23,29], very long separation time, which increased over 32 min [16], lower sensitivity and accuracy with regard to LOD and LOQ [12,13,19,20,22,24–26,28]. Furthermore, the validation merits were not studied [15–17,29]. All the published methods used univariate methods for optimization of the extraction procedure, which was incapable of measuring the interaction among the studied variables. On the other hand, multivariate analysis (experimental design) has the privilege of monitoring all variables and studying the effects encountered among them.

The purpose of this study is to simultaneously separate and precisely estimate TMP, CIP, CTX, DOX, TYL, SMZ, FLU, and CST, which are commonly used antibiotics in poultry farms in Ismailia, Egypt. We use an experimental design as a multivariate analytical tool to determine the best extraction procedure with the highest % of recovery and best chromatographic conditions for the targeted compounds separation. Due to the rising concerns regarding food safety and human health protection, quality control departments in food industries require a fast, accurate, and cost-effective tool for analyzing food products. This gives rise to the importance of HPLC-UV as a quality control tool found in most food factories. As animal-derived food is considered a very complex matrix containing high ratios of proteins and fats, which may affect the separation and quantitation of the targeted analytes. Thus, the proposed work also aimed to use LC/MS/MS as a quantitative tool for residual analysis of the targeted compounds in real broiler samples obtained from local markets in Ismailia governorate, Egypt.

2. Materials and Methods

2.1. Instrumentation

We used the HPLC (Shimadzu, Kyoto, Japan) instrument model LC-10 ADVP pump, SCL-10 AVP controller, DGU-12 A Degasser, Rheodyne 7725i injector with 5 or 20 µL loop, and an SPD-10AVP UV-VIS detector. A DALIAN REPLETE® Hong Kong column oven was used. The Class-VP software was used for data acquisition.

Experimental design, data analysis, response surfaces, and graphs were made using Design Expert Version 7.1 (Stat-Ease Inc., Minneapolis, MN, USA).

We used an ACQUITY UPLC H-Class equipped with Xevo TQD mass spectrometer (Waters, Milford, MA, USA). The ACQUITY UPLC H-Class consisted of a quaternary solvent manager QSM, sample manager FTN, and column manager CM (including column heater). The data acquisition was made using the Masslynx V4.1 SCN918 software.

2.2. Chemicals and Reagents

TMP, DOX, TYL, SMZ, FLU, and CST were gifted by ADWIA pharmaceuticals company (10th of Ramadan, Egypt) and certified to be above 99.3% purity. The CIP and CTX (99.5% purity) were gifted by the Egyptian International Pharmaceutical Co. (EIPICO) (10th of Ramadan, Egypt). HPLC grade acetonitrile and methanol (Fisher Scientific, Loughborough, UK). Double distilled water was filtered through (0.45 µm) cellulose membrane filters. The formic acid was of analytical grade.

2.3. Sample Preparation

2.3.1. Tissue Samples Fortification

To prepare enriched broiler samples (muscle and liver), a suitable volume of the standard compounds ranging from 30 to 300 µg kg^{-1} was spiked with 2 g of ground blank broiler tissues. After spiking, the broiler samples were left for 30 min at ambient temperature to permit effective interaction between the added drugs and the chicken samples before the extraction step.

2.3.2. Collection of Samples

In total, 20 fresh chicken chests and liver (10 of each) were collected from some markets in Ismailia governorate, Egypt. After collection, the samples were taken to the laboratory and preserved at −80 °C for further extraction and examination by the chromatographic method.

2.3.3. Extraction Procedure

The chicken sample (muscle and liver) (2 g of each) was homogenized and then transferred to a 15 mL polypropylene centrifuge tube. The sample was mixed with Twelve mL (0.2% formic acid in acetonitrile), vortexed for 5 min, sonicated for 5 min, followed by centrifugation for 5 min at 3200 rpm at ambient temperature. The supernatant was separated into a 25 mL volumetric flask. The residue was mixed with twelve mL of methanol and vortexed for 5 min, sonicated for 5 min, followed by centrifugation for 5 min at 3200 rpm at ambient temperature. The supernatant was collected and mixed with the first extract in the same flask and evaporated to dryness. The residue was reconstituted in 1 mL acetonitrile and 0.5 mL methanol, followed by cold centrifugation (at 4 °C) at 5000 rpm for 10 min. The supernatant was filtered by a syringe nylon filter (0.22 µm) into dark vials for assay.

2.3.4. Experimental Designs

In the current extraction procedure, investigation of the significance of four independent factors on the recoveries of the studied compounds was performed using a two-level Full factorial design (FFD). FFD uses 2^{k-p} experiments, where k is the number of factors studied, and p is the degree of fractionality of the FFD ($p < k$) [36]. The FFD matrix is given in Table S1.

In the current extraction procedure, four factors (Table S1) were investigated, including acetonitrile amount (mL), methanol amount (mL), formic acid (%), and extraction time (min). All experiments were carried out randomly three times. The selected response factors were the percentage of recoveries of the eight studied drugs (R%(TMP), R%(CIP), R%(CTX), R%(DOX), R%(TYL), R%(SMZ), R%(FLU) and R%(CST) (Table S1). From the results of the FFD, optimization of the extraction procedure was made using CCD. It was found that during screening, the studied factors were significant. Table S2 indicates the performed experiments and their responses.

2.4. Chromatographic Conditions for HPLC-UV Method

Detailed HPLC separation and quantification parameters are described in Table 1.

Table 1. The chromatographic conditions for the HPLC-UV method.

Parameters	Conditions	
Column	A 150 × 4.6 mm (i.d.) Phenomenex® (5 m particle size) reversed-phase C_{18}	
Mobile phase	A: 0.1% formic acid in water B: acetonitrile	
Gradient	Time (min.)	B%
	0–5	10%
	5–10	35%
	10–15	55%
	15–20	90%
	20–30	10%
Flow rate	1 mL min^{-1}	
Column temperature	30 °C	
Injection volume	20 µL	
Detector wavelength	260 nm	

2.5. Experimental Design

2.5.1. Screening Experiments for HPLC-UV Method Using FFD

Two-level FFD studied three independent factors that affect separation quality. The factors were: (a) acetonitrile (%) in each step of the gradient elution program, (b) column temperature (°C), and (c) flow rate. Screening designs can identify significant main effects instead of interaction effects. [36]. Upon application of the experimental design, minimizing the number of variables is recommended to simplify response models and decrease variability. The matrix for FFD is represented in Table S3. All experiments were carried out randomly three times. Selected response factors were (a) the resolution of critically separated peaks TMP and CIP peaks, CIP and CTX peaks, CTX and DOX peaks, and DOX and TYL peaks, (b) the first peak (TMP) retention time, and (c) the last peak (CST) retention time.

2.5.2. Optimization of the HPLC-UV Method

Based on the FFD results, central composite design (CCD) was designed using only significant factors. It is used to study the main factors and interaction effects. [37]. The studied factors were significant, and Table S4 records the obtained experiments and responses. For each analyte under the investigation domain, the locations of the highest HPLC-UV response were identified using surface plots created by a quadratic polynomial equation. The optimal conditions were attained by selecting the best value for each HPLC-UV response.

2.6. LC/MS/MS Analysis

Real samples were analyzed using LC/MS/MS Waters instrument. The separation was achieved using gradient separation. The detailed conditions are set out in Table 2.

Table 2. The chromatographic conditions for LC/MS/MS method.

Parameters	Conditions
Column	ACQUITY UPLC HSS T3 C_{18}, 1.8 µm, 50 mm × 2.1 mm column
Mobile phase (Gradient)	A: 0.1%(v/v) formic acid in water B: 0.1% (v/v) formic acid in methanol
Flow rate	0.3 mL min^{-1}
Column temperature	35 °C
Detector	Electrospray ionization (ESI)/positive mode
Voltage	Cone voltage: 42 V Capillary voltage: 2200 V
Drying gas flow rate	650 L hr^{-1}
Cone gas flow rate	50 L hr^{-1}

The mass spectrometry index for analytes monitoring is listed in Table 3.

Table 3. LC-MS/MS parameters of monitoring the TMP, CIP, CTX, DOX, TYL, SMZ, FLU, and CST compounds.

Compound Name	Precursor Ion (*m*/*z*)	Product Ion (*m*/*z*)	Dwell Time (s)	Cone Voltage (V)	Collision Energy (V)	Polarity
TMP	291.30	230.20	0.20	42	30	+Ve
CIP	332.10	314.10	0.20	42	22	+Ve
CTX	456.10	396.20	0.20	42	15	+Ve
DOX	445.20	428.20	0.20	42	20	+Ve
TYL	916.50	174.10	0.20	42	40	+Ve
SMZ	254.10	156.00	0.20	42	12	+Ve
FLU	262.10	244.00	0.20	42	15	+Ve
CST	578.50	101.46	0.20	42	35	+Ve

2.7. Standard and Calibration Solutions

For the preparation of stock solutions, (25 mg) of TMP, CIP, CTX, DOX, TYL, SMZ, and CST were dissolved separately in (50 mL) of methanol, while (25 mg) of FLU was dissolved in (50 mL) acetonitrile. Stock solutions were stored at 4 °C. Stock standard solutions were diluted with methanol to prepare 10 mg L^{-1} working standard solutions of each drug. From this standard solution, blank chicken samples spiked in the range from 30 to 300 g kg^{-1} for the HPLC-UV method and from 0.01 to 20 g kg^{-1} for the LC/MS/MS method were used for the calibration curve. All solutions were stored at −20 °C.

3. Results and Discussion

3.1. Samples Extraction

FFD was used to screen the factors affecting extraction. Table S5 presents the ANOVA of investigated factors and effects. When an independent factor has a p-value < 0.05, this suggests a significant effect on the given response. In Figure 1, perturbation plots are shown; which display the independent factor effect on a particular response, with all factors maintained constant at a reference point. The steepest slope shows the sensitivity to a particular factor [38].

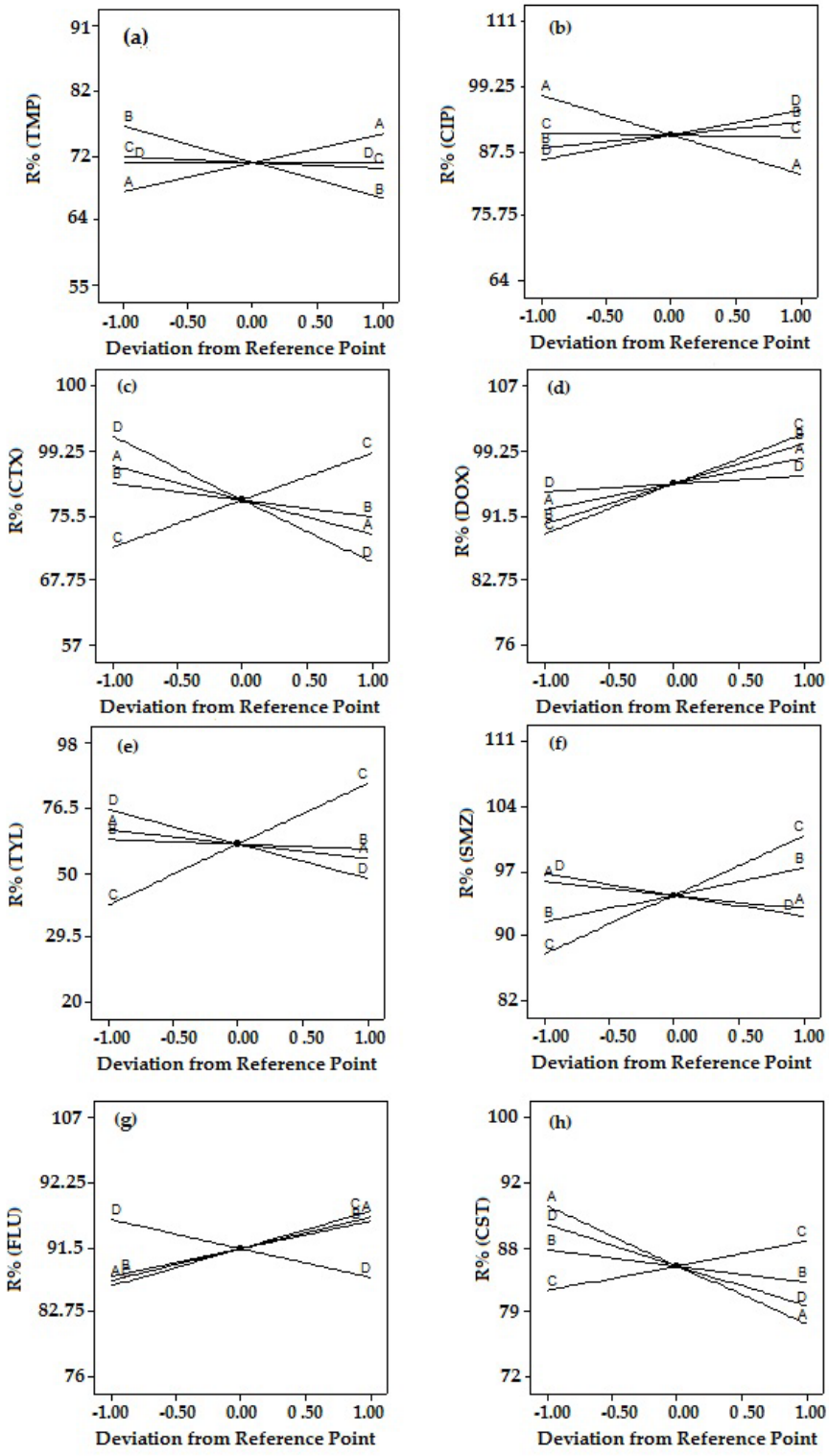

Figure 1. Perturbation plots for screening of factors affecting the extraction procedure, showing the effect of these factors on the recovery percentage of (**a**) TMP, (**b**) CIP, (**c**) CTX, (**d**) DOX, (**e**) TYL, (**f**) SMZ, (**g**) FLU and (**h**) CST, where A is methanol amount, B is acetonitrile amount, C is formic acid (%) and D is the time of extraction.

The results from the ANOVA and perturbation plots indicated that the amount of methanol (MeoH mL) had the most significant effect on the recovery percentage of TMP (R%(TMP)), CIP (R%(CIP)), DOX (R%(DOX)), FLU (R%(FLU)) and CST (R%(CST)). The

amount of acetonitrile (ACN mL) had the most significant effect on the recovery percentage of TMP (R%(TMP)), DOX (R%(DOX)), SMZ (R%(SMZ)) and FLU (R%(FLU)), the percentage of formic acid (Formic acid%) had the highest significant effect on the recovery percentage of CTX (R%(CTX)), TYL (R%(TYL)), SMZ (R%(SMZ)) and FLU (R%(FLU)), and finally the time of extraction (min) had the most significant effect on all selected responses except R%(TMP) and R%(DOX). R^2_{adj} was >0.85 in all cases, indicating that the experimental data fit properly [36].

The results of CCD optimization are given in Table S6. The results revealed that the amount of methanol (mL) had the greatest significant effect on R%(FLU), the amount of acetonitrile (mL) had the greatest significant effect on R%(CTX), R%(DOX), R%(SMZ), R%(FLU) and R%(CTX), the percentage of formic acid (Formic acid%) had the greatest significant effect on R%(TMP) and R%(TYL). Finally, time of extraction (min) had the greatest significant effect on R%(TMP), R%(CTP), and R%(CST). The response surface of the desirability function is given in Figure S1.

Optimal conditions were 12 mL of methanol and 12 mL of 0.2% formic acid in acetonitrile used as extracting solvent, and the time of extraction was 40 min, which gave the best recovery of the studied compound achieving a desirability value of 0.69.

3.2. HPLC-UV Method

3.2.1. Screening Experiments by FFD

Table S7 provides the ANOVA of the studied factors and effects. In Figure 2, perturbation plots are shown. The results from the ANOVA and perturbation plots indicated that the percentage of acetonitrile (ACN%) had the highest significant effect on all investigated responses except on critically separated peaks resolution, TMP peak, and CIP peak (Rs (TMP-CIP)). Column temperature had the highest significant effect on the resolution of the TMP peak and CIP peak (Rs (TMP-CIP)), CIP peak and CTX peak (Rs (CIP-CTX)), CTX peak and DOX peak (Rs (CTX-DOX)), and DOX peak and TYL peak (Rs (DOX-TYL)), and finally flow rate had the highest significant effects on all studied responses. R^2_{adj} was >0.85 in all cases, proving the proper fit of experimental data [36].

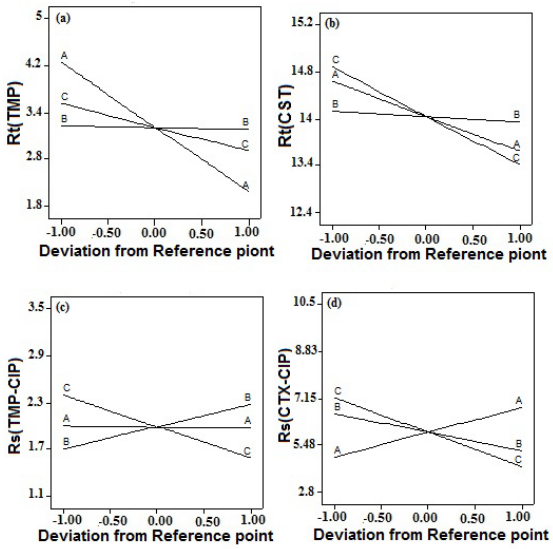

Figure 2. *Cont.*

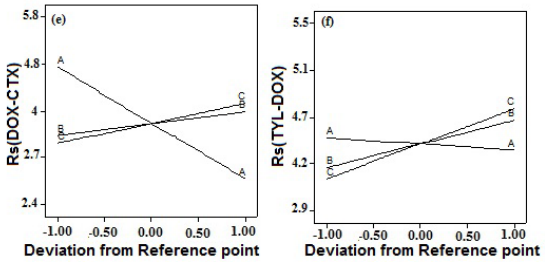

Figure 2. Perturbation plots for screening of factors affecting HPLC-UV method describing the effect of the studied factors on the responses (**a**) retention time of (TMP), (**b**) retention time of (CST), (**c**) resolution of (TMP-CIP) peaks, (**d**) resolution of (CIP-CTX) peaks, (**e**) resolution of (CTX-DOX) peaks, and (**f**) resolution of (DOX-TYL) peaks, where A, is acetonitrile percentage (ACN%), B, is column temperature and C, is flow rate.

3.2.2. Optimization by CCD

Results of the CCD are given in Table S8, which shows that percentage of acetonitrile and flow rate significantly affect all selected responses except on Rs (TMP-CIP), and column temperature (°C) significantly affects Rt (TMP) and Rs (FLU-CST). Response surfaces are given in Figures S2–S4. The response surface of the desirability function is given in Figure S5, and the desirability value was 0.65. The optimum final conditions are shown in Table S8. The chromatogram of Figure 3 represents separation using the final optimum conditions.

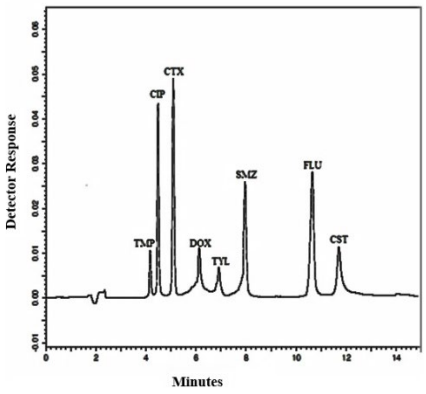

Figure 3. HPLC-UV chromatogram of 20 µL injection of synthetic mixture of 4 µg mL^{-1} of TMP, CIP, CTX, DOX, CST, 6 µg mL^{-1} of TYL, and 2 µg mL^{-1} of SMZ, and FLU.

The blank chicken sample (Figure 4a) chromatogram displayed no interference between the peaks of the studied compounds. Figure 4b gives a spiked blank chicken sample with antibiotics of interest after extraction. System suitability results are shown in Table S9, indicating the effect of the optimized extraction conditions where no interferences occur.

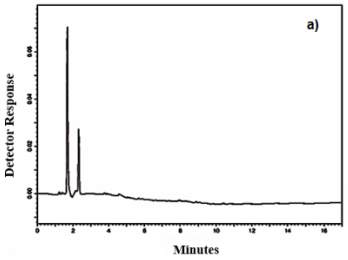

Figure 4. *Cont.*

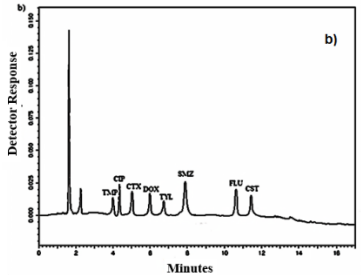

Figure 4. (**a**) Blank chicken sample HPLC-UV chromatogram and (**b**) chromatogram of a blank chicken sample spiked with 300 μg kg^{-1} of the studied compounds after extraction.

3.3. Methods Validation

The methods were validated according to the ICH guidelines [39] through different parameters as follows:

3.3.1. Linearity and Range

A matrix-matched calibration curve judged the linearity for the HPLC method on seven concentration levels ranging between 30–300 μg kg^{-1} for all the studied compounds except 40–300 μg kg^{-1} for TYL.

For the LC/MS/MS method, linearity ranged from 0.01 to 20 g kg^{-1} for all targeted analytes. The linearity parameters were shown in Tables S10 and S11, respectively

3.3.2. Detection and Quantitation Limit

Detection (LOD) and quantitation limits (LOQ) for the chromatographic methods were calculated using the S.D. of the response and the slope of the calibration curve. The values of LOD and LOQ are provided in Tables S10 and S11, indicating the high sensitivity of the HPLC and LC/MS/MS methods.

3.3.3. Accuracy and Precision

Repeatability and accuracy were tested by spiking the blank chicken muscle and liver extracts using three different concentration levels from each drug and repeating them three times during the day (intra-day precision). Intermediate precision and accuracy were examined by analysis of three concentrations from each compound three times on three days (inter-day precision). Accuracy is indicated by the relative error (RE%), while precision is indicated by the relative standard deviation (RSD%).

Tables S12 and S13 represent intra and inter-day precision and accuracy. The developed HPLC-UV and LC/MS/MS methods were precise as R.S.D.% values were <5% and accurate as RE% was found to be less than ±5%.

3.3.4. Selectivity

The selectivity of the proposed methods was assessed by preparing five mixtures of the studied compounds within the linearity range at different concentrations. The mixtures were analyzed according to previously discussed HPLC-UV and LC/MS/MS methods. Appropriate recoveries (Table S14) were generated, revealing the good selectivity of the methods for the simultaneously studied compounds.

3.3.5. Stability

TMP, CIP, CTX, DOX, TYL, SMZ, FLU, and CST standard solutions stability was tested by leaving the standard solutions at room temperature and in the refrigerator. The solutions have no chromatographic changes for a day at room temperature and for a month in the fridge at 4 °C.

3.4. Real Sample Analysis

Poultry meat and liver are consumed at a very high rate in Egypt due to their low price compared to red meat. Thus, it is very important to carefully determine the safety and clearance of chicken flesh from the residues of the antibiotics.

3.4.1. HPLC-UV Analysis

There is no reported method for determining these selected antibiotics, neither simultaneously in the same chosen matrix nor in Egypt.

This HPLC-UV chromatographic study is the first one used in the concurrent determination of TMP, CIP, CTX, DOX, TYL, SMZ, FLU, and CST concentrations in broiler chicken muscles and liver in Egypt and to compare the results with international limits with simple and fully optimized sample treatment, good sensitivity, and short elution time.

In total, 10 samples (each composed of fresh chicken breast and liver) of broiler chicken were collected from the retail markets and analyzed under the previously described conditions. The findings of HPLC-UV analysis are illustrated in Table 4, which indicates the presence of antibiotics in 5 samples out of 10. The range of detected compounds is from 3.5 to 11.8 μg kg^{-1}, which is lower than the permitted limits MRL (100 μg kg^{-1} for TMP, CIP, CTX, DOX, TYL, and SMZ, 150 μg kg^{-1} for CST and 200 for FLU).

Table 4. Analysis of TMP, CIP, CTX, DOX, TYL, SMZ, FLU, and CST residue in chicken samples by HPLC-UV method.

Broiler Samples		Drug Concentration (μg kg^{-1})							
		TMP	**CIP**	**CTX**	**DOX**	**TYL**	**SMZ**	**FLU**	**CST**
1	Meat	-	4.00	-	5.75	-	-	4.5	8.65
	Liver	-	7.65	-	-	-	-	5.90	7.95
2	Meat	-	-	-	-	-	-	-	-
	Liver	-	-	-	-	-	-	-	-
3	Meat	-	-	-	11.80	-	-	-	-
	Liver	5.51	-	-	10.50	6.65	10.20	-	7.60
4	Meat	-	-	5.00	-	7.20	-	4.25	-
	Liver	6.70	6.75	8.85	-	11.00	3.50	-	4.10
5	Meat	-	-	-	-	-	-	-	-
	Liver	-	-	-	-	-	-	-	-
6	Meat	5.50	-	4.50	-	-	8.15	-	11.00
	Liver	8.0	-	-	-	8.60	9.90	-	8.10
7	Meat	-	-	-	11.65	-	-	-	8.10
	Liver	7.52	-	-	9.55	-	-	-	9.90
8	Meat	-	-	-	-	-	-	-	-
	Liver	-	-	-	-	-	-	-	-
9	Meat	-	-	-	-	-	-	-	-
	Liver	-	-	-	-	-	-	-	-
10	Meat	-	-	-	-	-	-	-	-
	Liver			-	-	-	-	-	-

(-): Not detected.

3.4.2. LC/MS/MS Analysis

The reported LC/MS/MS did not determine the selected drugs simultaneously. TYL was determined [27,28,36] but with a difficult extraction procedure and low sensitivity.

Some antibiotics were detected in the liver chicken matrix only with poor % recovery and less sensitive LOD and LOQ [23]. CIP also was determined in chicken meat using very long extraction procedures and large organic solvent consumption [27].

Figure 5 and Table 5 show the amount of residue of the studied antibiotics in real samples, indicating the trace concentration of drugs in five samples within permitted limits.

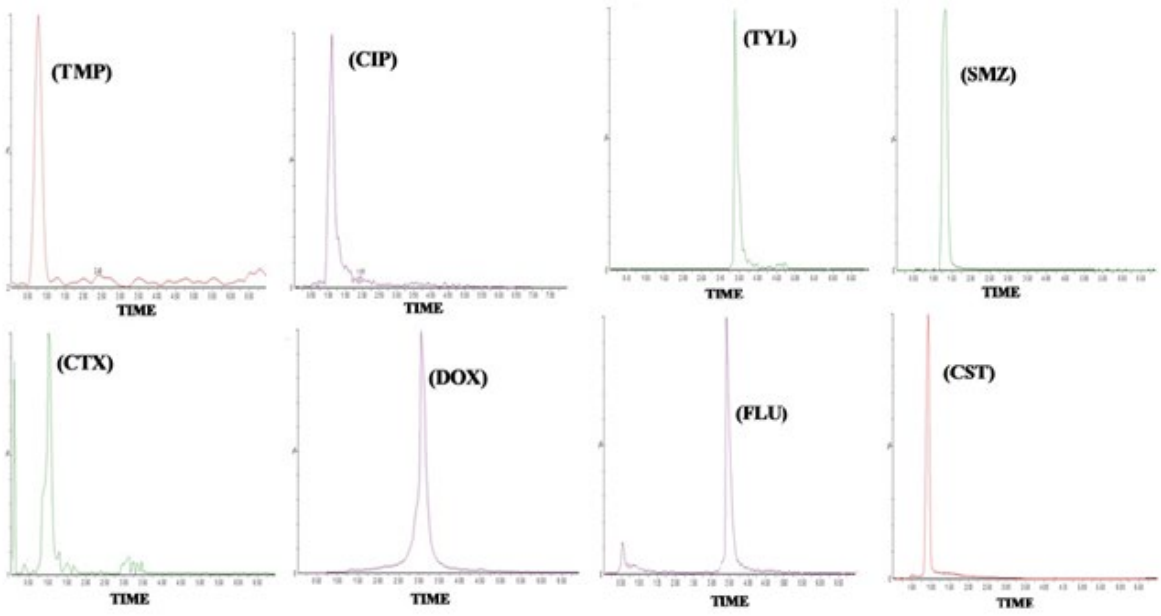

Figure 5. LC/MS/MS chromatogram of TMP, CIP, TYL, SMZ, CTX, DOX, FLU, and CST.

Table 5. LC/MS/MS analysis of TMP, CIP, CTX, DOX, TYL, SMZ, FLU, and CST residue in the chicken sample.

Broiler Samples		Drug Concentration (μg kg^{-1})							
		TMP	**CIP**	**CTX**	**DOX**	**TYL**	**SMZ**	**FLU**	**CST**
1	Muscle	2.00	4.40	-	5.10	-	-	5.10	9.20
	Liver	-	8.50	1.09	1.20	2.60	-	6.00	8.50
2	Muscle	-	-	-	-	-	-	-	-
	Liver	-	-	-	-	-	-	-	-
3	Muscle	4.00	-	-	11.20	-	-	ND	2.30
	Liver	6.00	-	-	9.45	5.50	9.60	1.10	8.00
4	Muscle	4.22	2.30	4.40	-	6.30	2.00	4.00	1.20
	Liver	7.40	6.00	8.30	2.30	10.10	4.50	-	4.80
5	Muscle	-	-	-	-	-	-	-	-
	Liver	-	-	-	-	-	-	-	-
6	Muscle	6.20	1.20	5.00	-	2.00	7.20	-	10.20
	Liver	8.70	1.90	2.30	-	9.20	10.30	-	7.50
7	Muscle	2.20	-	-	10.40	-	-	-	8.50
	Liver	8.10	-	2.40	8.89	-	-	-	10.20

Table 5. *Cont.*

Broiler Samples		Drug Concentration (μg kg^{-1})							
		TMP	**CIP**	**CTX**	**DOX**	**TYL**	**SMZ**	**FLU**	**CST**
8	Muscle	-	-	-	-	-	-	-	-
	Liver	-	-	-	-	-	-	-	-
9	Muscle	-	-	-	-	-	-	-	-
	Liver	-	-	-	-	-	-	-	-
10	Muscle	-	-	-	-	-	-	-	-
	Liver			-	-	-	-	-	-

(-): Not detected.

4. Conclusions

Effective, quick gradient HPLC-UV and LC-MS/MS techniques were developed to concurrently determine TMP, CIP, CTX, DOX, TYL, SMZ, FLU, and CST, which are extensively used antibiotics in Ismailia Governorate, Egypt poultry farms. The chromatographic conditions, sample pretreatment, and extraction procedures were optimized using experimental design. The proposed methods confirm the experimental design flexibility, time-saving, and minimal experiments. The methods reflected good validated results and were utilized successfully to analyze the studied antibiotics in chicken muscle and liver samples. The residues of targeted drug levels ranged from 3 to 11.8 μg kg^{-1} for HPLC-UV and from 1.10 to 11.20 for LC/MS/MS. These residues are within the acceptable limits of the European Union for each antibiotic, indicating the broiler chicken's suitability and safety for edible use. The proposed methods could be used as a fast-monitoring tool to ensure food safety in poultry industries, quality controlling, and accurately assessing the safety and suitability of chicken-derived food found in the Egyptian Market.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations9120427/s1, Figure S1: Response surface obtained for desirability function for different two factors interaction for optimization of extraction procedure; Figure S2: Response surfaces related to the interaction effects of the percentage of acetonitrile and column temperature: (a) retention time of (TMP) peak, (b) retention time of (CST) peak, (c) resolution (TMP-CIP) peaks, (d) resolution (CIP-CTX), (e) resolution (CTX-DOX) peaks, and (f) resolution (DOX-TYL) peaks; Figure S3: Response surfaces related to the interaction effects of the percentage of acetonitrile and mobile phase flow rate: (a) retention time of (TMP) peak, (b) retention time of (CST) peak, (c) resolution of (TMP-CIP) peaks, (d) resolution of (CIP-CTX) peaks, (e) resolution of peaks (CTX-DOX) peaks, and (f) resolution of (DOX-TYL) peaks. The column temperature was kept constant at 30 °C; Figure S4: Response surfaces related to the interaction effects of the column temperature and mobile phase flow rate: (a) retention time of (TMP) peak, (b) retention time (CST) peak, (c) resolution of (TMP-CIP) peaks, (d) resolution of (CIP-CTX) peaks, (e) resolution of (CTX-DOX) peaks and (f) resolution (DOX-TYL) peaks. The percentage of acetonitrile was kept constant at 10%; Figure S5: Response surface obtained for the desirability function for different two factors interaction for optimization of HPLC-UV method; Table S1: (a) Factors examined in the screening phase for factors affecting extraction procedure FFD, (b) Design matrix. (c) The corresponding experimental response values for FFD; Table S2: CCD for optimization of the factors affecting the extraction procedure. (a) Design matrix, (b) the corresponding experimental response values; Table S3: (a) Factors examined in the screening phase of HPLC-UV separation conditions (FFD), (b) design matrix, (c) the corresponding experimental response values for factorial design; Table S4: CCD for HPLC response optimization. (a) Design matrix and (b) the corresponding experimental response values; Table S5: ANOVA results for FFD for screening of the factors affecting the extraction procedure. A 5% level of significance was desired; Table S6: ANOVA results for CCD for optimization of the factors affecting the extraction procedure; Table S7: ANOVA results for the factorial design for screening factors affecting HPLC-UV method; Table S8: ANOVA results for CCD for optimization of HPLC-UV method; Table S9: The system suitability test results of the HPLC-UV for estimation of

TMP, CIP, CTX, DOX, TYL, SMZ, FLU and CST; Table S10: Characteristic parameters of the calibration equations for the HPLC-UV method for simultaneous determination of TMP, CIP, CTX, DOX, TYL, SMZ, FLU, and CST in the chicken sample; Table S11: Characteristic parameters of the calibration equations for LC-MS/MS method for concurrent estimation of TMP, CIP, CTX, DOX, TYL, SMZ, FLU, and CST in the chicken sample; Table S12: Intra-day and inter-day precision of the HPLC-UV method estimated by recovery of TMP, CIP, CTX, DOX, TYL, SMZ, FLU, and CST from spiked chicken tissues; Table S13: Intra-day and inter-day precision of the LC-MS/MS method estimated by recovery of TMP, CIP, CTX, DOX, TYL, SMZ, FLU, and CST from the chicken sample; Table S14: Determination of the TMP, CIP, CTX, DOX, TYL, SMZ, FLU, and CST in laboratory prepared mixtures using the proposed (a) HPLC -UV (b) LC/MS/MS methods.

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