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Bufferized solvent extraction and HPLC fluorometric detection method for sarafloxacin in pig and chicken muscles

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ABSTRACT: In this study, a method for the detection of sarafloxacin in pig and chicken muscles was developed using HPLC-FLD as a regulatory residue technique. Good extraction efficiency was achieved using a mixture of 1% orthophosphoric acid–0.2 M $MgCl_2$ in water and acetonitrile as an extraction solvent, and *n*-hexane partitioning and centrifugation for cleanup was used in the absence of dehydration. Specificity, linearity, detection and quantification limits, recovery, accuracy and precision were all validated, and all results were sufficient for the SARA regulatory residue method in pig and chicken muscles. The method developed and described herein was not only simple but also reliable, and was applied to market samples to determine their residue contents. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: sarafloxacin; HPLC-FLD; pig; chicken; muscle

Introduction

Veterinary drugs such as antibiotics, anthelminthics and pesticides are used extensively for the treatment and protection of animals against diseases in the global livestock industry. Sarafloxacin (SARA) is a fluoroquinolone (FQ) antibacterial agent that is used for the control of early mortality in growing turkeys and broiler chickens (Codex of Federal Regulations, 1997). SARA is the first FQ approved in the United States for use in food-producing animals, but was recently withdrawn from the market owing to concerns about microbial resistance (Chu *et al*., 2002; Rodríguez Cáceres *et al*., 2010). In the Republic of Korea, SARA is not currently registered, but its maximum residue limits (MRLs) were established as 10–80 µg/kg for poultry by the Korea Food and Drug Administration (KFDA). Despite the establishment of MRLs for veterinary drugs, foodstuffs of animal origin could be exposed to residual risk as the result of illegal use, as well as farmers' misuse, overuse and general lack of knowledge. Hence, a regulatory residue method is necessary for the monitoring of potentially unsafe residues resulting from unapproved use, so that government authorities can act more positively to ensure the safety of livestock products.

This study was sponsored by the KFDA, a governmental institution that is in charge of the creation of MRLs and regulatory residue methods for pesticides and veterinary drugs. The KFDA was tasked with developing a regulatory residue method for SARA in chicken and pig muscles. A variety of methods for the simultaneous determination of the FQs, including SARA, in pig or chicken muscles have been reported on the basis of liquid chromatography with fluorometric (Rose *et al*., 1998; Holtzapple *et al*., 1999; Posyniak *et al*., 1999; Yorke and Froc, 2000; Ramos *et al*., 2003; Schneider *et al*., 2007), ultraviolet (Barrón *et al*., 2002; Hermo *et al*., 2005; Christodoulou *et al*., 2007; Tsai *et al*., 2009), electrochemical (Rodríguez Cáceres *et al*., 2010) or mass spectrometric detection (Schneider and Donoghue, 2002; Bailac *et al*., 2006; Clemente *et al*., 2006; Kaufmann *et al*., 2008). In these previous studies, non-routine sample preparation techniques including immunoaffinity (Holtzapple *et al*., 1999), microwave (Hermo *et al*., 2005), dispersive extraction (Tsai *et al*., 2009) and capillary electrophoresis chromatographic separation (Barrón *et al*., 2002) have been applied, but their regular use is rather limited, owing to their non-routine nature. Although diverse solid-phase extraction (SPE) cartridges (strong cation exchange, Rose *et al*., 1998), C_{18} (Ramos *et al*., 2003; Christodoulou, *et al*., 2007) and styrene or polystyrene divinylbenzene copolymers (Posyniak *et al*., 1999; Rodríguez Cáceres *et al*., 2010) were used in the sample preparation, the SARA recovery values ranged from 51 to 69% (Rose *et al*., 1998) or from approximately 70 to 90% (Posyniak *et al*., 1999; Ramos *et al*., 2003; Rodríguez Cáceres *et al*., 2010), except in the study of Christodoulou *et al*. (2007) (96.7–102.8%). Other extraction methods without SPE cartridge

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Abbreviations used: CIP, ciprofloxacin; ENRO, enrofloxacin; FQ, fluoroquinolone; MRLs, maximum residue limits; SARA, sarafloxacin.

cleanup yielded recovery results of 71% (Yorke and Froc, 2000) or between 74.1 and 85.7% (Schneider *et al*., 2007). Mass spectrometry usually evidences high sensitivity but requires more instrument time; this requirement can occasionally exceed instrument availability in routine surveillance laboratories (Nochetto *et al*., 2009). The principal objective of this study was to devise an improved method without SPE cleanup which would be effective and sensitive for the determination of SARA in chicken and pig muscles, and which could be applied as a regulatory residue method.

Experimental

Chemicals and Reagents

Sarafloxacin hydrochloride (SARA, 93.5%) was purchased from Dr Ehrenstorfer GmnH (Augsburg, Germany). Enrofloxacin (ENRO, 99.9%) and ciprofloxacin (CIP, 99.9%) were obtained from Riedel-de Haën (Seelze, Germany). HPLC-grade acetonitrile (MeCN) and analytical-grade *n*-hexane, sodium hydroxide and anhydrous sodium sulfate were provided by Merck (Darmstadt, Germany). Analytical-grade orthophosphoric acid and magnesium chloride were purchased from Sigma-Aldrich (Missouri, USA).

Standard Solutions

Stock solutions of SARA and ENRO were prepared in methanol at a concentration of 100 μg/mL, whereas CIP was melted with a mixture of 0.025 M orthophosphoric acid (pH 3 adjusted using sodium hydroxide)–MeCN (8:2, v/v). Working solutions were prepared via serial dilutions of stock solution of SARA with 0.025 M orthophosphoric acid (pH 3)–MeCN (8:2, v/v) at eight different levels. The SARA concentrations of the working solutions were 0.25, 0.5, 1, 1.5, 2, 2.5, 5 and 10 μg/mL. ENRO and CIP were used in the pig and chicken muscles, respectively, as internal standards. Standard solutions were stored at −24°C in amber bottles.

Preparation of Calibration Curves in Matrices

Calibration curves of SARA were constructed by spiking 0.1 mL of each standard working solution (eight different concentrations) into blank pig or chicken muscle samples (5 g) in order to obtain final concentrations of 5, 10, 20, 30, 40, 50, 100 and 200 μg/kg. The internal standards, ENRO and CIP, were also added to the pig and chicken muscles, respectively. These calibration samples were obtained via the sample preparation procedures described below, in triplicate, and then analyzed via HPLC-FLD.

High-performance Liquid Chromatography

The HPLC system utilized in this study consisted of a Shimadzu (Japan) model SCL-10A *VP* system controller, model LC-6 AD binary pump, a CTO-20A column oven, a spectrofluorometric detector (RF-10 A) and the *CLASS VP* computing program. An Aqua C_{18} 200 Å (250 × 4.6 mm i.d., 5 μm particle size, Phenomenex, California, USA) column protected by an AQ C_{18} analytical guard cartridge (4 × 3 mm, Phenomenex) was used to separate the FQs from the sample extracts flowed in mobile phase with 0.025 M orthophosphoric acid (pH 3)–MeCN (78:22). The 20 μL samples were carried by the mobile phase into the column, which was kept in an oven at 40°C at a flow rate of 1 mL/min. The FQs were detected with a fluorescence detector at an excitation wavelength of 275 nm and an emission wavelength of 460 nm. All solvents were filtered (0.45 μm) and degassed prior to use.

Sample preparation

Pig and chicken muscles were purchased from a supermarket located in Gwangju (Republic of Korea) and confirmed as a blank via a preliminary trial. The samples were then chopped into pieces and separately homogenized with a blender. The homogenized samples were stored in a freezer at −24°C until analyzed. An approximately 5 g representative sample of homogenized muscle was placed into a 50 mL high-density polyethylene centrifuge tube along with ENRO (for pig) or CIP (for chicken), and 20 mL of a mixture of 1% orthophosphoric acid–0.2 M $MgCl_2$ in water and MeCN (1:4, v/v) was added. The sample was shaken in a vortex mixer for 1 min, followed by 30 min in a mechanical shaker (EYELA, Japan), and centrifuged for 10 min at 6000 rpm at 4°C. The supernatant was then transferred to a 50 mL conical polypropylene tube and mixed vigorously with 8 mL of *n*-hexane for 3 min to remove fatty materials. The aliquot (8 mL) of the bottom layer was then transferred into a 100 mL evaporating flask and evaporated to 1 mL with a rotary vacuum evaporator at a bath temperature not exceeding 50°C. The concentrated extract was then transferred to a graduated 5 mL polypropylene tube and the evaporating flask was washed with 0.025 M orthophosphoric acid (pH 3)–MeCN (8:2, v/v). The wash extract was combined with the extract in the graduated polypropylene tube, and the total volume was adjusted quantitatively to 2 mL. The aliquot (1 mL) of the extract was then transferred into another 2 mL microcentrifuge tube and centrifuged for 10 min at 9000 rpm. The aqueous layer was filtered (0.2 μm, PTFE), and then injected into the HPLC-FLD.

Results and Discussion

Optimization of Sample Extraction

Sample extraction procedures were optimized from the preliminary experiments, which were conducted via sequential trials consisting of the spiking of SARA into chicken muscle at 100 μg/kg, vigorous shaking with 20 mL of extraction solvent (acid–MeCN mixture, 1:4, v/v) for 30 min, 10 min of centrifugation at 6000 rpm at 4°C, mechanical shaking of the supernatant with *n*-hexane, dehydration of the aqueous aliquot with 7 g of anhydrous sodium sulfate, evaporation to 1 mL with a rotary vacuum evaporator, reconstitution with 0.025 M orthophosphoric acid (pH 3)–MeCN (8:2, v/v), centrifugation of the aliquot for 10 min at 9000 rpm at 4°C and HPLC-FLD detection. Since the extraction with buffer resulted in a turbid extract and required further cleanup (Yorke and Froc, 2000; Schneider *et al*., 2007), different acid buffers or their various concentrations mixed with MeCN were evaluated as extraction solvents. Unfortunately, relatively low recoveries were obtained from 1% orthophosphoric acid, 2% acetic acid, 2% formic acid and 5% acetic acid: 75.5, 74.0, 70.9, and 76.5%, respectively. In the results of the tests with various concentrations from 0.1 to 5.0% of orthophosphoric acid, 1% orthophosphoric acid was selected as an acid buffer (Fig. 1). However, because the recovery yields were low as before, 0.2 M $MgCl_2$ was mixed with 1% orthophosphoric acid, in accordance with the methods described by Schneider *et al*. (2007), so that the presence of magnesium ions improved the recovery of the FQs in chicken muscles. A mixture of 1% orthophosphoric acid–0.2 M $MgCl_2$ in water and MeCN (1:4, v/v) was applied as an extraction solvent, but its recovery was slightly increased, to 78.4%. As another attempt to increase recovery, dehydration with anhydrous sodium sulfate was skipped; this induced dramatically enhanced recovery, to 90.8%. This result was similar to that of the study of Roybal *et al*. (1997), in which it was reported that an insufficient introduction of anhydrous sodium sulfate exerted a negative effect on SARA recovery in milk. Anhydrous sodium sulfate absorbs not only moisture, but also SARA in the extracts, because SARA is soluble in acidic aqueous solvents.

In the reconstitution step, the ratio of MeCN (0.025 M orthophosphoric acid (pH 3)–MeCN, 5:5, v/v) and the pH of 0.025 M orthophosphoric acid [0.025 M orthophosphoric acid (pH 1.5)–MeCN, 8:2, v/v] were assessed; however, the chromatography

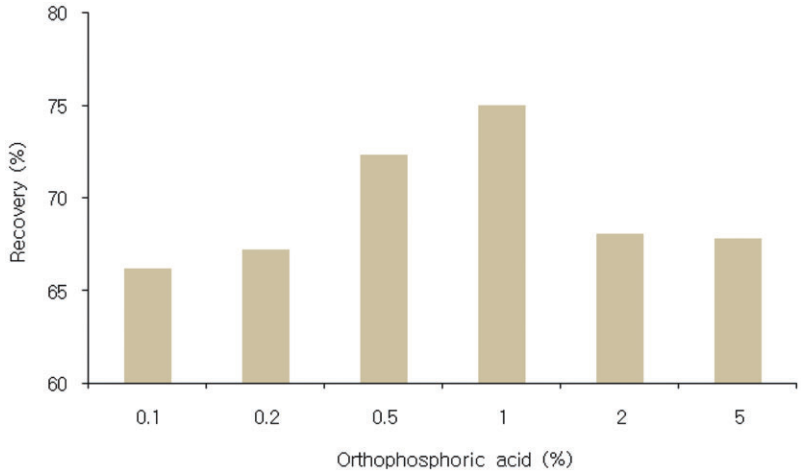

Figure 1. Effect of various percentages of orthophosphoric acid in the mixture with MeCN (1:4, v/v) on the extraction recovery of SARA in chicken muscles.

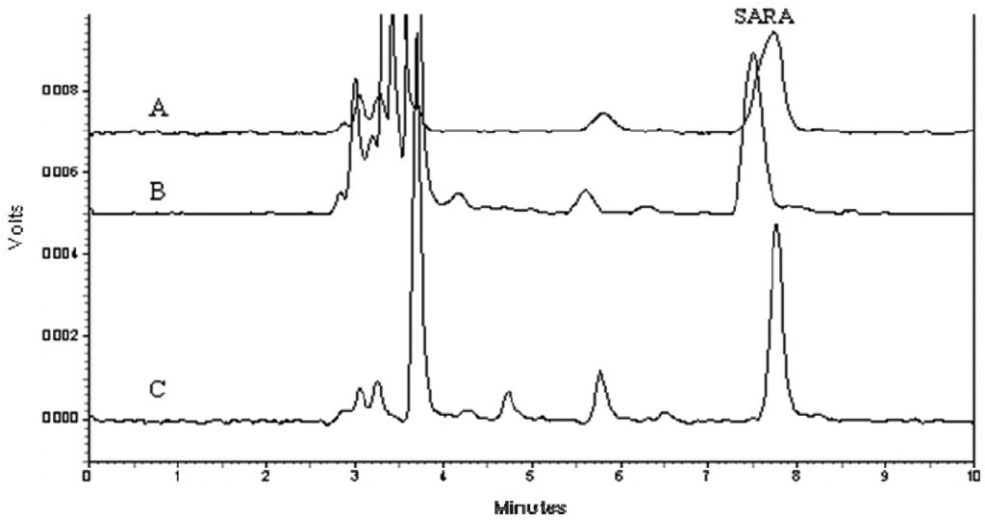

Figure 2. Effect of various reconstitution solvents on peak symmetry of SARA in chicken muscle extract: (A) 0.025 M orthophosphoric acid (pH 3)–MeCN (5:5, v/v); (B) 0.025 M orthophosphoric acid (pH 1.5)–MeCN (8:2, v/v); (C) 0.025 M orthophosphoric acid (pH 3)–MeCN (8:2, v/v).

results were unacceptable, due to an asymmetric peak of SARA. However, a mixture of 0.025 M orthophosphoric acid (pH 3) and MeCN (8:2, v/v) generated a symmetric and sharp peak (Fig. 2), and it was possible to precipitate the remaining sample co-extracts after the previous shaking with *n*-hexane to defat the extract. These defatting and/or deproteinizing steps without SPE rendered the extraction procedure more rapid, simple, and selective, and also enhanced the specificity of the chromatographic method.

Chromatographic Analysis

To detect SARA, ENRO and CIP on a HPLC-FLD, 0.025 M orthophosphoric acid (pH 3)–MeCN (78:22, v/v) as a mobile phase and an AQUA™ C_{18} polar-endcapped column were utilized in this study, in a slightly modified version of the previous study of Marazuela and Moreno-Bondi (2004). In that study, it was determined that AQUA™ endcapped with hydrophilic (polar) functional groups enabled the separation of polar compounds in highly aqueous mobile phases, thereby permitting a fast column equilibration, as compared with the conventional C_{18} column method. Good sensitivity and separation for the three FQs were noted under the above-described analytical conditions.

Ideally, an internal standard should exhibit physicochemical properties similar to those of the analyte (Garcia *et al*., 2000). For this reason, ENRO and CIP (both of which have chemical structures similar to SARA) were utilized as internal standards. The two FQs were efficiently extracted and separated from the muscle samples. ENRO was selected for the pig muscles and CIP for the chicken muscles, owing to interferences in each sample.

Method Validation

Specificity. The specificity of the method developed herein was evaluated by testing representative blank muscle samples ($n = 3$) to confirm the absence of potential interfering compounds at the

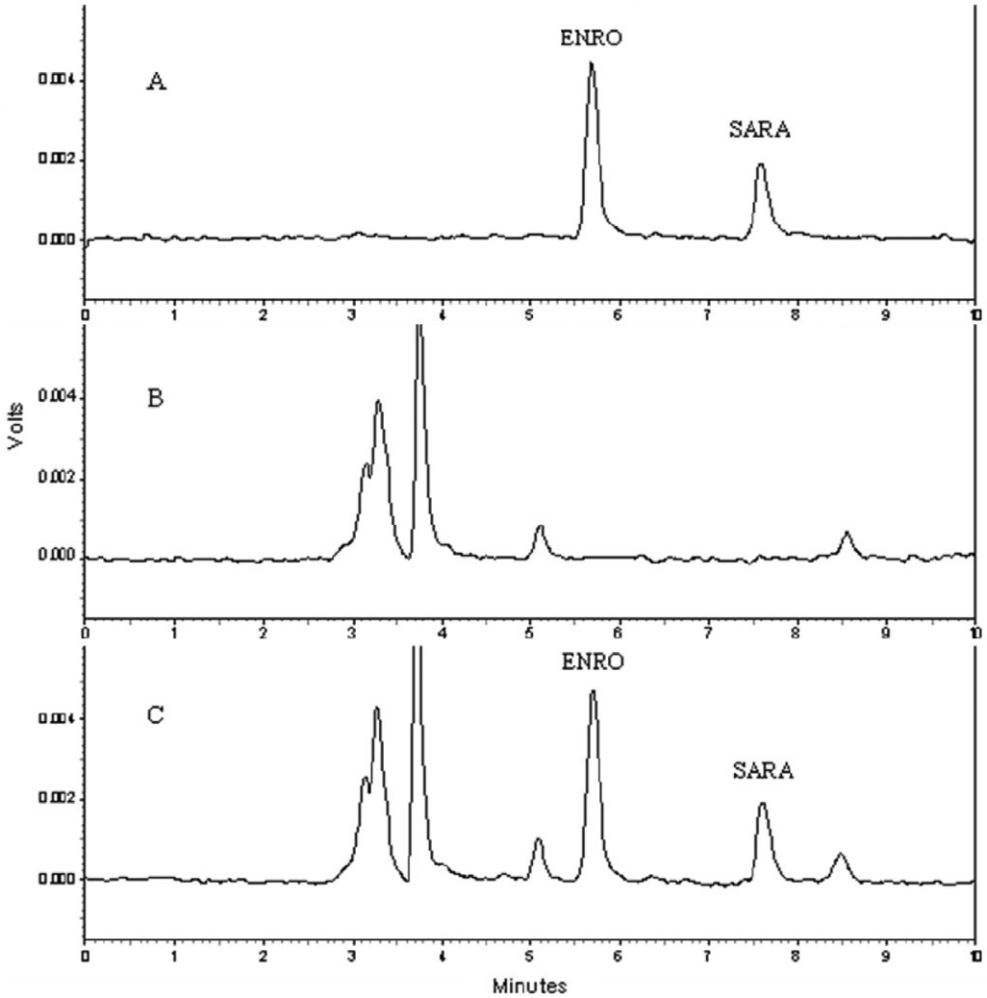

Figure 3. Typical chromatograms of standard ENRO (IS) and SARA at 0.01 and 0.02 μg/mL (A); blank pig muscle (B); and spiked pig muscle at 0.01 and 0.02 mg/kg (C).

elution times of the analytes in the same chromatographic run as is shown in the blank chromatograms. Figures 3 and 4 show representative chromatograms of blank muscles and spiked muscles with mixtures of SARA and ENRO or CIP. Retention times were 4.79, 5.66 and 7.58 for CIP, ENRO, and SARA, respectively. According to our results, no interfering endogenous peaks appeared at these retention times, and the retention times of the tested FQs at the spiked samples were completely consistent with that of the standard solution. All of the FQs were eluted as separate symmetric peaks. Additionally, the run time was adequate within the capacity of numerous samples assessed on a daily basis.

Linearity. Matrix-matched calibration curves were constructed by determining the peak area ratio of SARA to the internal standards at eight specific points. The linearity of the method was well demonstrated over a concentration range of 5–200 μg/kg, with an r^2 value of ≥0.999.

Limit of detection and quantification. Limits of detection (LOD) and limits of quantification (LOQ) were determined on the basis of the standard deviation of the response and the slope. The LOD and LOQ are calculated by multiplying σ/S by 3.3 and 10 respectively, where S is the slope of the calibration curve and σ is the standard deviation of blank samples' responses (ICH, 1996). The LOD and LOQ in the pig muscles were 1.1 and 3.4 μg/kg, and 1.7 and 5.1 μg/kg in chicken muscle, respectively; these values were lower than the MRL of SARA, according to measurements conducted in the Republic of Korea. Therefore, sensitivity was thoroughly evaluated in order to investigate SARA residues in the pig and chicken muscles.

Recovery. Recovery was evaluated with pig and chicken muscles spiked at 5, 10, and 20 μg/kg, with six replicates per concentration. The recovery values for the pig and chicken muscles are listed in Table 1. The average recoveries (means ± SD) ranged from 94.4 ± 4.6 to 97.8 ± 5.1% in the pig muscles and from 95.8 ± 4.8 to 106.0 ± 5.3% in the chicken muscles, respectively. The ranges of RSDs were from 4.8 to 6.2% in the pig muscles and from 4.2 to 5.0% in the chicken muscles.

Accuracy and precision. Precision was determined by assessing reproducibility and repeatability. Repeatability was evaluated via intra-laboratory analyses (intra- and inter-day) in this study, because reproducibility is difficult to achieve in the context of inter-laboratory analyses. Intra-laboratory analyses were conducted via intra-day analysis, using spiked pig and

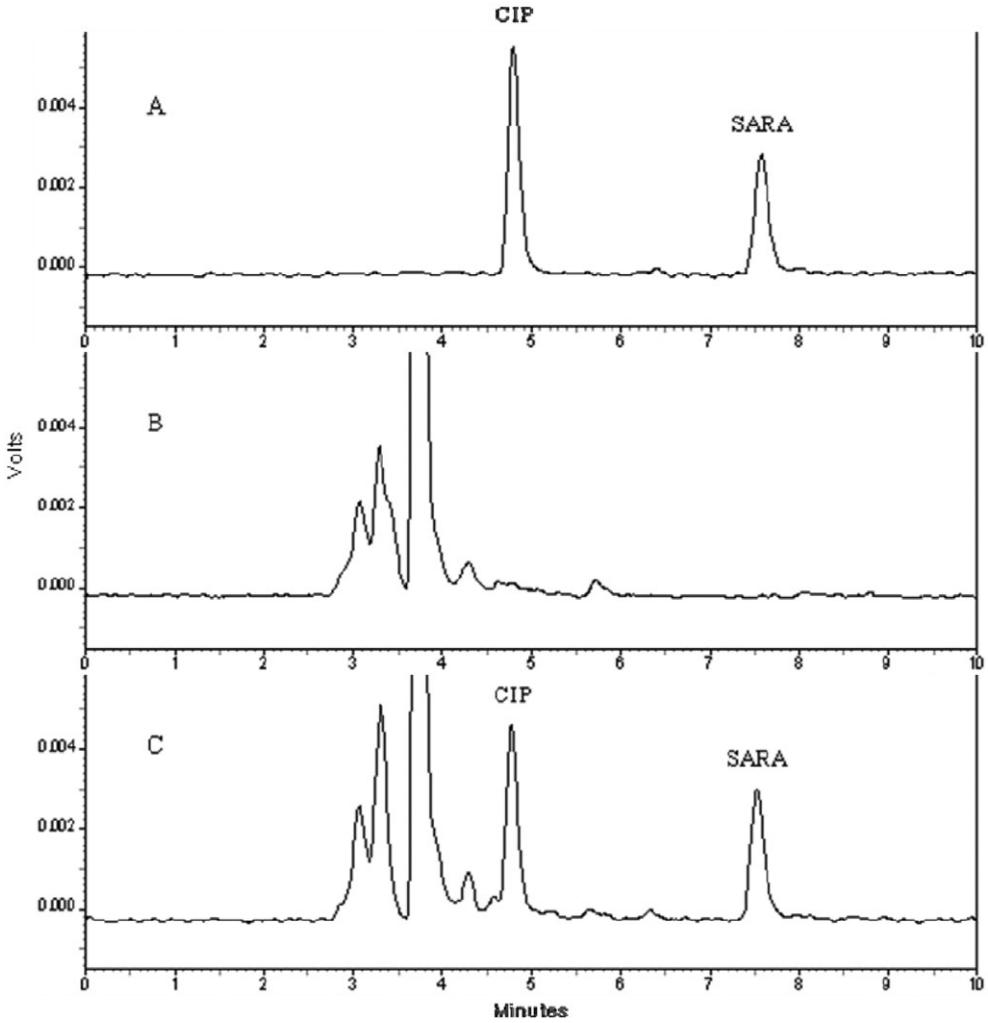

Figure 4. Typical chromatograms of standard CIP (IS) and SARA at 0.01 and 0.03 μg/mL (A); blank chicken muscle (B); and spiked chicken muscle at 0.01 and 0.03 mg/kg (C).

Table 1. Recovery, limit of detection, and limit of quantification of SARA in pig and chicken muscles using HPLC-FLD

Muscle	Concentration (μg/kg)		Recovery (M[a] ± SD, %)	RSD (%)	LOD (μg/kg)	LOQ (μg/kg)	MRL[b] (μg/kg)
Pig	5		95.1 ± 5.9	6.2	1.1	3.4	Not established
	10		94.4 ± 4.6	4.8			
	20		97.8 ± 5.1	5.2			
Chicken	5	(MRL/2)	95.8 ± 4.8	5.0	1.7	5.1	10
	10	(MRL × 1)	106.0 ± 5.3	5.0			
	20	(MRL × 2)	97.1 ± 4.0	4.2			

[a] Mean of six replicates.
[b] KFDA in the Republic of Korea.

chicken muscles at three different concentrations (20, 50, and 100 μg/kg) for one day; inter-day analyses were conducted at the same concentrations for three separate days. These concentrations were higher than the recovery concentrations, but were included in the linear range; all experiments were conducted in six replicates per specified concentration. The accuracy was described as the percentage of recovery by comparing the measured concentration to its true value, and the precision was expressed as a relative standard deviation (RSD). Table 2 shows the results of the accuracy and precision of the developed method. The averages of intra- and inter-day accuracy ranged from 87.3 to 99.6% and from 92.8 to 96.4%, whereas the intra- and inter-day precisions were 3.4–9.4 and 4.2–8.9%, respectively. The accuracy and precision values were excellent and were consistent with the range provided in the Codex guidelines (Codex, 1993).

Table 2. Intra-day and inter-day accuracy and precision of SARA in pig and chicken muscles

Muscle	Concentration (μg/kg)	Mean calculated concentration (μg/kg)	Accuracy (%)	RSD (%)
Pig	*Intra-day precision and accuracy (n = 6)*			
	20	19.1	95.6	4.9
	50	49.8	99.6	3.4
	100	90.3	90.3	5.2
	Inter-day precision and accuracy (n = 18)			
	20	19.2	96.0	5.1
	50	48.2	96.4	6.4
	100	92.8	92.8	4.2
Chicken	*Intra-day precision and accuracy (n = 6)*			
	20	17.8	88.9	9.4
	50	43.6	87.3	4.6
	100	89.0	89.0	5.4
	Inter-day precision and accuracy (n = 18)			
	20	18.9	94.3	8.9
	50	47.1	94.2	7.9
	100	95.1	95.1	7.3

Applicability to Market Samples

The method developed herein was applied to evaluate SARA in market-obtained pig and chicken muscles that were purchased from the six major cities in the Republic of Korea. All determination processes were conducted in triplicate, but no SARA residue was detected in any of the samples.

Conclusion

A method for the determination of SARA residues using HPLC-FLD was developed in this study, using pig and chicken muscles. This method, interestingly, achieved excellent validation results using MeCN bufferized with 1% orthophosphoric acid and 0.2 M $MgCl_2$, and *n*-hexane cleanup. *n*-Hexane cleanup obviated the need for other preparative columns or SPE cartridges without sacrificing high recovery levels, and resulted in a quick, effective and economic cleanup procedure. Since the developed method not only fulfills all the criteria required for validation according to the Codex guidelines but is also simple and cost-effective, this method may prove to be a useful regulatory and routine analytical method for SARA in pig and chicken muscles.

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