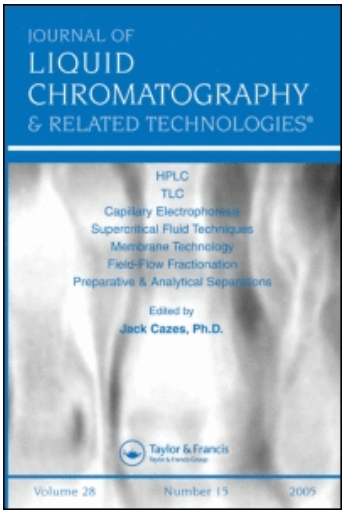

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DETERMINATION OF SULFAMETHAZINE AND ITS MAJOR METABOLITES IN EGG ALBUMIN AND EGG YOLK BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A quantitative liquid chromatographic method for the determination of sulfamethazine (SMZ), N4-acetyl sulfamethazine (N4-acetyl-SMZ), and desamino sulfamethazine (desamino-SMZ) in egg albumin and egg yolk is described. Egg albumin or yolk was homogenized in acetonitrile and centrifuged. The supernatant was evaporated to dryness and residue reconstituted in the mobile phase. Albumin extract was directly analyzed by high performance liquid chromatography (HPLC). Hexane was added to the yolk sample, vortex mixed, and centrifuged to separate the layers. The top hexane layer was removed and a small amount of salt was added to break the emulsions. The lower aqueous layer was analyzed by HPLC. The HPLC system included a reversed phase column, a gradient mobile phase of 5-15% acetonitrile and 0.01M phosphate buffer, and a UV detector set at 268 nm. The recovery of SMZ and N4-acetyl-SMZ, both fortified at 1 ppm levels, from egg albumin was 101 and 88% and from egg yolk was 79 and 91%, respectively. The recovery of desamino-SMZ at 2 ppm fortification level from egg albumin and egg yolk was 84 and 63%, respectively.

The method was applied to detect the presence of SMZ and its potential metabolites in eggs collected after dosing hens with SMZ. Parent drug, SMZ, was the major compound transferred to both egg albumin and yolk. Small concentration of N4-acetyl-SMZ metabolite were also detected in some eggs; however, no desamino-SMZ or other metabolites were detected.

INTRODUCTION

Sulfamethazine or 4-amino-N-(4,6-dimethyl-2-pyrimidine) benzene sulfonamide is approved for use in boiler chicks for the prevention and treatment of various bacterial infections.[1] It is, however, not approved for use in laying chickens. The unauthorized or extralabel use of sulfamethazine in hens can leave violative residues in eggs and other edible products. The residue may include a parent drug, SMZ, as well as its metabolites. The major metabolites of sulfamethazine that have been reported and identified[2,3,4] include N4-acetyl-SMZ, desamino-SMZ, N4-D-glucosyl-SMZ, and N4-(1-deoxy-D-glucuronyl)-SMZ. Their structures are shown in Figure 1.

A number of methods for the determination of sulfonamides, including sulfamethazine, in eggs and other edible animal products have been reviewed.[5,6,7] A review of the literature shows that several other procedures use ODS-cartridge,[8] amino cartridge,[9] and supercritical fluid extraction[10] as cleanup for eggs and other tissues, prior to HPLC analysis. Recently HPLC with chemiluminescence detection was also utilized for the determination of sulfamethazine in chicken serum and eggs.[11] Supercritical fluid extraction of sulfamethazine and its metabolites from meat tissue was also recently reported.[12] These methods, however, are deficient in the determination of sulfonamide metabolites in eggs; particularly, the simultaneous determination of sulfamethazine and its major metabolites in egg albumin and yolk requires a renewed effort. The present paper describes a procedure for the simultaneous determination of sulfamethazine and its major metabolites in chicken egg albumin and egg yolk, using simple liquid-liquid extraction and clean-up followed by HPLC analysis.

EXPERIMENTAL

Equipment

The LC system consisted of Hewlett-Packard (HP) Model 1050 system (Palo Alto, CA, USA) fitted with a quaternary pump, an auto sampler, a column heater, a solvent bottle holder with a helium purge, an HP computer, VECTRA 486/66XM with HP ChemStation software (DOS series), HP laser jet 4 plus printer, and an HP variable wavelength detector set at 268 nm.

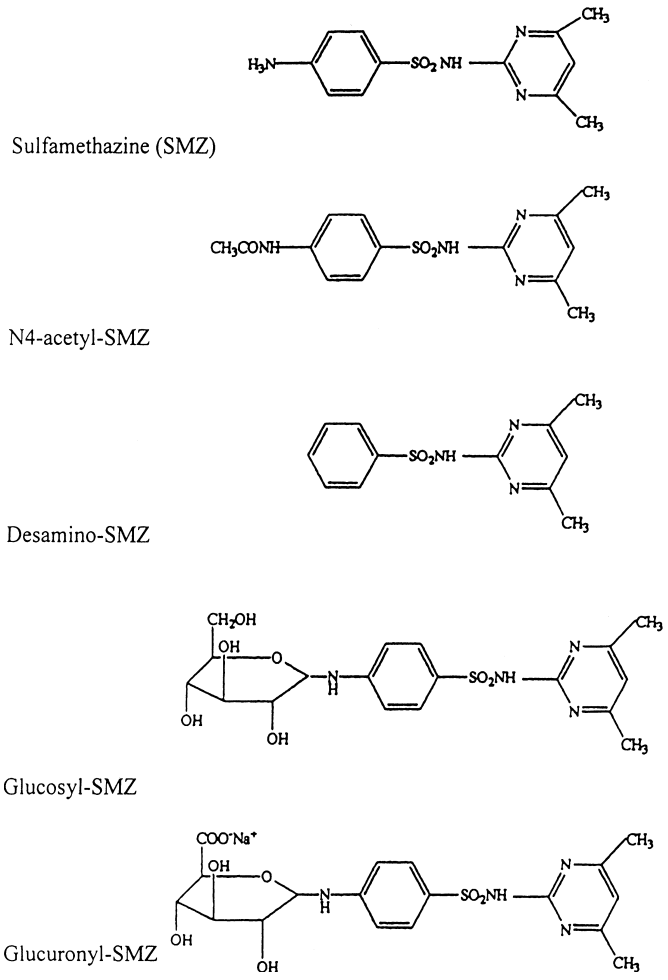


Figure 1. Structures of sulfamethazine and its major metabolites.

The analytical (150 mm x 4.6 mm) and guard (7.5 X 4.6 mm) columns employed were LiChrosorb RP select B and LiChrosorb RP-8 (Alltech Associates, Deerfield, IL, USA), having a packing of 5 μm particle size. Both the analytical and guard columns were in a column heater set at 35°C. All centrifugations were carried out at 4100 RCF using swing-out rotor (M4) in a Jouan CR 422 bench top refrigerated centrifuge (Jouan Inc., Winchester, VA, USA) set at 4°C for 15 minutes. Polypropylene tubes, Falcon round bottom (14 mL), and conical bottom (15 mL) were used (Corning Glass Works, Corning, NY, USA). All transfers were made with Eppendorf digital pipettes.

Chemicals and Reagents

Glass distilled organic solvents (Burdick & Jackson Laboratories, Muskegon, MI, USA) and water from Milli-Q plus ultrapure water system (Millipore Corporation, Bedford, MA, USA) were used. All chemicals were of HPLC grade, except where noted. Sulfamethazine was obtained from Sigma Chemical Company (St. Louis, MO, USA). Metabolites, N4-acetyl-SMZ, Desamino-SMZ, Glucosyl-SMZ, and Glucuronyl-SMZ were a gift, provided by Dr. Gaylord Paulson of ARS/USDA, Metabolism and Radiation Research Laboratories, Fargo, ND, USA.

The HPLC Mobile Phase

A stock solution of potassium dihydrogen phosphate, 0.5 M was prepared by weighing 68 g and transferring with water to a 1- liter glass volumetric flask; additional water was added to the mark, mixed and refrigerated until used. A mobile phase buffer, 0.01 M KH_2PO_4, was prepared by transferring 20 mL of 0.5 M stock buffer into a 1-liter flask; additional water was added to reach the mark and mixed. No pH adjustment was made. The gradient mobile phase was used and consisted of an initial combination of 5% acetonitrile in 0.01 M phosphate buffer for 5 minutes, gradient to 15% acetonitrile in 15 minutes, held for 20 minutes, and gradient back to 5% acetonitrile in 35 minutes. A post equilibration time of 5 minutes was used before next injection.

Preparation of Standard Solutions

The primary stock standard solutions of SMZ, N4-acetyl-SMZ, and desamino-SMZ were prepared by weighing 20.1, 4.5, and 5.2 mg and transferring with methanol into 100, 50, and 50 mL volumetric flasks, respectively. Additional methanol was added to bring to the mark to give 201, 90, and 104 μg/mL concentration levels, respectively. The secondary stock solutions of about 10 μg/mL were prepared by transferring 0.5, 1.1, and 0.85 mL of the primary stock of SMZ, N4-acetyl-SMZ, and desamino-SMZ, respectively. Other dilutions were made as appropriate. All solutions were refrigerated until used.

Egg Albumin and Egg Yolk Samples

Control eggs were obtained from white Leghorn hens. The albumin and yolk were separated, transferred into 50 mL polypropylene tubes, and stored at -20°C, if not used immediately. Egg albumin and egg yolk samples were fortified with secondary stock solutions by weighing 1 g sample into 14 mL Falcon round bottom polypropylene tubes and adding 100 μL of 10.05 ppm

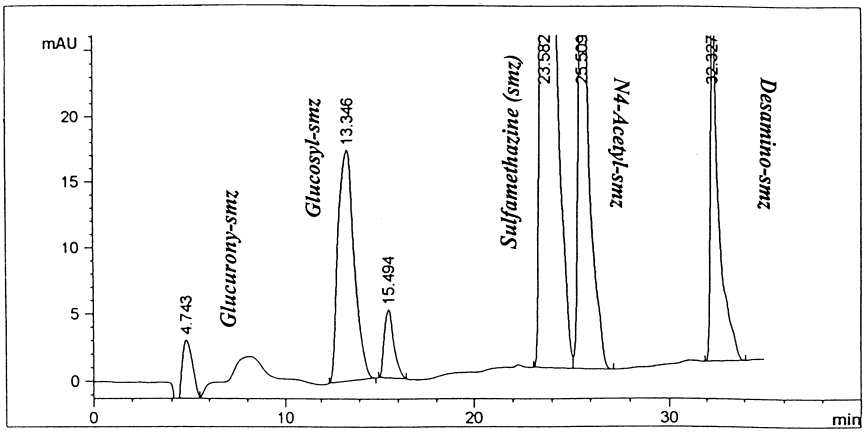


Figure 2. HPLC chromatogram of a mixture of SMZ and its metabolites: glucuronyl-SMZ, glucosyl-SMZ, N4-acetyl-SMZ, and desamino-SMZ.

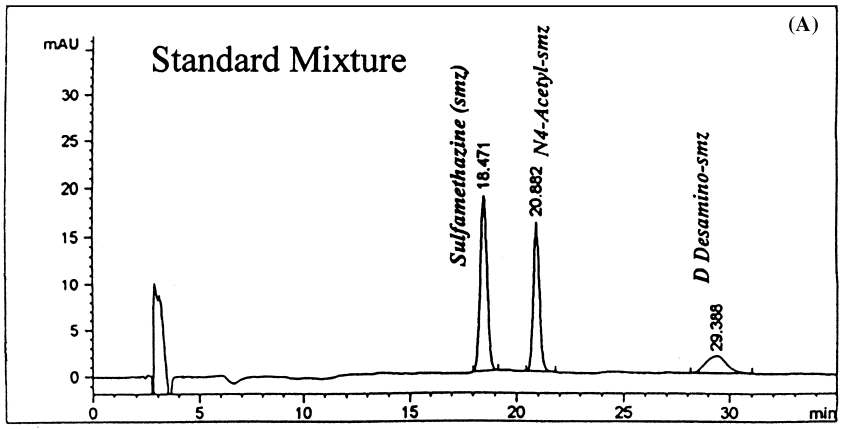
SMZ, 100 µL of 9.9 ppm N4-acetyl-SMZ, and 200 µL of 8.84 ppm desamino-SMZ and then vortex mixed to give fortification levels of about 1, 1, 2 ppm, respectively. For incurred eggs, a white Leghorn hen was fed a single dose of 75 mg SMZ/kg body weight by capsule.

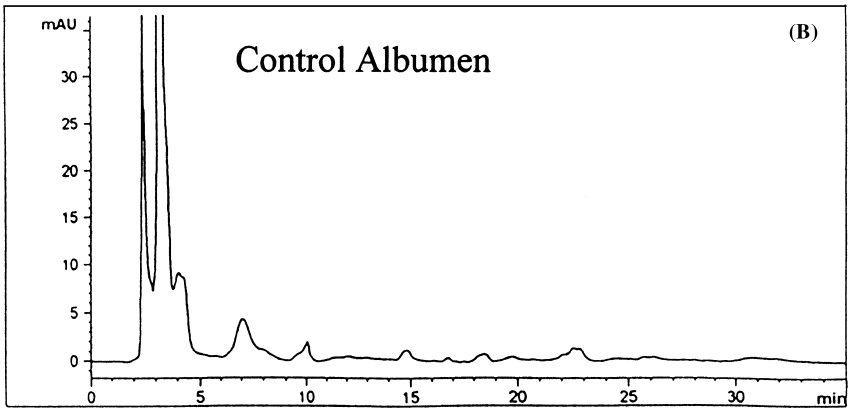
The eggs were collected over a period of 9 days after drug administration. The albumin and yolk were separated from each egg and stored at -20°C, if not analyzed immediately.

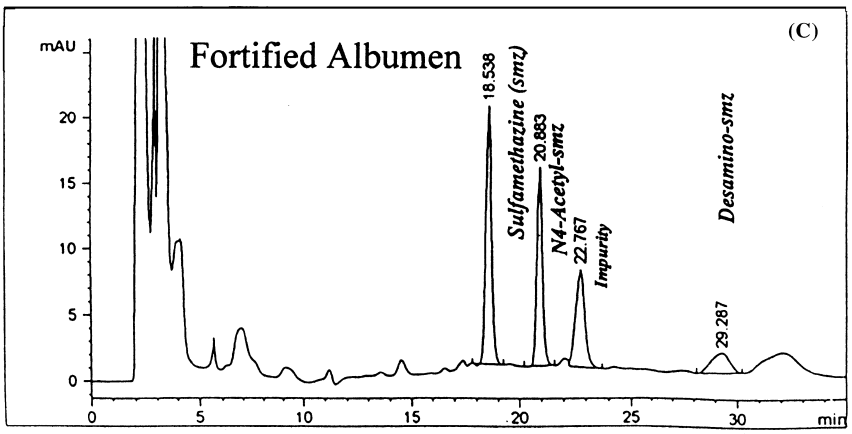
Sample Extraction and Clean-up

A 1-g portion of egg albumin or egg yolk sample (control, fortified, or incurred) was weighed in to 14 mL falcon polypropylene centrifuge tubes. Four mL of acetonitrile was added to each sample and homogenized with Omni TH homogenizer (Omni International, Inc., Gainesville, VA, USA), using disposable probes, for one minute. The samples were centrifuged at 4100 RCF for 15 minutes at 4°C and the supernatant was transferred into 15 mL conical polypropylene centrifuge tubes. The pellet was loosened with the wooden side of the Q-tip and rehomogenized with two mL of acetonitrile and centrifuged.

The supernatant was added to the previous one, and the combined extracts were evaporated to dryness with an N-evaporator. The residue was dissolved in one mL mobile phase. The albumin extract was analyzed directly by HPLC. Four mL of hexane were added to the yolk extract, vortex mixed, and centrifuged to separate the layers. The upper hexane layer was removed with







Pasteur pipette and discarded. About 250 mg of NaCl was added to the lower aqueous layer to break the emulsion. The sample was vortex mixed and centrifuged. An aliquot of the lower layer was transferred with Pasteur pipette to a sample vial or vial insert and analyzed by HPLC.

HPLC Analysis

Three calibration standard mixtures of SMZ (0.5, 0.75, and 1 μg/mL), N4-acetyl-SMZ (0.5, 0.75, and 1.0 ppm) and desamino-SMZ (1.0, 1.5, and 2.0 ppm) were prepared from their 10 ppm secondary stock standards by transferring the appropriate aliquot and bringing the total volume to 1 mL with the mobile phase (15% acetonitrile /buffer). A new set of calibration standards was run before each set of fortified or incurred samples were assayed. Injections of 75 μL standards or samples were made into the LC column.

Figure 2 shows a chromatogram of a standard mixture of SMZ and four of its metabolites, glucuronyl-SMZ, glucosyl-SMZ, N4-acetyl-SMZ, and desamino-SMZ, eluted in the decreasing order of their polarity. Both glucuronyl and glucosyl conjugates were unstable in methanol solution; the former degraded back to 86% SMZ and the latter to 6% SMZ and 17% to an unknown compound.

RESULTS AND DISCUSSIONS

Recovery of SMZ and Its Metabolites from Egg Albumin and Egg Yolk

Parts a, b, and c of figure 3 show typical chromatograms of 75 ul injection of standard mixture (SMZ, N4-acetyl-SMZ, and desamino-SMZ), control egg albumin extract and fortified albumin extract, respectively. Parts a and b of figure 4 show typical chromatograms of control egg yolk and fortified egg yolk extracts. There is an impurity peak, a doublet, in the egg yolk samples at about 17 minutes, but does not interfere with the elution of SMZ peak. Sulfamethazine, N4-acetyl-SMZ, and desamino-SMZ peaks are also well separated from each other and from endogenous interfering compounds in egg albumin and yolk. External standard curves in the range of 37-69 ng for SMZ and N4-SMZ, and 66-121 ng for desamino-SMZ were constructed from the HPLC analysis and found to be linear. The r^2 in the range of 0.928- 0.999 for SMZ, N4-acetyl-SMZ, and desamino-SMZ were obtained. These curves were used to quantitate fortified egg albumin and egg yolk samples.

Figure 3 (left). HPLC chromatgrams of (A) standard mixture of SMZ (68.5 ng), N4-acetyl-SMZ (67.5 ng), and desamino-SMZ (120.5), (B) control egg albumin, and (C) egg albumin fortified with SMZ, N4-acetyl-SMZ, and desamino-SMZ.

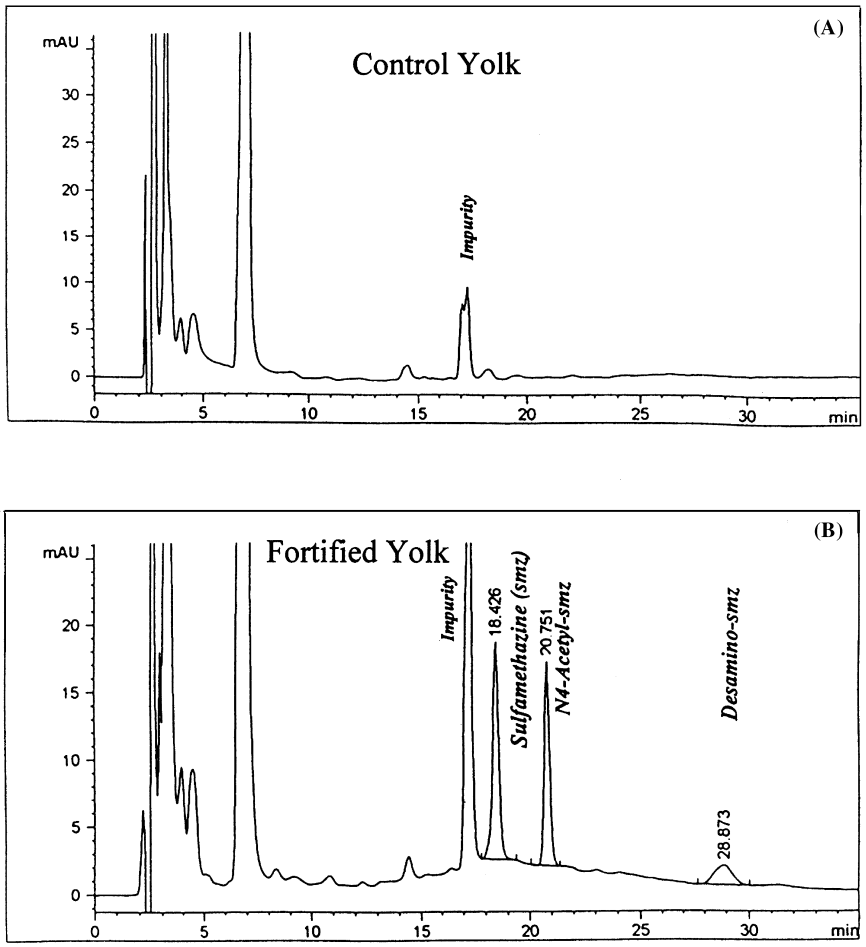


Figure 4. HPLC chromatograms of (A) Control egg yolk; (B) egg yolk fortified with SMZ, N4-acetyl-SMZ, and desamino-SMZ.

The recoveries of SMZ and two of its metabolites from egg albumin are listed in table 1. The average recoveries of SMZ and N4-acetyl-SMZ, both fortified at 1 μg/g, were 101 and 88%, respectively with coefficient of variation (CV) of 5% for both. The average recovery for desamino-SMZ, fortified at 2 μg/g, was 84% with CV of 9%. Both the recoveries and CV for the three compounds from egg albumin are well within the acceptable FDA guidelines for accuracy and precision.[13] Table 1 also lists recoveries from egg yolk. The average recoveries of SMZ and N4-acetyl-SMZ, both fortified at 1 μg/mL, were

Table 1

Recovery of SMZ, N4-Acetyl-SMZ, and Desamino-SMZ from Fortified Egg Albumin and Egg Yolk

Sample	SMZ From Albumin	SMZ From Yolk	N4-Acetyl-SMZ From Albumin	N4-Acetyl SMZ From Yolk	Desamino-SMZ From Albumin	Desamino-SMZ From Yolk
1	93.8	84.8	81.4	93.6	74.7	70.6
2	99.2	82.9	87.5	94.1	78.8	62.4
3	99.2	71.5	87.5	89.0	78.8	62.4
4	104.9	75.2	84.5	92.2	92.8	61.6
5	106	81.5	91.7	87.8	92.8	56.4
6	102.8	81.0	92.2	91.1	87.6	61.6
Average	101.2	79.2	87.5	91.3	84.3	63.2
Std. Dev.	4.7	5.1	4.1	2.5	7.8	4.9
% CV	4.6	6.4	4.7	2.8	9.3	7.7

79 and 91% respectively, with corresponding CV's of 6 and 3%. The average recovery of desamino-SMZ, fortified at 2 μg/g, was 63% with CV of 8%. With the exception of recovery for desamino-SMZ from egg yolk, these results also are within the Food and Drug Administration (FDA) guidelines for acceptable accuracy and precision. Lower recovery of desamino-SMZ suggests its binding with the highly lipid components in the yolk.

Analysis of Incurred Egg Albumin and Egg Yolk Samples

The incurred egg albumin and yolk samples were assayed to validate the overall procedure for the detection of SMZ, N4-acetyl-SMZ, and desamino-SMZ. Parts a and b of figure 5 show typical liquid chromatgrams of incurred egg albumin and egg yolk extracts. Table 2 lists SMZ and N4-acetyl-SMZ concentrations detected from incurred eggs collected over 216 hours after dosing SMZ to a hen. No desamino-SMZ or other metabolites were detected in any of the incurred egg albumin or egg yolk samples. Only 48 hour egg albumin contained a significant amount of N4-acetyl-SMZ, however, less significant amounts were detected in the yolks of eggs collected at 48 - 120 hours (table 2).

Forty eight hour post dose egg albumin sample contained the highest concentration of SMZ, 18.7 μg/g; SMZ concentration declined to 0.322 μg/g at 96 hours. The 48-hour sample also contained 0.632 μg/g of N4-acetyl-SMZ, which was undetectable in other albumin samples. The highest concentration of SMZ, 5.3 μg/g, also accumulated in the 48 hour post dose egg yolk sample and declined to 0.106 μg/g in 216 hour sample.

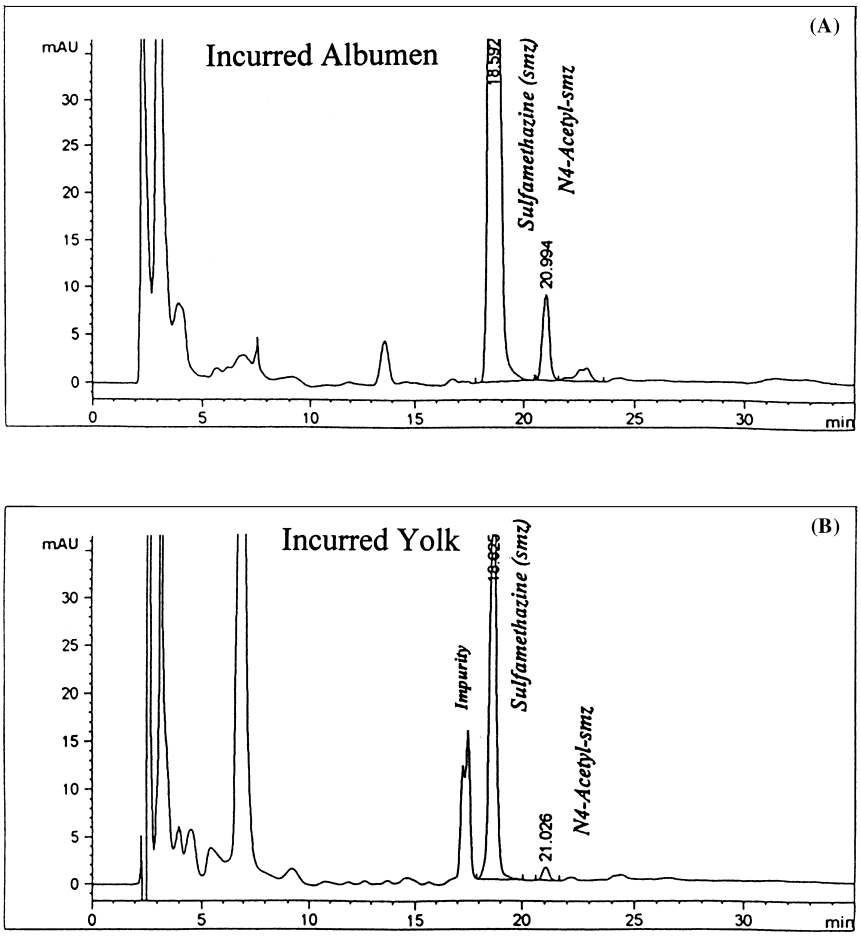


Figure 5. HPLC chromatograms of (A) incurred egg albumin (B) incurred egg yolk.

Small concentrations of N4-acetyl-SMZ were also present in 72 hour (0.198 μg/g), 84 hour (0.181 μg/g), 96 hour (0.0389 μg/g), and 120 hour (0.045 μg/mL) egg yolk samples. These values are outside the standard curve; however, they were detectable. These result suggest that the parent drug, SMZ, is the marker residue in both egg albumin and egg yolk, since relatively low concentrations (<4%) of acetyl metabolite was detected in both albumin and yolk samples. Additionally, SMZ appears to be depleted somewhat faster from egg albumin (96 hours) than from egg yolk (216 hours). These different patterns of drug depletion between egg albumin or yolk are probably associated

Table 2

Concentration of SMZ and N4-Acetyl-SMZ in Incurred Egg Albumin and Yolk

Egg Collected-hours after dosing	SMZ found in Albumin (ppb)	SMZ found in Yolk (ppb)	N4-Acetyl-SMZ found in Albumin (ppb)	N4-Acetyl-SMZ found in Yolk (ppb)
48	18744	5313	632	ND*
72	1427	2301	ND*	198
84	390	1567	ND*	181
96	323	729	ND	39
120	ND*	663	ND*	45
216	ND*	106	ND*	ND*

* ND = not detected.

with their dissimilar formation. Because albumin is synthesized and excreted within days, whereas egg yolk develops over a period of weeks,[14] SMZ may be stored in developing yolk for a longer period of time. This has also been demonstrated for other drugs.[15] However, it must be noted that these data reflect the results from a single hen and may not reflect the rate of depletion of SMZ from egg albumin and yolk of the larger population of hens. In this study we were interested only in methods development and its application to incurred egg albumin and egg yolk samples. Additional work on the transfer of radiolabeled parent drug and its metabolite in egg albumin and yolk from several hens, administered with C14-SMZ, will be reported in the near future.

CONCLUSIONS

A gradient HPLC procedure for the separation of SMZ, and its major metabolites, N4-acetyl-SMZ, desamino-SMZ, glucosyl-SMZ, and glucuronyl-SMZ has been developed. A simple extraction and clean-up method to accurately and precisely recover SMZ and its major metabolites from fortified and incurred egg albumin and yolk was also developed. Parent SMZ was the major compound transferred into egg albumin and yolk of the eggs collected from the dosed hens. Only N4-acetyl-SMZ metabolite, in lower concentrations, was detected in some egg albumin and egg yolk samples.

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