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Gas chromatographic determination of incurred chloramphenicol residues in eggs following optimal extraction

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Abstract

The existing method for analysis of chloramphenicol (CAP) residues in animal tissues was optimized to extract spiked-labelled and unlabelled chloramphenicol from freeze-dried egg albumen and yolks. Although recoveries of CAP were essentially the same for both albumen and yolk, the standard deviation was narrow for albumen compared to yolk. There was no statistical difference in recoveries of spiked CAP from whole liquid or freeze-dried (powdered) eggs. The method was validated with eggs of chickens given CAP in drinking water. No loss of CAP occurred during freeze-drying. The method has the potential of being used routinely for monitoring CAP in eggs and egg products.

1. Introduction

Although chloramphenicol (CAP), a broad-spectrum antibiotic, has no reported adverse effect on animal health, it has been shown to be toxic to humans. CAP causes dose-related suppression of bone marrow which results in many related diseases such as leucopenia [1,2]. Two potentially fatal adverse effects of CAP are aplastic anemia and gray syndrome [3]. There is also evidence that prolonged topical use of CAP in the human eye causes aplastic anemia [3–5]. In one documented case, a daily dose of 2 mg over 40 days caused death [4]. These and many other cases are of major concern to consumers and regulatory officials since the effect(s) of consuming small amounts of CAP via food is unknown.

CAP is a very effective veterinary drug and is used to control diseases such as salmonellosis [6], mastitis and other cattle diseases [7–9]. Further, CAP is also used to treat diseases of animal pathogen, which have become resistant to other commonly used antibiotics [10,11]. In poultry, CAP has been recommended for the treatment of *Salmonella* infections [12] and prevention of secondary infections associated with chronic respiratory diseases and "blue-comb" [13].

In view of the high toxic effects of CAP to humans, it has been subject to strict control in many countries around the world. In some countries, it is still used under very controlled conditions. The USA [14] and Canada [15] have banned the use of CAP for food-producing

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animals. The World Health Organization recommended that CAP not be used for treatment of food animals [16].

Various chromatographic techniques have been developed to detect and quantitate CAP residues in food products including eggs. The methods employ gas chromatography (GC), liquid chromatography (LC), thin-layer chromatography (TLC), and involve many clean-up steps prior to analysis. Methods developed up to 1984 have been reviewed by Allen [17]. In recent years, modification of previous methods and employment of immunoassay techniques have been reported [18,19]. Capillary GC [20] and GC–mass spectrometry (MS) [21], LC and LC–MS [23–25] have been used as confirmatory tools.

Most of the reported methods for analysis of CAP residues are for spiked samples, and not validated fully for actual (incurred) samples [26,27]. As our work was near completion, Samouris et al. [25] reported a high-performance liquid chromatography technique for detection of incurred CAP residues in eggs of chickens given chloramphenicol in feed at a concentration of 800 mg/kg for 1 day. Our work in poultry involves 9 CAP drinking water concentrations over 10 consecutive days.

This paper details an optimum condition for the extraction of chloramphenicol residues from incurred eggs. The validity of the extraction technique is supported by the use of ^{14}C-labelled chloramphenicol. Data are provided which show that freeze-drying of liquid eggs caused no loss of CAP residues.

2. Experimental

2.1. Reagents and solutions

The solvents ethyl acetate, methanol, hexane and cyclohexane (distilled-in-glass grade or equivalent) were purchased from Caledon Labs. (Georgetown, Canada).

Chloramphenicol (unlabelled) was purchased from Aldrich (Milwaukee, WI, USA) and [^{14}C]Chloramphenicol (dichloroacetyl 1,2-^{14}C, >98% radio purity) was obtained from NEN Products, a Division of DuPont.

The derivatizing reagent Sylon HTP (No. 3-3403) was purchased from Supelco Canada (Toronto, Canada) and consisted of hexamethyldisilazane (HMDS)–chlorotrimethylsilane(TMCS)–pyridine (3:1:9).

Sodium chloride solution (4%) was prepared by dissolving the appropriate amount of NaCl (ACS grade) in filtered/distilled deionized water.

The scintillation cocktail was ICN Biomedicals' (Irvine, CA, USA) CytoScint, a ready-to-use, environmentally safe preparation.

2.2. Preparation and storage of standard solutions

Stock solutions were prepared by dissolving a known amount of CAP in methanol in an acid-rinsed volumetric flask and stored at 0°C. Similarly, intermediate and analytical standard solutions of CAP were prepared by appropriate dilution of stock and intermediate solutions, respectively. The concentration of analytical CAP solutions ranged from 12.5 to 250 pg/ml.

Supplier's [^{14}C]CAP solution (in ethanol) was diluted with methanol to give a working solution of 222 dpm/μl (0.650 ng CAP/μl). A calculated volume of the working solution was added to the sample to study the solvent extraction efficiency and the effect of freeze-drying on the residue level in eggs.

2.3. Animal treatment

Adult White Leghorn hens (1.6 to 2.1 kg body mass) had free access to drinking water containing CAP at 0, 1, 5, 10, 25, 50, 100, 150 and 200 mg/ml for 10 consecutive days followed by a withdrawal period of 10 days. Eggs were collected daily and stored at 4°C until analyzed after freeze-drying (powdered).

2.4. Processing of eggs

Eggs from individual treatment group were divided into two groups of 5–6 eggs each. One group was set aside for development and valida-

tion of a Robotic sample preparation technique (this will be reported separately). The second set of eggs were broken, separated into albumen and yolk, combined on group and date basis. The separated and pooled albumen and yolk were mixed thoroughly with a spatula and freeze dried before analysis. Freeze dryer, a Virtis Model 50 SRC (Fisher), was operated at 450 mTorr vacuum (1 Torr = 133.322 Pa), shelf temperature 20°C, condenser temperature −55°C and drying time 7 days.

2.5. Extraction of CAP from powdered eggs

The first step in the existing method ([28–30]; Fig. 1A) is the extraction of samples with 4% NaCl and ethyl acetate. However, in our hands, this step caused heavy emulsion formation resulting in considerable difficulties with separation and delays in analysis time. Steps shown in Fig. 1B did not form emulsion. Incurred freeze-dried samples were extracted by steps shown in Fig. 1B, which is a slight modification of the existing method for tissues.

2.6. Preparation of powdered spiked samples

A 1-g amount of freeze-dried control albumen or yolk, after mixing with a spatula, was weighed into a 50-ml Falcon centrifuge tube. Samples were spiked with the appropriate volume of unlabelled or labelled [^{14}C]CAP solution to produce samples containing 2, 10, 25 and 50 ng/g (ppb). Samples were mixed gently for even distribution and allowed to stand for 60 min prior to extraction. Spiked samples were extracted by the method detailed in Fig. 1B. Extracts containing [^{14}C]CAP were analyzed first by LSC followed by GC. Similarly, the whole liquid egg (albumen and yolk mixed together) was spiked with a known amount of unlabelled CAP and recovery studies were conducted. Recovery study was repeated with powdered whole eggs as well.

Liquid albumen and yolk of control eggs were spiked with a known amount of [^{14}C]CAP and freeze-dried as detailed above to observe any loss of ^{14}C during this process.

2.7. Derivatization

Derivatization conditions were established using [^{14}C]CAP. [^{14}C]CAP (equivalent to 2, 10, 50 ppb) was transferred to a centrifuge tube, Sylon HTP (200 μl) added to the tube, tube heated at 65°C for 20 min, volume reduced under gentle stream of nitrogen, residue redissolved in hexane and radioactivity measured before analysis by GC. Data showed that very little radioactivity was lost during the entire derivatization process. This procedure was used to derivatize extracts from spiked and actual samples.

2.8. Gas chromatograph and accessories

A Model 8500 Perkin-Elmer gas chromatograph equipped with (i) a DB-1701 column (30 m × 0.32 mm I.D., 25 μm film thickness), (ii) splitless injector with silanized glass insert, (iii) an electron-capture detector, (iv) a data handling system (PE Nelson 900 Series interface), and an autosampler (PE AS-100) was used. GC operating conditions were: injector at 280°C; detector at 330°C; oven temperature programmed from 100°C (0.5 min equilibrium time) to 250°C (at 20°C/min) and finally to 280°C (at 5°C/min) and hold for 15 min; carrier gas P-5 (methane–argon, 5:95) flow-rate 3 ml/min plus 57 ml/min make-up gas for a total of 60 ml/min.

2.9. Liquid scintillation counter

A Beckman scintillation System Model LS 3801 was used for radioactivity measurement using an external standard and correction for quenching.

2.10. Statistical analysis

All data were statistically analyzed using Microsoft Excel software (Version 5.0, Analysis ToolPak; Microsoft, Redmond, WA, USA). The relative standard deviation (R.S.D.) was calculated using the formula: standard deviation × 100/mean.

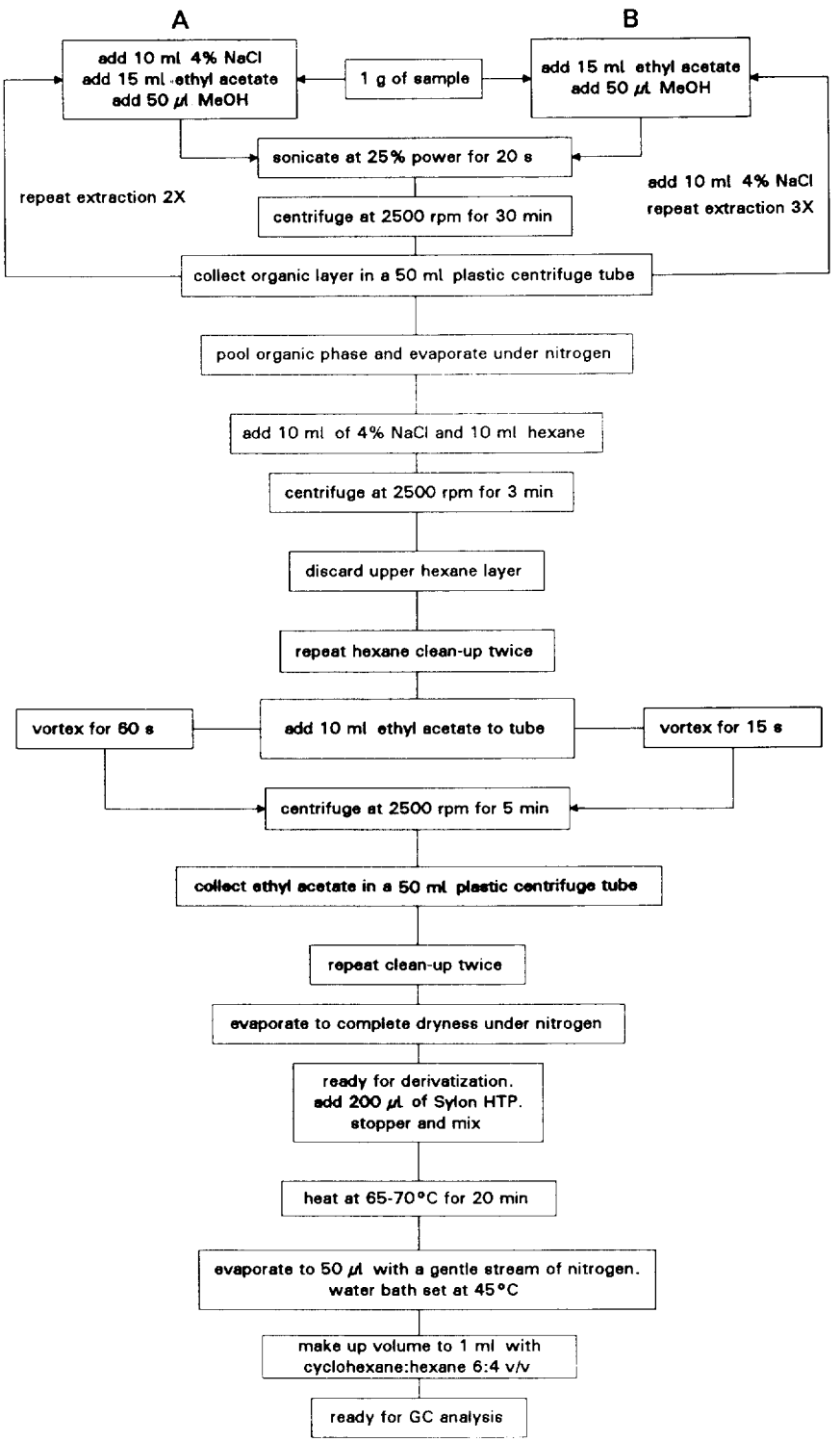

Fig. 1. Techniques for the extraction, isolation and derivatization of chloramphenicol from eggs. (A) Existing method; (B) modified method.

2.11. Miscellaneous supplies

Further supplies used were: (a) glassware: volumetric flasks, pipettes, disposable pipettes, 15-ml centrifuge tubes; (b) polypropylene centrifuge tubes; (c) autosampler vials/caps/crimper; (d) microsyringes: Hamilton various sizes; (e) solvent evaporator N-Evap Model 111 (Organomation Associates); (f) mixer: Vortex, Braun adjustable; (g) centrifuge: IEC clinical; (h) balances: (Mettler AE 160, BB 2400).

3. Results and discussion

CAP is a broad-spectrum antibiotic which is highly effective in treating many diseases of farm animals. However, CAP showed adverse reactions in humans and was banned (USA and Canada) from use on milk, meat and egg-producing animals. Since CAP is less expensive and possesses high efficacy for treatment of animal diseases that are not resistant to other registered antibiotics, there exists a situation where CAP can be misused and/or abused. In recent years, considerable efforts have been made world-wide to develop efficient and cost-effective methods to monitor CAP residues in milk, meat and eggs to protect humans from its severe adverse effect.

Currently, CAP in eggs and egg products are analyzed following the method described for determination of CAP in milk and tissues [28–30]. As part of the ongoing effort of monitoring drug residues in eggs, we reviewed all available techniques. One of the major difficulties of the existing solvent extraction technique is the formation of heavy emulsion. This step is difficult, tedious and time consuming.

In our hands, the use of the existing extraction technique as detailed in Fig. 1A gave poor and inconsistent recoveries of spiked CAP from powdered eggs (freeze-dried). It is assumed that this was, in part, due to the formation of heavy emulsion during extraction. To overcome the emulsion formation, we investigated various alternatives and observed that emulsion formation can be eliminated, if the first extraction is done with ethyl acetate without 4% NaCl, followed by further extraction with 4% NaCl. When our investigation was already completed, Kijak [21] and Munns et al. [31] also reported extraction of CAP from milk and shrimp first with ethyl acetate without the use of 4% NaCl. In addition, the extraction for 15 s is sufficient rather than 1 min. The modified method is shown in Fig. 1B. Recoveries of spiked [^{14}C]CAP from powdered albumen and yolk by two methods are recorded in Table 1. The recoveries of spiked CAP from

Table 1
Recoveries of [^{14}C]CAP from spiked powdered eggs by methods described in Fig. 1A and B

Sample	Spiked at ng/g (ppb)	Recoveries (%)[a]			
		Fig. 1A		Fig. 1B	
		LSC	GC	LSC	GC
Albumen	50	52.7 ± 9.1	104.5 ± 15.3	86.5 ± 5.2	70 ± 4.1
	25	45.4 ± 3.3	39.9 ± 9.3	79.7 ± 3.0	73.7 ± 10.9
	10	72.0 ± 11.1	51.4 ± 10.1	85.3 ± 6.0	73.1 ± 7.0
	2	54.3 ± 28.7	70.0 ± 30.3	101.5 ± 12.7	94.7 ± 11.3
Yolk	50	86.1 ± 2.4	71.5 ± 17.6	79.1 ± 11.0	70.3 ± 5.8
	25	58.2 ± 3.5	33.7 ± 4.5	70.6 ± 6.1	68.2 ± 20.1
	10	87.3 ± 1.5	86.0 ± 14.7	87.6 ± 3.0	72.3 ± 3.5
	2	82.6 ± 3.8	142.4 ± 10.1	74.3 ± 2.7	76.5 ± 13.1

[a] Mean ± R.S.D., $n = 3$–5.

Table 2
Recoveries of spiked CAP from the whole egg (liquid and powdered)

Sample	Spiked at ng/g	Recoveries (%)[a]
Liquid	25	79.0 ± 3.5
	10	84.0 ± 6.7
	5	77.5 ± 7.7
	2	106.0 ± 22.0
Powder	25	73.0 ± 7.0
	10	79.0 ± 6.0
	5	92.0 ± 3.0
	2	87.0 ± 7.0

[a] Mean ± R.S.D., $n = 6$.

Table 3
Effect of freeze-drying of eggs on [^{14}C]CAP residues

Sample	Spiked at ng/g[a]	Recoveries (%)[b]
Albumen	50	103.5 ± 5.1
	25	100.6 ± 7.6
	10	99.6 ± 7.2
	5	82.9 ± 12.1
	2	109.1 ± 5.9
Yolk	50	109.1 ± 6.6
	25	108.9 ± 5.9
	10	90.5 ± 9.0
	5	94.5 ± 9.8
	2	96.6 ± 2.7

[a] Liquid albumen and yolk were spiked with a known amount of [^{14}C]CAP, mixed with a glass pipette and freeze-dried in a commercial freeze-drier for 4 days.
[b] Mean ± R.S.D., $n = 5$ or 6.

powdered egg samples shown in Table 2 have greater reproducibility (R.S.D.) for the modified method (Fig. 1A). The data from two procedures, when compared used an F-test-two-samples for variance (Microsoft Excel, 1993), show a significant difference $p = 0.084$ and 0.003 for albumen and yolk, respectively. The calibration curves, representing mean peak areas versus concentration, show excellent linearity for concentration from 2 to 200 pg. The correlation coefficient (r) was 0.9995, and the limit of detection was established at 0.5 ppb (twice the noise level).

Recoveries of CAP (Table 2) from liquid and powdered whole eggs show no significant difference in the two types of egg handling. However, the advantage of powdered eggs is that they can be stored for a long period and be used for quality control.

No information is available on the stability of CAP during the freeze-drying process. Our data in Table 3 showed that there was no loss of CAP during the freeze-drying process.

Chromatograms of standard CAP and extracts of blank albumen and yolk after derivatization with Sylon HTP are shown in Fig 2. Silylated CAP elutes in a reasonable time of under 15 min. The blank samples (albumen and yolk) did not exhibit significant interferences (less than twice the baseline noise level) in that region.

A few pooled powdered eggs (albumen and yolk separately) were analyzed to check the validity of the modified method (Fig. 1B). Data shown in Table 4 are based on liquid eggs. The average water content in albumen was 70.5%, and that in yolk was 42.3%. These values were used in recalculating the concentration of CAP in incurred liquid eggs. The literature value for water content is 88 and 56% for albumen and yolk, respectively [32]. Data in Table 4 showed that residues in yolk were considerably higher than albumen. It also appears that residues of CAP in albumen decreased rapidly when treated water supply was removed. However, the residues in yolk appeared to persist. A similar result was reported by Samouris et al. [25]. This may, in part, be due to lipid content of yolks. It is well documented that lipophilic substances, such as pesticides having halogen moiety, are deposited preferentially in yolk [33,34].

A Robotic extraction technique was developed with spiked samples which was validated by analysis of 60 albumen and yolk samples containing incurred residues from the feeding trial. Data will be reported separately [35].

4. Conclusions

On the basis of data compared here for the existing and modified extraction techniques, it is

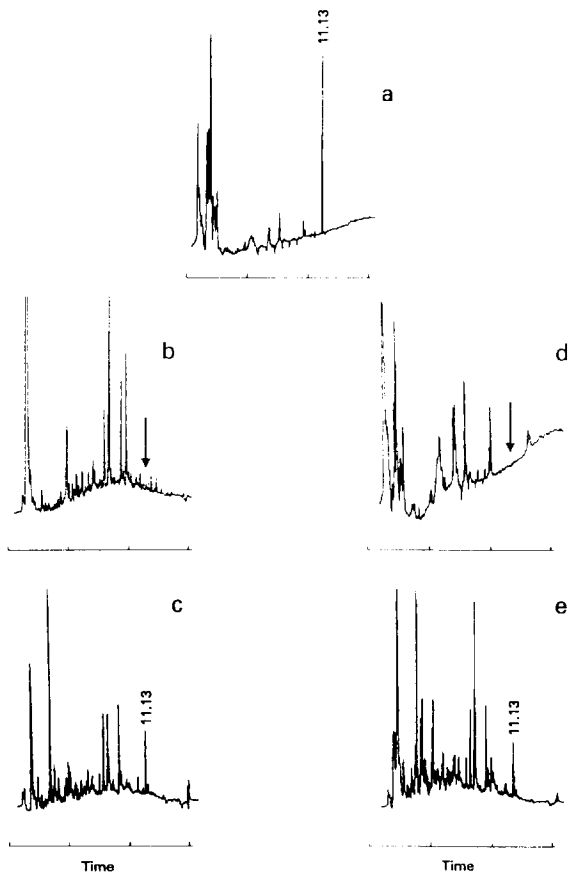

Fig. 2. Gas chromatograms after treatment with silylating agents: (a) CAP standard; extract of (b) blank albumen, (c) incurred albumen; extract of (d) blank yolk, (e) incurred yolk.

concluded that the modified technique is superior and is reproducible. Any advancement leading to fast, accurate, reproducible, environmentally friendly and less costly method will always be welcomed. One factor which is very important in any method development is the availability and affordability of the analytical system by developing countries for monitoring food supplies before they are exported, thus safeguarding the health of consumers globally. We believe that the modified method has the potential for being employed routinely to monitor CAP residues in eggs and other biological samples worldwide.

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Table 4
Concentration of CAP in albumen and yolk (liquid basis)[a] of eggs from laying hens given CAP in water

Day[b]	Concentration in water (mg/ml)	Albumen (ng/kg)[c]	Yolk (ng/kg)[c]
8	25	26.55 ± 4.14	78.02 ± 4.90
7	50	65.55 ± 0.23	166.28 ± 6.63
8	100	271.89 ± 2.77	477.81 ± 7.73
− 2[d]	200	26.69 ± 9.78	1834.65 ± 7.35

[a] Data from analysis of powdered incurred albumen and yolk were converted to liquid basis.
[b] Refers to number of days from the start of treatment of CAP in water.
[c] Mean ± R.S.D., $n = 4$.
[d] Refers to days after water was removed. Laying hens had free access to drinking water containing CAP for 10 continuous days.

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