

# Detection of Chloramphenicol Residue in Bovine Meat Using Liquid Chromatography Mass Spectrometry

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## ABSTRAK

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Kloramfenikol (chloramphenicol = CAP) adalah antibiotika berspektrum luas yang telah dilarang penggunaannya di banyak negara oleh karena menimbulkan efek samping yang serius terhadap kesehatan manusia. Instrumentasi yang digunakan dalam mendeteksi CAP dalam pangan harus mampu menunjukkan batas kemampuan terendah yang dibutuhkan (minimum required performance limit = MRPL) pada 0,3 ng/g. Tujuan dari penelitian ini adalah untuk mengembangkan metoda deteksi residu CAP pada daging sapi menggunakan kromatografi cair spektrometri masa (KCSM) dan mengetahui keberadaan residu CAP pada 36 sampel daging sapi dari pasar lokal dan 16 sampel daging impor. Kolom yang digunakan adalah kolom fasa terbalik C18 dan dipisahkan menggunakan fasa gerak campuran air-asetonitril (1:1) pada laju alir 0,2 mL/menit serta diionisasi dengan electron spray ionisation (ESI) ion negatif. Hasil validasi metoda untuk parameter uji linearitas menunjukkan nilai koefisien korelasi ( $R^2$ ) sebesar 0,9981 untuk kalibrasi dengan konsentrasi 0,125; 0,25; 0,63; 1,00 dan 2,00 ng/g. Uji perolehan kembali dari fortifikasi pada tiga tingkat konsentrasi yang berbeda (0,25; 0,50 dan 1,00 ng/g) adalah 77,5; 97,3 dan 83,4% untuk masing-masing konsentrasi fortifikasi. Nilai batas decision dan batas kemampuan masing-masing adalah 0,15 ng/g dan 0,17 ng/g. Hasil analisis terhadap 52 sampel yang diuji menunjukkan bahwa residu CAP terdeteksi pada 9 sampel pada kisaran konsentrasi 0,14 hingga 2,70 ng/g dan 6 diantaranya melebihi MRPL. Oleh karenanya, untuk menjamin penyediaan pangan yang aman bagi konsumen, kewaspadaan dan monitoring terhadap residu CAP pada pangan asal ternak perlu ditingkatkan.

**Kata Kunci:** Kloramfenikol, Residu, KCSM, Daging Sapi

## ABSTRACT

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Chloramphenicol (CAP) is a broad spectrum antibiotic that has been banned in many countries due to its serious side effect to human. Detection of CAP residue in food has been determined to a minimum required performance limit (MRPL) of 0.3 ng/g. The purpose of this research was to conduct the analysis of CAP residue in bovine meat by using LCMS and to study the presence of CAP residue in marketed bovine meat samples. LC separation was done on a Shimpack column C<sub>18</sub> with ammonium acetate 10 mM/water as mobile phase, and ESI-MS analysis in negative ion mode. The coefficient of determination,  $R^2 = 0.9981$  at concentration of 0.125, 0.25, 0.63, 1.00 and 2.00 ng/g. Recovery at three fortification levels (0.25, 0.50 and 1.00 ng/g) was in the range 77.5, 97.3 and 83.4%. The decision limit and the detection capability were 0.15 ng/g and 0.17 ng/g respectively. Analysis results of 52 marketed samples showed that CAP residue were detected in 9 samples in the concentration range of 0.14 to 2.70 ng/g and 6 among those positive samples were above the MRPL value. Therefore, it is important to increase the awareness and also to monitor regularly CAP residues in food originated from animal to provide safe food for the consumers.

**Key Words:** Chloramphenicol, Residue, LCMS, Bovine Meat

## INTRODUCTION

Chloramphenicol (CAP) is a broad-spectrum antibiotic with historical veterinary uses in all major food-producing animals and with current uses in humans and companion animals. It is effective against both Gram-positive and Gram-negative organisms, rickettsiae, chlamydiae, and mycoplasmas. CAP has

shown serious side effect to human, cause bone marrow depression and a severe aplastic anemia (Reeves 2012). The International Agency for Research on Cancer (IARC) in 1990 assigned CAP in group 2A and considered as "probably carcinogenic to humans".

Low doses of administered CAP are still able in resulting residues in edible tissues from treated food of animal source. Consumers of milk, meat, aquaculture

products, honey and eggs might be exposed to potentially harmful levels of drug residues which also contribute to the development of resistant strains of bacteria and too serious health problems (Ferguson et al. 2005). For these reasons, the use of CAP was banned by the European Union (EU) and other countries includes the USA, Canada, Australia, Japan and China, due to the potential health risk posed by its traces in food. Therefore, there are no withdrawal times and no safe residue levels in meat, eggs, milk and other food with animal origin. The EU has determined for CAP minimum required performance limit (MRPL) of 0.3 ng/g (part per billion = ppb) and it represents a minimum content of an analyte in a sample which at least to be detected and confirmed by the applied method (EC 2003). Whereas, Indonesian government determined for CAP maximum residue level (MRL) of 10 ng/g in meat, egg and milk (DSN 2000).

Even CAP was banned in some countries, it may still be in use in some developing countries including Indonesia (Latif 2004) because of its low cost (Mottier et al. 2003; Ferguson et al. 2005). Therefore, despite legal prohibition on its use in domestic animals, there is evidence suggesting that CAP may be widely used in cattle industry.

Numerous chromatographic methods for determining CAP in various matrices have been reported such as GC with electron capture detector (Pfenning et al. 2000) and HPLC (Posyniak et al. 2003; Samouris et al. 2003). The GC-ECD analysis required derivatization which lengthens the preparation procedure. Meanwhile, the GC-MS technique is unsuitable for CAP detection at level less than 2 ng/g due to insufficient detector sensitivity and selectivity in the electron impact mode (Gantverg et al. 2003). The HPLC-UV method was often disturbed by interfering peaks and did not provide any structural or fragmentation information of the target compound. Those methods also have a main drawback of difficulties in reaching MRPL requirement of 0.3 ng/g. The use of liquid chromatography tandem to MS (LC/MS) offers a rapid, simplified, specific and sensitive (Penney et al. 2005; Rodziewicz & Zawadzka 2008) even not as superior as LCMSMS that provides more complete ionization precursors at lower levels than LC/MS that useful in confirmation purpose.

Nowdays, the use of LCMSMS is common to determine CAP residues in meat, poultry meat, seafood, egg, honey, milk, plasma and urine (Ronning et al. 2006; Siqueira et al. 2009). However, as far as we know, no study concerning the determination of CAP in animal products which detected by liquid chromatography (LC) has been published in Indonesia at concentration lower than the MRPL (0.3 ng/g). The lowest detection limit of CAP reported in Indonesia was 16.5 ng/mL detected by HPLC (Salita 2011) and

0.31 ng/g by LCMS which meant still above the recommended MRPL (Muchlisa 2012).

In order to fulfill the MRPL requirement for CAP detection, this study aimed to demonstrate the ability of LCMS to detect CAP residue in bovine meat at least at the level of 0.3 ppb and also to determine the presence of its residue in local meat samples marketed in Indonesia.

## MATERIALS AND METHODS

### Chemicals

Acetonitrile (HPLC grade), acetonitrile p.a, hexane and ammonium acetate were obtained from Merck (Darmstadt, Germany). Chloramphenicol (P/N C1919) standard was used as CAP reference standard and purchased from Sigma-Aldrich (St.Louis, MO, USA). The stock solution of the CAP reference standard was prepared in acetonitrile at 1000 µg/L and was stored at -20°C. Dilutions of these stock solution were all prepared in Milli-Q water and stored at 4°C. The stability of CAP stock solution at 4°C is at least 6 months. An ultra high purity (UHP) water is processed by Elga Purelab (Lab Water, Veolia Water Solution & Technologies, UK).

### Sample extraction

The method for detecting the CAP residue in meat was adopted from Penney et al. (2005). A 5 g homogenized blended meat sample was placed in a 50 mL polypropylene centrifuge tube. The sample was then added with 12 mL acetonitrile and then vortexed for 30 seconds. The sample was eventually centrifuged at 3000 rpm for 10 minutes and the supernatant was transferred to a second polypropylene tube. Another additional 12 mL acetonitrile was added to the remaining sample and the process was repeated. The acetonitrile extracts were combined and added with 10 mL hexane. The extract was then shaken slowly and the hexane layer was aspirated to waste. This step was repeated 2 times. The extract was evaporated to dryness at temperature of 60°C. The residue was dissolved in 1 mL mobile phase of 10 mM ammonium acetate-acetonitrile (60 : 40) and passed through a 0.45 µm (22 mm) Acrodisc PTFE syringe filter (Waters Corp. Milford, USA) into an LC auto-sampler vial.

### LCMS conditions

The LCMS system consisted of a Shimadzu LC/MS 2010 EV single quadropole with electrospray (ESI) probe, Shimadzu AutoSampler and Shimadzu Solvent Delivery Modules (Shimadzu Corp. Kyoto, Japan).

Separation was achieved by a Shimpack column C<sub>18</sub> (150 mm x 2.1 mm) (Shimadzu Corp.) in combination with the guard pre-column. The mobile phase was a binary gradient mobile phase of an UHP water (100% A) and an acetonitrile HPLC grade (70% B) at flow rate of 0.2 mL/min. Injection volume (simplo) of 10 µL for standards and samples was applied, and the temperature was maintained at 30°C. Negative ion mode electrospray ionization (ESI) and selected ion monitoring (SIM) of two masses (*m/z* 321 and 323) were used. Heater block temperature was 200°C, gas temperature 250°C, curved desolvation line (CDL) temperature 250°C, drying gas flow 0.1 L/min, nebulizing gas flow (N<sub>2</sub>) 1.5 L/min. The diagnostic fragment ions of the parent ion of deprotonated molecular ion [M-H]<sup>-</sup> (*m/z* 321) and the corresponding isotopic ion (*m/z* 323) were chosen following the previous study (Krivohlavek et al. 2007) that obtained the most obvious areas of CAP found at *m/z* 321 and 323. In this study, *m/z* 321 was chosen as the quantifying ion.

### Method validation

A method validation is the process of demonstrating that analytical procedures are suitable for their intended use. The validation method includes the determination of linearity (calibration curve), accuracy (recovery study) and limit of detection (LOD) and limit of quantitation (LOQ) and conducted in accordance to the performances criteria (Ellis 2008).

### Analysis of marketed samples

A total of 36 of bovine meat samples from traditional and modern markets in Sukabumi, Bandung and Jakarta collected in July-August 2010 and 16 imported samples received in February and March 2011 were analysed using the validated method. Meat samples were stored at -20°C before analysis.

## RESULTS AND DISCUSSION

### LCMS detection

Mass spectrometry in conjunction with chromatographic separation (LCMS) is a very powerful combination for identification of an analyte in the extract. Structural information obtained from LCMS generated in the mass spectra from protonated or deprotonated molecules resulting from the compound ions combining with the mobile phases (Jeannota et al.

2000). CAP were first analyzed in negative ESI-MS mode to select characteristic ions as the precursors and detected of two *m/z* 321 (Figure 1) which represents the deprotonated molecule [M-H]<sup>-</sup> of the isotope of CAP and *m/z* 323, the isotopic ion which corresponds to the <sup>37</sup>Cl isotope (Penny et al. 2005). The ESI source is suitable for analysis because of the polar nature of CAP.

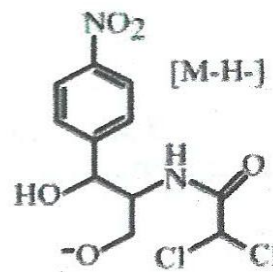


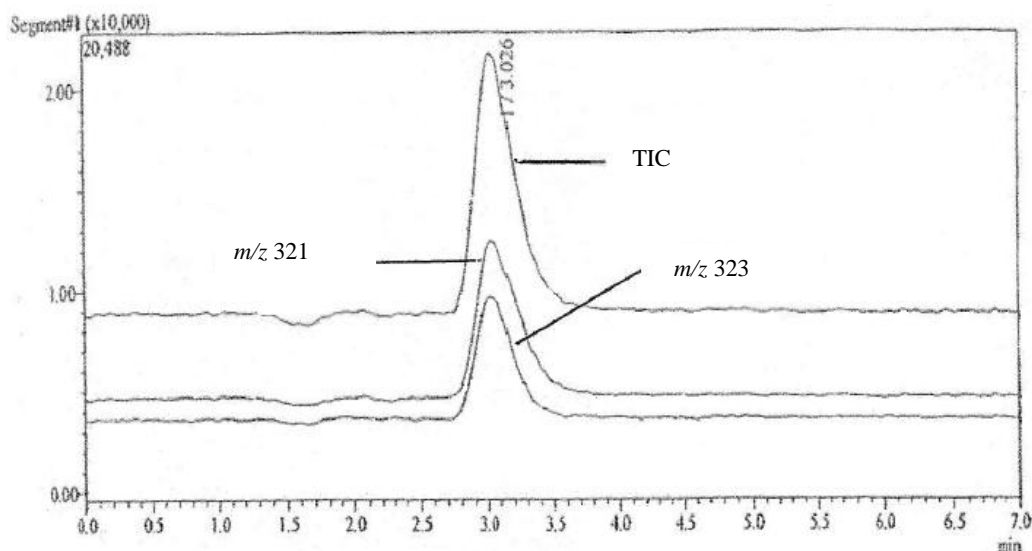
Figure 1. CAP precursor ion *m/z* 321 (Siqueira et al. 2009)

A confirmation method for determination CAP in bovine meat using the LC/MS that developed in this research offered simplicity without the use of solid phase extraction (SPE) step for purification. For identification and confirmation purposes of CAP both *m/z* 321 and 323 ions are necessary to give signal at the same retention time with the analyte in the standard solution and have the same value of the ion intensity ratio both in sample and in sample solution (Marghitas et al. 2010). Therefore confirmation is very specific and can be calculated as ordinary chromatographic separation by comparing to its standard.

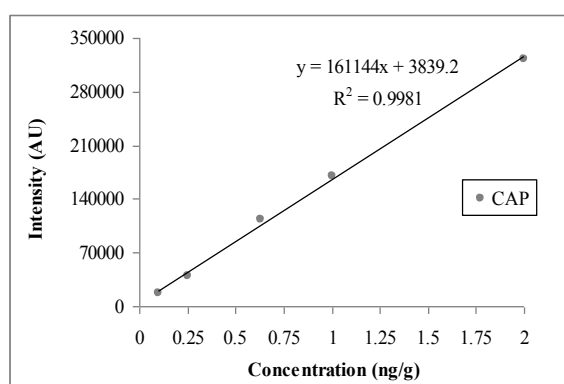
Specificity was determined by analysing 10 injections of CAP standard solution and blank samples. The peaks should represented *m/z* 321 and 323. No interfering peaks were observed at the retention time around 3.0 minutes. This allows for clear identification and quantification of all analytes. Typical chromatogram of standard solution containing 1.25 ng/g CAP shown in Figure 2.

### Linearity, recovery and limit of reporting level

Linearity response was evaluated by injecting in 3 replicates of CAP solutions containing A calibration plot was constructed by plotting peak area against concentration as can be seen at Figure 3. Regression analysis revealed a linear relationship between response and concentration with a correlation coefficient of 0.9981. The regression equation was  $y = 161144x + 3839.2$ .



**Figure 2.** Chromatogram of a standard solution containing 1.25 ng/g of CAP



**Figure 3.** Calibration curve of CAP standard solution on several concentrations

The accuracy of analysis method can be evaluated from the recoveries at three different spiked levels of 0.25, 0.50 and 1.00 of CAP standards into blank samples ( $n = 3$ ) and subjected to the complete analytical procedure in determining the recovery of the method as presented on Table 1.

The recoveries gave a satisfactory results in the range of 77.5% to 97.3% and reproducibility (RSD) in the range of 2.9 to 6.2%. These values comparable and more sensitive compared to the study conducted by Penney et al. (2005) for spiked levels of 0.5 to 3.0 ng/g CAP and obtained the recoveries within 83 to 90%.

The limit of reporting level (LRL) for the method, established as the lowest spiked level for which recovery and precision were reasonable at 7 replicates, was 0.125 ng/g. All results below the LRL (0.125 ng/g) will be assigned as not detected (ND). The method

decision limit ( $CC_{\alpha}$ ) and the detection capability ( $CC_{\beta}$ ) were evaluated in accordance with the guidelines in 2002/657/EC. The  $CC_{\alpha}$  was expressed as the concentration corresponding to the lowest calibration level (0.125 ng/g) plus  $2.33 \times$  standard deviation (SD) of the y intercept calculated.  $CC_{\beta}$  was calculated as  $CC_{\alpha} + 1.64$ -fold the standard deviation (SD) of the within-laboratory reproducibility at the  $CC_{\alpha}$ . The  $CC_{\alpha}$  and  $CC_{\beta}$  were obtained at 0.15 ng/g and 0.17 ng/g respectively. These were both well below the MRPL of 0.3 ng/g. Those values were also lower than those reported by Penney et al. (2005) nor Siqueira et al. (2009). However, its performance was not as good as the research conducted by Krivohlavek et al. (2007) who used the same brand of the LCMS and was able to detect the CAP residue in milk and performed a LOQ of 0.1 ng/g.

### Monitoring CAP residue in marketed beef samples

In order to verify the practical applicability of the validated method, analysis of CAP in 37 meat samples was conducted. The samples collected from local markets in West Java island of Sukabumi municipal (8 samples), Bandung city (9 samples), Jakarta city (20 samples) and 16 imported bovine meat (no information on the origin country) from Tanjung Priok Port.

The overall results revealed that the contamination occurred among 9 (17.3%) samples, i.e. 4 samples collected from Bandung and Jakarta contained CAP in concentration range of 0.15 to 1.92 ng/g and 5 imported samples at the concentration range of 0.14 to 2.70 ng/g, but was not detectable for sample collected from

**Table 1.** Recoveries for CAP detection in bovine meat

CAP spiking concentration (ng/g)	Measured recovery (ng/g)			Mean recovery (%)	RSD (%)
	I	II	III		
0.25	82.4	77.2	72.8	77.5	6.2
0.50	97.4	101.6	93.0	97.3	4.4
1.00	82.6	86.1	81.5	83.4	2.9

**Table 2.** CAP residues in bovine meat from local markets and imported samples

Location	n samples analysed	n and % of CAP detected samples	CAP concentration in detected samples (ng/g)
Sukabumi	7	None	ND
Bandung	9	1 (11.1%)	0.15
Jakarta	20	3 (15.0%)	0.16, 0.47 and 1.92
Imported meat	16	5 (56.2%)	0.14, 0.94, 1.29, 1.91 and 2.70
Total	52	9 (17.3%)	0.14 to 2.70

ND = not detected ( $\leq 0.125$  ng/g)

Sukabumi. The rest of 43 samples (82.7%) did not show any CAP residue and safe for human consumption. Six among 9 positive samples were above the MRPL (0.3 ng/g) in the range of 0.47 to 2.70 ng/g CAP. The imported samples (usually from Australia or New Zealand) indicated as positive samples which CAP concentration higher than the local samples.

The study above was different from the study conducted in Brazil (Siqueira et al. 2009) that shown none among 646 samples analysed above 0.1 ng/g. A monitoring CAP residues conducted in Croatia (Bilandzic et al. 2011) on 109 bovine meat samples showed that the contamination levels of the positive samples were under the MRPL. A monitoring program conducted in Oman also presented the occurrence of CAP residues in goat and sheep meat (Mahgoub et al. 2006) meant that CAP was being u not only in cattle industry but also in other animals such as chicken.

Although the contamination level of positive samples shown in this study below the Indonesian regulation of 10 ng/g, it is still important to increase the awareness and to monitor regularly to avoid the occurrence of CAP residues in food originated from animal. The probability for contamination of CAP residue might be arose from illegal use. Other possibility on its occurrence may be through ingestion of naturally occurring CAP from the environment. There was an evidence of the occurrence CAP naturally in Artemesia family plant originated from Mongolia at levels ranging from 0.1 to 450  $\mu\text{g}/\text{kg}$  in (Berendsen et al. 2010), and also that CAP produced in the soil and the plants (grass) absorbed CAP through their root systems.

The results of this study demonstrated that beef samples for human consumption marketed in some cities in West Java, contained rare case of CAP residue. The seemingly rare occurrence of CAP residue in the meat probably indicates of good application of veterinary practices by the farmers. On the other hand, high concentration of CAP residue in small portion of the samples analysis, could suggest a high risk of illegal use of chloramphenicol in beef industry so that it needs a control on the distribution of the illegal drug.

## CONCLUSION

A detection method for determination CAP in bovine meat could be achieved using the LC/MS. In this research decision limit ( $CC_{\alpha}$ ) and detection capability ( $CC_{\beta}$ ) were obtained at 0.15 ng/g and 0.17 ng/g respectively. These are both well below the MRPL of 0.3 ng/g.

The application of this method for monitoring of CAP in 52 bovine meat marketed in Indonesia demonstrated that 4 out of 36 local samples and 5 out of 16 imported samples were contained CAP, and 6 among 9 positive samples were above the MRPL in the range of 0.47 to 2.70 ng/g. Therefore, it is important to increase the awareness and also monitor regularly the CAP residues in food originated from animal. to provide safe food for consumers. It is also a necessity to expand the investigation on the occurrence to other type of food originated from animal such as liver, chicken meat, egg and milk.

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