# Comparison of Liquid Chromatography with Microbial Inhibition Assay for Determination of Incurred Amoxicillin and Ampicillin Residues in Milk

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A comparison was made between liquid chromatography (LC) methods and a microbial inhibition (MI) method (*Bacillus stearothermophilus* disk assay) for the determination of amoxicillin and ampicillin residues in milk from cows dosed with the drugs. One cow was injected intramuscularly with ampicillin, and a second cow received an intramammary infusion of amoxicillin. Milk samples were collected at various depletion time periods following administration of the drugs. The LC methods using formaldehyde and salicylaldehyde derivatization were applied in the determination of ampicillin and amoxicillin residues, respectively. The LC salicylaldehyde derivatization method was also applied to mixed milk samples for determination of both antibiotics, and the results were in agreement with those determined separately. The MI method was applied to each type of incurred milk. No significant differences were found between the LC and MI assay methods for residue levels within the reliable detection range of the MI method. The LC method was more sensitive than the MI method for residues <10 ng/mL.

**Keywords:** *LC; amoxicillin; ampicillin; incurred residues; milk; microbial method*

## INTRODUCTION

Amoxicillin and ampicillin are among the most commonly used $\beta$-lactam antibiotics in dairy farming for treating bacterial infection. Improper use of antibiotics may lead to trace residues in milk and food supplies and cause human health hazards. The U.S. official tolerance level for both amoxicillin and ampicillin in milk is 0.01 ppm (10 $\mu$g/L or 10 ppb; U.S. Code of Federal Regulations, 1991), and routine monitoring of residue levels in milk is mandatory (U.S. Food and Drug Administration, 1995a). At present, several commercial test kits are widely used for the detection of antibiotic residues in milk (Harik-Khan and Moats, 1995). These tests include the microbial or enzyme inhibition assay, microbial receptor assay, and various immunoassays (Senyk et al., 1990). However, these test kits are generally used for screening purposes, i.e., the detection of residues at or above the regulatory action level. These tests usually are rapid but not quantitative, selective, or specific.

A number of liquid chromatographic (LC) methods have been reported for the determination of ampicillin, amoxicillin, and other $\beta$-lactam antibiotics in milk (Harik-Khan and Moats, 1995; Moats, 1990, 1994; Moats and Harik-Khan, 1995; Straub and Voyksner, 1993; Straub et al., 1994; Terada and Sakabe, 1985; Tyczkowska et al., 1994; Voyksner et al., 1991). These procedures generally involve the extraction/deproteinization of milk with an acid, buffer, and/or organic solvent, cleanup by solid phase extraction or LC separation, and derivatization as needed, followed by LC determination using UV/PDA or LC/MS. However, many of these methods have detection limits of 30−100 ppb (Terada and Sakabe, 1985), which are higher than regulatory action levels. Using perfusive-particle LC combined with ultrasonic nebulization electrospray mass spectrometry, Straub et al. (1994) were able to lower the detection limits to ∼20−30 ppb for ampicillin and amoxicillin. Harik-Khan and Moats (1995) reported an integration procedure utilizing a gradient LC for separating $\beta$-lactam antibiotics from milk followed by activity tests using commercial kits and further identification and quantitation using an analytical LC-UV method. Milk samples spiked with antibiotics were used in most studies cited above. Zomer et al. (1995) developed an LC−receptorgram method combining LC separation with microbial receptor quantitative assay (Charm II) for $\beta$-lactam antibiotics in spiked and incurred milk. Anderson et al. (1996) used an LC method with automated LC cleanup and a microbial receptor assay for determination of the depletion of amoxicillin and ampicillin residues in milk from cows administered the antibiotics. Literature information concerning the analyses of incurred residues is very limited.

Recently, we developed a rapid LC method with precolumn formaldehyde derivatization for determination of ampicillin residues in milk (Ang and Luo, 1997). This method was a modification of a method for the determination of amoxicillin residues in fish muscle tissues (Ang et al., 1996) to eliminate the cleanup and concentration steps. However, this rapid method was applicable only for the determination of ampicillin but not for amoxicillin, due to the low sensitivity of the amoxicillin derivative and interfering substances in milk. Subsequently, we developed another procedure using salicylaldehyde as the derivatizing agent followed

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S0021-8561(97)00307-5 CCC: $14.00 

by LC with fluorescence detection (Luo et al., 1997). Both amoxicillin and ampicillin spiked in milk could be determined with high sensitivity (limit of detection was <2 ppb) and with good recoveries (>80%) at spiking levels of 5, 10, and 20 ppb.

The objective of the present study was to evaluate our two LC methods, with formaldehyde or salicylaldehyde derivatization, for the analyses of incurred amoxicillin and ampicillin residues in milk. Samples were also analyzed by a microbial inhibition (MI) method—*Bacillus stearothermophilus* disk assay (BSDA), a commonly used official method in regulatory laboratories for the detection of $\beta$-lactam antibiotic residues in milk. The comparison between the results of two approaches provides further information regarding the applicability of the LC methods for determination of incurred amoxicillin and ampicillin residues in milk.

## MATERIALS AND METHODS

**Incurred Milk Samples.** The dosing of cows with antibiotics was performed at the Center for Veterinary Medicine, U.S. Food and Drug Administration (Beltsville, MD). These milk samples were collected for the purpose of evaluation of analytical methodologies for determination of incurred antibiotic residues in milk, not for obtaining data on depletion of antibiotic residues following administration of the drugs. Thus, only one cow was tested for each of the dosing operations. A lactating Holstein cow (designated cow 1) was given ampicillin trihydrate at 11 mg (in 50 mL) per kilogram of the cow's body weight as a single intramuscular dosage. The dosage was given in the hind quarters using four injection sites. Milk samples were collected prior to dosing (2 L as control milk) and at 8, 24, 32, 48, 56, 72, and 96 h postdosing. All samples were taken from the total milk collected at each milking. Another lactating Holstein cow (cow 2) was given amoxicillin trihydrate (62.5 mg/10 mL of plastet), infusing one plastet in each quarter of the udder, for a total of 250 mg of drug administered (intramammary infusion). Milk samples were collected as above except that postdose samples were collected at 8−104 h postdosing.

All milk samples were collected in polyethylene "zip-lock" bags, labeled, double-bagged, and stored in the walk-in cooler prior to subsampling. Samples of 50-mL aliquots were placed in disposable polyethylene tubes, screw-capped and shipped frozen to the U.S. Food and Drug Administration, National Center for Toxicological Research laboratory at Jefferson, AR. Upon receipt, all samples were stored at −70 °C until used. Samples were thawed at 4 °C overnight before aliquots were taken for assays. Portions of milk from each source and control milk were mixed to provide additional samples containing incurred residues of both amoxicillin and ampicillin at target levels of 5, 10, and 20 ng/mL for each antibiotic.

**Chemicals and Apparatus for LC Analysis.** Reference standard amoxicillin trihydrate and ampicillin reference standards were purchased from the U.S. Pharmacopeial Convention (Rockville, MD). Standard stock solutions of amoxicillin and ampicillin at 1 mg/mL were prepared in water and stored at 4 °C for up to 1 month. Appropriate dilutions were made daily for preparation of intermediate and working solutions. Salicylaldehyde (ACS grade) and 1-pentanesulfonic acid, sodium salt (ACS grade), were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI). Sodium tungstate (ACS grade) was obtained from Sigma Chemical Co. (St. Louis, MO). All solvents were of HPLC grade and supplied by J. T. Baker, Inc. (Phillipsburg, NJ). Trichloroacetic acid was obtained from Aldrich; formaldehyde and potassium phosphate, monobasic, were of ACS grade. Water was deionized and passed through a carbon filter (Milli-Q water purification system, Waters Corp., Milford, MA).

The solid phase extraction system consisted of $C_{18}$ cartridges (Sep-Pak Vac 3 $cm^3$, 500 mg sorbent) and a vacuum manifold. Both were obtained from Waters. The HPLC system consisted of a Waters 600E pump and pump controller, a Model 7125 injector (Rheodyne, Cotati, CA) with a fixed-volume (100 $\mu$L) loop, a Waters 470 scanning fluorescence detector, and a Millennium 2010 Chromatogram Manager (version 2.1, Waters) for data processing and system monitoring operations. The analytical column, Prodigy 5 $\mu$m, ODS-3, 4.6 mm × 250 mm, was obtained from Phenomenex Co. (Torrance, CA).

**LC Procedure.** The procedure for determination of ampicillin residues in milk from cows injected with ampicillin was basically the same as that reported by Ang and Luo (1997). After thawing and thorough vortex mixing, 2-mL aliquots (in triplicate) of each milk sample (control or incurred samples) were deproteinized with 0.5 mL of trichloroacetic acid solution (20%) and 0.5 mL of acetonitrile. After mixing and centrifugation, antibiotic residues were extracted into the clear liquid phase. The extracts were reacted with trichloroacetic acid and formaldehyde solutions at 100 °C for 30 min to form fluorescent derivatives, which were then determined by LC with fluorescence detection. The excitation and emission wavelengths were 346 and 442 nm, respectively. The mobile phase was an isocratic system consisting of 80% phosphate buffer (0.05 M, pH 5.6) and 20% acetonitrile with a flow rate of 1 mL/min.

The method of Luo et al. (1997) was followed for the determination of amoxicillin residues in milk samples from cows infused with the antibiotic and for determination of both amoxicillin and ampicillin residues from mixed milk samples. Briefly, 5 mL of milk (control milk or incurred samples) was extracted/deproteinized using phosphate, sodium tungstate, and $H_2SO_4$. After centrifugation and filtration, an ion-pair reagent (1-pentanesulfonic acid) was added, and the extract was cleaned up and concentrated using the preconditioned $C_{18}$ cartridge. The antibiotics were eluted with 60% methanol and concentrated by vacuum evaporation. Derivatization of antibiotics was performed by adding salicylaldehyde and trichloroacetic acid and heating at 100 °C for 45 min.

A modified mobile phase was used to remove some late elution peaks before the next injection. It was a gradient system consisting of solvent A (32% acetonitrile and 68% phosphate buffer of 0.02 M $KH_2PO_4$, pH 5.5) and solvent B (80% acetonitrile and 20% water). Solvent A was used for 0−11 min at 1 mL/min. From 12 to 22 min, 63% solvent A and 37% solvent B were used at 1.5 mL/min. From 23 to 30 min, 100% solvent A was used at a flow rate of 1.5 mL/min. The flow rate was reduced to 1 mL/min before the next injection. The excitation and emission wavelengths were 357 and 445 nm, respectively.

**Microbial Inhibition (MI) Method.** All chemicals, reagents, and disks were supplied by Charm Science Inc. (Malden, MA). General operation procedures and apparatus were based on the method of the *B. stearothermophilus* disk assay (U.S. Food and Drug Administration, 1995a,b). The quantitative aspect of the analysis was based on the quantitative disk method for $\beta$-lactam antibiotics in milk in the *Official Methods of Analysis* (AOAC International, 1995), and the computation procedure was adapted from the U.S. Food and Drug Administration (1974). A brief description of the method including modifications is as follows:

Standard stock solutions (1 mg/mL) and intermediate standard solutions were prepared in water. Appropriate amounts of intermediate solutions were added to 5-mL aliquots of the control milk to yield 4, 8, 10, 20, 30, and 40 ng/mL as working standards. The 10 ng/mL concentration was used as the reference standard. Six disks per plate were used for the standard at each concentration level. For the generation of a standard curve, 90-$\mu$L aliquots of the reference standard (10 ng/mL) were added to three alternate disks and one of the other standard concentrations was added to the other three disks on the same culture plate. A positive control of penicillin solution (Bacto PM Positive Control, Difco Laboratories, Detroit, MI) was added to the center disk. Three culture plates were used for each concentration point. This procedure resulted in 45 determinations for the 10 ng/mL point and 9 determinations for each of the other points on the standard curve.

Incurred milk samples were tested following the same procedure; sample or reference standard was added to three alternate disks of each plate. Four plates were used for each

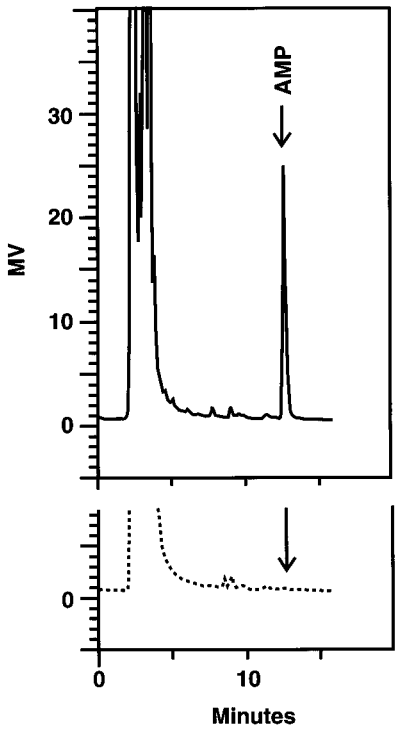


**Figure 1.** LC chromatograms of incurred ampicillin residue at 13.6 ng/mL in milk. Ampicillin was determined as the formaldehyde derivative. LC conditions are described in the text. Solid line is incurred milk, and dotted line is the control.

incurred sample. A separate plate with three disks was used for the control milk. After the plates had been incubated at 64 ± 2 °C for ~3 h, the inhibition zones were measured according to the standard procedure (U.S. Food and Drug Administration, 1995a). The average of 45 readings of the 10 ng/mL concentration was used as the correction point for the curve. The average value obtained for each concentration point was corrected, and standard curves were constructed. For the adjustment of zone sizes of samples, the average of 48 readings of the 10 ng/mL concentration was used as the correction point. The average of each plate was corrected in relation to the reference value of standard, and the corresponding concentration was calculated.

**Statistical Analysis.** The Statistical Analysis System (SAS, 1996) was used for all computations including the construction of the BSDA standard curves and the estimation of sample residue levels. The General Linear Model was used to determine factors associated with the residue concentrations found. The analytical method, type of residues, and postinjection period were used as independent variables, and the concentration found was the dependent variable.

## RESULTS AND DISCUSSION

**Ampicillin.** A typical LC chromatogram of ampicillin residues in the milk of cow 1 is shown in Figure 1. Control milk had only trace interfering peaks, similar to those reported previously for controls (Ang and Luo, 1997). The results of LC and MI assay analyses of incurred ampicillin in milk from cow 1 are presented in Table 1. Residues in control milk were not detectable by the LC method, i.e., below the limits of detection of 0.5 ng/mL for ampicillin. The amount of ampicillin in milk as determined by the LC method ranged from 31.9 ng/mL (8 h postdosing) to 0.6 ng/mL (56 h postdosing). Because the residue level in the 72 h postdosing sample was very low, no other samples collected after 72 h were analyzed. The coefficients of variation (CVs) of LC analyses for all samples from cow 1 were ≤7.23%.

**Table 1. LC Method vs MI Method for Determination of Ampicillin Residues in Milk from Cow 1 following Administration of the Drug**

| | ampicillin in milk,[a] ng/mL | | | | | |
|---|---|---|---|---|---|---|
| | LC method | | | MI method | | |
| postdosing time (h) | mean | SD | CV (%) | mean | SD | CV (%) |
| 8 | 31.9 | 2.31 | 7.23 | 32.1 | 2.13 | 6.62 |
| 24 | 14.1 | 0.55 | 3.93 | 12.9 | 0.70 | 5.42 |
| 32 | 5.5 | 0.29 | 5.39 | <4[b] | | |
| 48 | 1.1 | 0.07 | 6.74 | <4[b] | | |
| 56 | 0.6 | 0.02 | 3.63 | <4[b] | | |
| 72 | <0.5[c] | | | NA[d] | | |

[a] $n = 3$ for LC method; $n = 4$ for MI method. [b] Limit of detection = 4 ng/mL. [c] Limit of detection = 0.5 ng/mL. [d] Not analyzed.

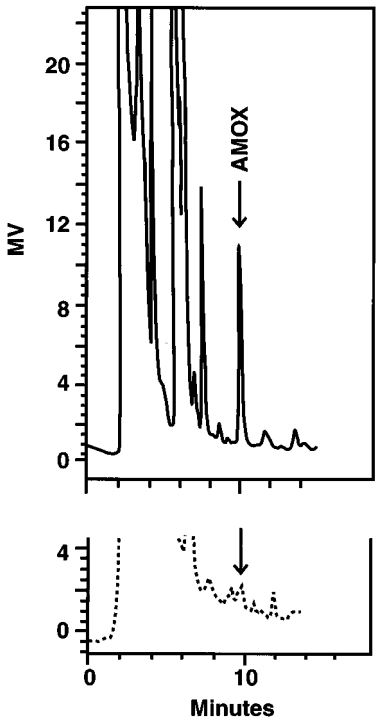


**Figure 2.** LC chromatograms of amoxicillin residue at 12.8 ng/mL in milk. Amoxicillin was determined as salicylaldehyde derivative. LC conditions are described in the text. Solid line is incurred milk, and dotted line is the control.

By the MI assay, ampicillin residues were detected in only the 8- and 24-h postdosing milk samples. Milk collected between 32 and 56 h postdosing was analyzed, but the levels of ampicillin residues were below the limit of detection (4 ng/mL). Thus, the 72-h postdosing samples were not analyzed. It was clear that the MI method was not as sensitive as the LC method for the determination of ampicillin at trace levels. The CVs of the MI assay were ≤6.62% for ampicillin. Statistical analysis of the data showed no significant difference (at 5% level) between the two methods for the determination of incurred ampicillin within the detectable range of the MI method.

**Amoxicillin.** A typical LC chromatogram of the amoxicillin residues in milk from cow 2 is shown in Figure 2. Control milks had some interfering peaks at <1.1 ng/mL, which was the detection limit for amoxicillin. More peaks were observed as the background signal for the analysis of amoxicillin by the LC salicylaldehyde method (Figure 2) than for the analysis of ampicillin by the LC formaldehyde method (Figure 1). The difference was due to the dilution factors used. The

milk extract for ampicillin determination was diluted to half of its original concentration, whereas the milk extract for amoxicillin determination was concentrated 2.5 times before the LC injection. Retention times and peak heights of the background signals varied from run to run; the levels of these peaks were at the detection limit.

The amoxicillin content as determined by the LC method was high (968 ng/mL) in the milk of cow 2 collected at 8-h postdosing, which was most likely due to the method of administration (intramammary infusion). However, after 24 h, the level of amoxicillin decreased dramatically and further reduced to the limit of detection (1.1 ng/mL) at the 72-h postdosing sampling interval. No samples collected after 72-h postdosing were analyzed. The CVs among replicate analyses of amoxicillin by the LC method for all samples of cow 2 were <8.3%.

By the MI method, there was no activity observed for the amoxicillin standard at 4 ng/mL. Thus, the effective working range for amoxicillin was between 8 and 40 ng/mL. The MI method detected antibiotic residues in milk collected at 24, 32, and 56 h postdosing of cow 2. The 8-h postdosing milk sample was not analyzed by the MI method because the concentration (as analyzed by the LC method) was excessively above the accurate range of the microbial standard curve. The 24-h postdosing milk sample was diluted with control milk to half of its original concentration before the microbial assay to produce an inhibition zone size within the effective range. A slightly higher value for amoxicillin residues was found in 56-h postdosing milk samples as compared to the 48-h counterpart. However, the value of 5.63 ng/mL (56-h postdosing milk) was extrapolated from the standard curve. Both the 48- and 56-h postdosing milk samples contained <10 ng/mL of amoxicillin, which is the regular detection level of this antibiotic in milk by the MI method. The CVs of the microbial assay of amoxicillin were generally <8%. Statistical comparison of the amoxicillin results of samples collected at 24-, 32-, and 48-h postdosing showed that no significant differences were found between the two methods (at 5% level) on residue concentrations.

**LC Analysis of Mixed Milk.** In the development of an analytical method for regulatory purposes, it is essential that the new method is validated with samples containing incurred residues ranging from 0.5 to 2 times the tolerance level. Thus, in this study, the LC method was tested for mixed milk samples prepared to contain approximately 5, 10 (tolerance level), and 20 ng/mL of each of the two antibiotics. Values predetermined by the LC methods (Tables 1 and 2) were used as a guide in the calculation of the resulting residue levels in mixed milk. Figure 3 shows the LC analysis of incurred amoxicillin and ampicillin residues in a mixed milk sample. The salicylaldehyde derivatives of amoxicillin and ampicillin were well separated from each other and from interfering compounds. The LC methods can be used as a determinative procedure to distinguish between amoxicillin and ampicillin. The peak at 15.5 min was due to the salicylaldehyde reagent rather than the milk. The mean recovery values of ampicillin determined in the mixed milk were 92.2%, 86.8%, and 87.4% for the targeted levels of 5, 10, and 20 ng/mL, respectively. The overall mean recovery value for ampicillin was 88.8% with a CV of 3.3%, indicating that the levels determined in the mixture were somewhat lower (11.2% less) than the target values. This probably was due to the fact that recovery values of the salicylaldehyde–LC method were generally lower (mean = 83.2%; Luo et al., 1997) than those of the formaldehyde–LC method (mean = 90%; Ang and Luo, 1997). The latter did not require a cleanup/concentration step. Data were not corrected for recovery.

The mean recovery values of amoxicillin determined in the mixed milk were 100%, 94.9%, and 100% of the target levels of 5, 10, and 20 ng/mL, respectively. The overall mean recovery of the three levels was 98.3% with a CV of 3.0%. Little differences were expected in this case because the same LC method was used for the determination of amoxicillin residues in both the mixed milk and the individual milk containing incurred amoxicillin. The salicylaldehyde derivatization–LC method was applicable to the determination of both ampicillin and amoxicillin simultaneously in incurred milk. The estimated residue levels in control milk extract were below their limits of detection (1.1 and 0.5 ng/mL for amoxicillin and ampicillin, respectively). The limits of quantitation were reported previously as 2.4 and 1.7 ng/

**Table 2. LC Method vs MI Method for Determination of Amoxicillin Residues in Milk from Cow 2 following Administration of the Drug**

| postdosing time (h) | amoxicillin in milk,[a] ng/mL | | | | | |
|---|---|---|---|---|---|---|
| | LC method | | | MI method | | |
| | mean | SD | CV (%) | mean | SD | CV (%) |
| 8 | 968 | 30.9 | 3.19 | NA[b] | | |
| 24 | 12.6 | 0.59 | 4.72 | 14.0 | 0.83 | 5.94 |
| 32 | 10.0 | 0.83 | 8.29 | 10.4 | 0.45 | 4.34 |
| 48 | 5.5 | 0.38 | 8.29 | 5.6[c] | 0.45 | 7.91 |
| 56 | 5.5 | 0.17 | 3.13 | 8.4 | 0.35 | 4.21 |
| 72 | <1.1[d] | | | NA[b] | | |

[a] $n = 3$ for LC method; $n = 4$ for MI method. [b] Not analyzed (concentration levels were too high or too low). [c] Value extrapolated from the standard curve. [d] Limit of detection = 1.1 ng/mL.

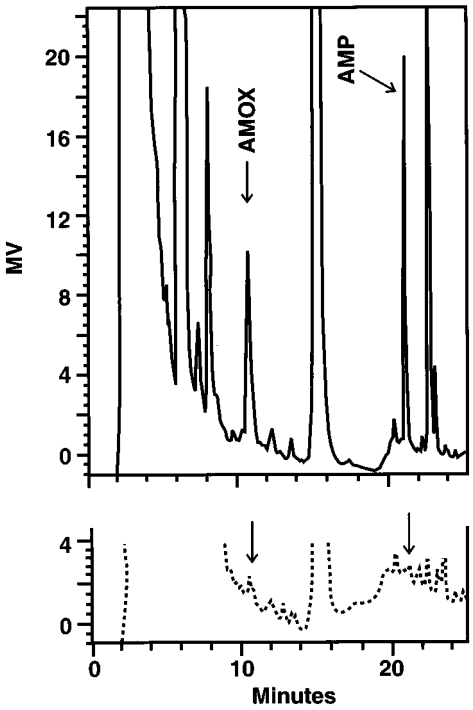


**Figure 3.** LC chromatograms of incurred ampicillin (8.6 ng/mL) and amoxicillin (9.7 ng/mL) residues in a mixed milk sample. Both ampicillin and amoxicillin were determined as salicylaldehyde derivatives. LC conditions are described in the text. Solid line is incurred milk, and dotted line is the control.

mL for amoxicillin and ampicillin, respectively (Luo et al., 1997). The variations on the background signal of the mixed milk were similar to those encountered when amoxicillin alone was present in the milk (cow 2). The possible causes have been addressed under Amoxicillin. To prolong the life of LC columns and to avoid any minor changes in the performance of LC columns, it is advisable to extract the salicylaldehyde derivatives with ethyl ether (three times), evaporate off the ether, and redissolve the extract in mobile phase before the LC analysis. This additional step, similar to the procedure of Ang et al. (1996), eliminated most of the acid in the extract, which could cause minor malfunction of the column after repeated usage.

In the investigation of Luo et al. (1997), only spiked milk was analyzed and no comparison was made between the LC and MI methods. Methods for extraction and determination of antibiotic residues in spiked milk may not be applicable to incurred residues; antibiotic chemicals may be bound to components in incurred milk and not as readily extractable as those chemicals spiked to milk. Furthermore, incurred antibiotic residues may exhibit in metabolite forms with different antimicrobial activities. New methods being developed should be evaluated using both spiked and incurred samples. Due to the difficulties in obtaining incurred samples, many studies on method development have used only spiked samples (Ang and Luo, 1997; Harik-Khan and Moats, 1995; Luo et al., 1997; Moats, 1990, 1994: Moats and Harik-Khan, 1995; Straub and Voyksner, 1993; Straub et al., 1994; Tyczkowska et al., 1994; Voyksner et al., 1991). In such cases, there would always be a concern and question as to the applicability of these methods to incurred residues. The present study provides indispensable information on the assessment of previously developed LC methods on their applicability to incurred residues in milk and their quantitative results as compared to those obtained by a commonly used MI method.

Antibiotics are commonly used to treat cows infected with mastitis and other illnesses. There is a concern that milk from mastitis cows interferes with some microbiological screening tests. We do not anticipate such problems would be encountered with the LC methods because they determine the antibiotics as chemical compounds, not as growth inhibition factors. Furthermore, the LC methods were developed as a determinative procedure for use in regulatory laboratories where bulk milk samples pooled from a number of farms are tested. The effect of any abnormal milk from a mastitic cow is much diluted. Nevertheless, we concur with a suggestion that mastitis milk should be tested in future studies and the effect from mastitis milk after dilution should be evaluated by further experiments.

**Conclusions.** The LC methods using formaldehyde and salicylaldehyde derivatization were applicable for determination of incurred amoxicillin and ampicillin residues, respectively, in milk. The salicylaldehyde–LC method was also applicable for the determination of both residues. No significant differences were found between the LC and MI results at levels detectable by the MI method. The LC methods were more sensitive than the MI method at levels <10 ng/mL. Results suggest that for practical purposes, i.e., detection of ampicillin and amoxicillin residues at 10 ng/mL in milk, the LC methods are comparable to the currently used MI method.

ACKNOWLEDGMENT

We thank Pak Chu and Jurgen von Bredow for providing the milk samples. W.L. was supported in part by an appointment to the ORAU Postgraduate Research Program at the National Center for Toxicological Research administered by the Oak Ridge Associated Universities through an interagency agreement between the U.S. Department of Energy and the U.S. Food and Drug Administration.

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Received for review April 14, 1997. Accepted August 6, 1997.[⊗]

JF970307Q

[⊗] Abstract published in *Advance ACS Abstracts*, October 1, 1997.