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An Analytical Method for Lasalocid Residues in Livestock Products

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ABSTRACT

A reverse phase high performance liquid chromatographic (RP-HPLC) method for the determination of lasalocid in the muscle and liver of chicken and cattle was developed. Lasalocid was extracted from samples with acetonitrile. After filtration, the extract was purified by liquid-liquid partition, concentrated by rotary evaporation, purified with a silica Sep-Pak cartridge, and finally determined by fluorimetric HPLC. The separation was performed on a Cosmosil 5C18-AR-II column (5 μm, 4.6 x 150 mm) with methanol/0.02M KH_2PO_4 (84/16, v/v) as the mobile phase, and lasalocid was detected at Ex 310 nm and Em 420 nm. Recovery studies were performed at the 0.05, 0.1 and 0.2 ppm fortification levels in each of the four commodities. Average recoveries were in the range of 93.17~95.61, 91.53~93.59, 96.93~97.70 and 90.42~95.92% from chicken muscle and liver, and bovine muscle and liver, respectively. Coefficients of variation for average recoveries were less than 5%. The limit of detection was 0.005 ppm in these four commodities. When 20 samples purchased from various markets in Taipei were analyzed, no lasalocid was detected.

Key words: **lasalocid, coccidiostat, livestock product, high performance liquid chromatography (HPLC).**

INTRODUCTION

Lasalocid (LA, 6-[7R-[5S-ethyl-5-(5R-ethyltetrahydro-5-hydroxy-6S-methyl-2H-pyran-2R-yl)tetrahydro-3S-methyl-2S-furanyl]-4S-hydroxy-3R,5S-dimethyl-6-oxononyl]-2-hydroxy-3-methylbenzoic acid), which belongs to the group of carboxylic polyether antibiotics, is produced by the fermentation of *Streptomyces lasaliensis* [1]. The chemical structure of this compound is characterized as a carboxylic acid ionophore and a number of cyclic ether moieties as shown in Figure 1. The carboxylic acid ionophore exhibits natural fluorescence, the intensity of which is highest when the carboxylic acid moiety is in the ionized form. It has been shown that ionophores are able to mitigate the transport of monovalent ions such as Na^+ and divalent ions such as Mg^{2+} across lipid membranes. This ability has aroused considerable interest and has led to the discovery of its biological activities [2, 3]. The formula of LA is $C_{34}H_{53}NaO_8$. The melting point is between

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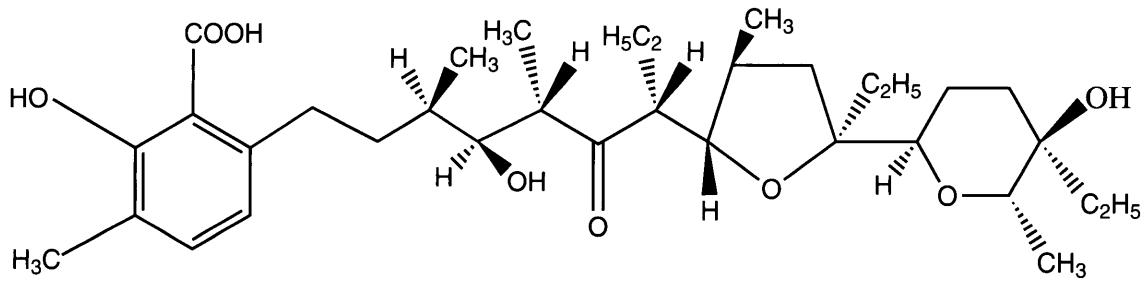

Figure 1. Structure of lasalocid.

191~192℃. LA can be dissolved in organic solvent but it is water insoluble. Its λ_{max} in isopropanol is 308nm and LD_{50} in mice (orally) is 146 mg/kg [4]. A premix product namely Avatex (Roche Products, Hertfordshire, UK) is now commercially available in Taiwan.

LA is allowed to be used in Taiwan as a feed additive to prevent coccidiosis of poultry and to increase the weight gain of cattle according to "The Rules of Use of Feeds Additives" announced by the Council of Agriculture in 1985 [5]. This compound can be added to poultry feeds at concentrations of 75~125 ppm but 3 days withdrawal of the drug is required. It can also be administered in cattle feeds at concentrations of 28~33 ppm for increasing the weight gain and feed conversion efficiency. There is no drug withdrawal requirement, but the minimal and maximal intake are limited to 250 and 360 ppm per head of cattle, respectively. A trial with 7-day-old broiler chickens demonstrated that when LA was introduced at levels of 0.1~0.5 ppm, residue levels in muscle, kidney, and liver were 0.006~0.008 ppm, 0.005~0.010 ppm, and 0.006~0.022 ppm, respectively. Residue levels in all three tissues dropped to below 0.005 ppm after one day withdrawal of the drug [6]. A feeding trial in which ^{14}C-labeled LA was administered to cattle showed low levels of radioactivity in all tissues except the liver [6].

According to "Tolerances for Residues of Animal Drugs" announced by the Department of Health of Taiwan in 1987, any LA residue in meat products is prohibited [7]. Foods containing LA are also prohibited in Japan [8]. However, residues of this drug in meat products are acceptable in USA at 1.2, 7.2, 1.2 and 4.8 ppm in chicken muscle, chicken liver, bovine muscle and bovine liver, respectively [9]. So far, in Taiwan, there is no standard analytical method for the detection of LA in foods, nor have LA residues in commodities been investigated. Before LA residue levels can be regulated in meat products in Taiwan, however, it is necessary to establish an analytical method for the determination of LA residue in chicken muscle, chicken liver, bovine muscle, and bovine liver. Commercial meat products also need to be analyzed, both to evaluate the established method and also to investigate the current LA residue levels in such products. All these data could be used as references for setting up a new regulation for LA residue level in foods.

Several analytical methods have been developed for the determination of LA in tissues. These include thin layer chromatography (TLC) [10, 11], fluorometric method [12], high performance liquid chromatography (HPLC) [2, 3, 6, 8, 13-17], gas chromatography / mass spectrometry (GC/MS) [17, 18], and liquid chromatography / mass spectrometry (LC/MS) [1]. The TLC method is used only for screening tests. The fluorometric method adopted by AOAC is commonly used for the analysis of animal feeds, but this method is not suitable for the analysis of animal tissues because of the presence of far more complex components which can easily interfere with the results [15]. The GC method is also not suitable for LA analysis because the high molecular weight (MW: 590.8) of LA and the complexity on its structure means that LA needs to be derivatized for GC analysis [15]. GC/MS and LC/MS are used to determine the presence of LA, but these methods

are not well documented. HPLC with fluorometric detection, on the other hand, is considered a reliable analytical method for LA analysis as evidenced by abundant published papers. Our preliminary study showed that although sample preparation for normal phase (NP)-HPLC method [1, 14, 15] is simple and rapid, the lack of cleanup procedure in this method easily leads to interference in the LA analysis and results in inaccuracy in quantification. Reverse phase (RP)-HPLC method [1, 3, 6, 8, 12, 16] includes a cleanup operation that can reduce the impurities so as to improve the sensitivity and recovery. The purpose of this study was to develop a simple and rapid RP-HPLC method for the LA analysis. We paid particular attention to the selection of low toxicity solvents. HPLC conditions, sample extraction and sample cleanup procedure were also studied.

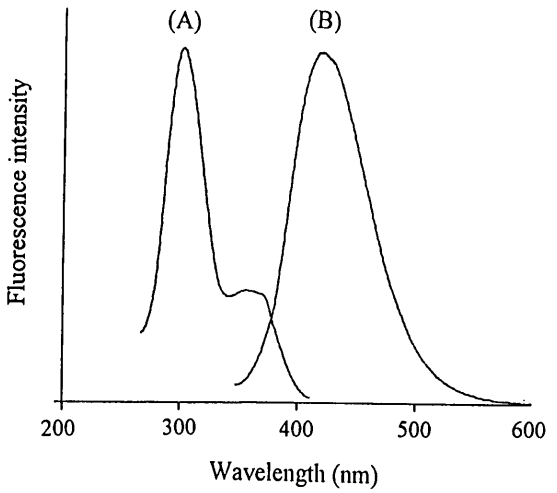

Figure 2. Fluorescence spectra of lasalocid dissolved in methanol/0.02M KH_2PO_4 (pH3.0) (9/1, v/v). (A) Excitation spectrum at 310nm, (B) emission spectrum at 420nm.

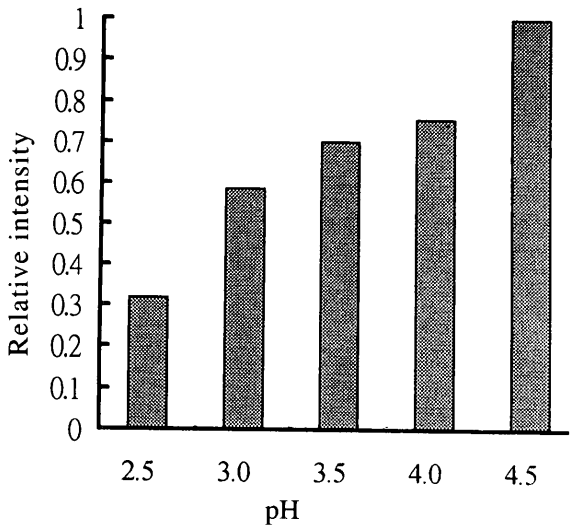

Figure 3. Effect of pH on fluorescence intensity of lasalocid in 0.02M phosphate buffer.

MATERIALS AND METHODS

I. *Tissue Sample Preparation*

Tissue samples of chicken muscle, chicken liver, bovine muscle, and bovine liver were obtained from local traditional markets and super-

Table 1. Comparison of recovery of 0.2 ppm lasalocid spiked into chicken muscle and liver extracted with different solvents

Sample	Extraction solvent	Recovery [a] (%)
Chicken muscle	Acetonitrile	95.47 (1.16) [b]
	Methanol	86.58 (4.12)
	Ethyl acetate	97.56 (2.17)
Chicken liver	Acetonitrile	93.09 (2.23)
	Methanol	83.82 (3.94)
	Ethyl acetate	81.60 (1.66)

[a] average of triplicate.
[b] value in the parenthesis is coefficient of variation (%).

Table 2. Comparison of lasalocid recovery from silica Sep-Pak cartridge eluted with different solvents

Eluent	Recovery [a] (%)
Chloroform/methanol (9/1) 10 ml	95.77 (3.24)[b]
Ethyl acetate 10 ml	69.42 (3.50)
Acetonitrile 10 ml and methanol 10 ml	96.03 (4.25)

[a] average of triplicate.
[b] value in the parenthesis is coefficient of variation (%).

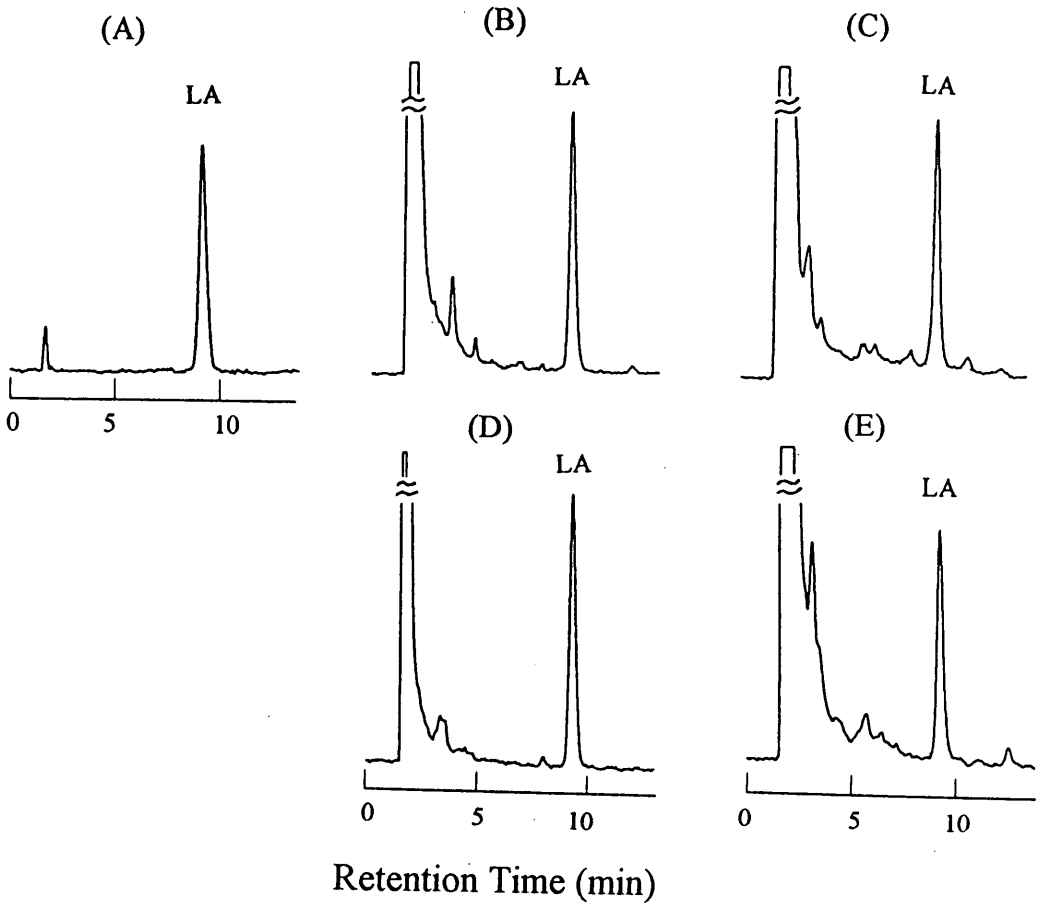

Figure 4. HPLC chromatograms of (A) standard solution of 0.125 mg/ml lasalocid (LA), (B), (C), (D) and (E): chicken muscle, chicken liver, bovine muscle and bovine liver spiked with 0.05 ppm lasalocid, respectively.

Table 3. Recoveries of lasalocid spiked into chicken muscle, chicken liver, bovine muscle and bovine liver

Sample	Spiked level (ppm)	Recovery [a] (%)
Chicken muscle	0.05	95.23 (2.87)[b]
	0.1	95.61 (1.76)
	0.2	93.17 (2.97)
Chicken liver	0.05	91.53 (3.45)
	0.1	91.74 (2.91)
	0.2	93.59 (4.01)
Bovine muscle	0.05	97.60 (1.64)
	0.1	96.93 (1.00)
	0.2	97.70 (2.97)
Bovine liver	0.05	90.42 (4.65)
	0.1	92.66 (4.35)
	0.2	95.92 (1.81)

[a] average of triplicate.
[b] value in the parenthesis is coefficient of variation (%).

markets during May 1997. Five samples of each tissue were collected. The tissue samples were cut into pieces, ground, thoroughly mixed, and then placed in a plastic bag and frozen at -20 ℃ for use as needed.

II. *Chemicals*

Lasalocid sodium as a standard was purchased from Hoffmann-La Roche Inc. (New Jersey, USA). The HPLC grade acetonitrile, methanol, ethyl acetate, hexane, and chloroform were obtained from Lab-Scan Asia Ltd. (Bangkok, Thailand). Reagent grade potassium dihydrogen phosphate was from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

III. *Preparation of Standard Solution*

Ten mg of LA standard was exactly weighed

Sample (5 g)
↓ homogenized w/CH_3CN (30 ml × 3)
↓ filtered
Filtrate
↓ partitioned w/ 50 ml of n-hexane saturated w/ CH_3CN
CH_3CN layer
↓ evaporated to dryness
Residue
↓ dissolved in n-hexane (3 ml × 2)
Silica Sep-Pak cartridge
↓ washed w/ $CHCl_3$ (5 ml × 2)
↓ eluted w/ CH_3CN (10 ml) and then MeOH (10 ml)
Combined CH_3CN w/ MeOH
↓ evaporated to dryness
↓ dissolved in MeOH (2 ml)
HPLC

Figure 5. Analytical procedures for lasalocid determination.

into a 100 ml volumetric flask and dissolved to volume in acetonitrile as a stock standard solution. This solution was then diluted as needed with acetonitrile to make up five standard solutions with concentrations of 0.125, 0.25, 0.5, 1, and 2 μg/ml.

IV. *Sample Extraction and Cleanup*

The sample was prepared according to the method of Ishikuro [8] with a slight modification. Defrosted tissue (5 g) and acetonitrile (30 ml) were homogenized by using a Nissei AM-3 Homogenizer for 1 min. The homogenates were then filtered through a Buechner Funnel. The residue was homogenized again with 30 ml of acetonitrile for 1 min and then filtered. This extraction procedure was carried out twice. Combined filtrates were placed in a separation funnel in which the acetonitrile-saturated hexane solution (50 ml) was added and shaken thoroughly for 5 min. The acetonitrile phase was collected and evaporated to dryness at 40℃ using a rotary evaporator. The residue in the bottle was dissolved in 3 ml of hexane and then transferred into a silica Sep-Pak cartridge (Waters Inc. 690 mg) which was activated with 5 ml of hexane before loading the sample. The bottle was rinsed again with 3 ml of hexane which was then loaded to the same silica Sep-Pak cartridge. The hexane which was passed through the cartridge was discarded. The cartridge was then washed with 5 ml of chloroform twice. The components of interest were eluted with 10 ml of acetonitrile followed by 10 ml of methanol. Combined elutes were evaporated to dryness at 40℃. The residue was dissolved in 2 ml of methanol and filtered through a 0.45 μm membrane prior to HPLC analysis.

V. *High Performance Liquid Chromatography (HPLC) Analysis*

HPLC analysis was performed by using a Shimadzu Cooperation HPLC system (Kyoto, Japan) equipped with a LC-10AT liquid pump system, an RT-551 spectrofluorometric detector, and a C-R4A Chromatograph data management system. The separation was carried out on a

Cosmosil 5C18-AR-II (5 μm, 4.6 x 150 mm i.d., Nacalai Tesque, Inc., Kyoto, Japan) column pumped with a mobile phase of methanol/0.02 M KH_2PO_4 (84/16, v/v) at an isocratic flow rate of 1.0 ml/min. The spectrofluorometric detector was set at excitation wavelength (Ex) 310 nm and emission wavelength (Em) 420 nm. The sample injection volume was 20 μl.

The standard curve of LA was made by averaging the peak areas of triplicate injection of five different LA concentrations. The equation of this standard curve was calculated as follows.

$Y = -7635.8857 + 515027.6940X$

Where X is the concentration in μg/ml and Y is area count. The correlation coefficient (r) was 0.9998. Quantification of LA in tissue samples was performed by comparing the HPLC peak area count of LA to the standard curve.

VI. *Recovery Test*

Recovery tests were carried out in triplicate by spiking the LA standard at three concentration levels (0.05, 0.1, and 0.2 ppm) into 5 g of chicken muscle, chicken liver, bovine muscle, or bovine liver. The samples were then prepared as described above, and recoveries of LA from each tissue sample were obtained by HPLC analysis.

VII. *Test for the LA Detection Limit*

After tissue samples were spiked with different concentration levels of LA standard and the residue of LA was analyzed by HPLC, the detection limit was evaluated by the peak signal / noise (S/N) ratio. An S/N ratio greater than 3 was considered as a detectable peak.

RESULTS AND DISCUSSION

I. *HPLC Analysis*

LA exhibits fluorescence with excitation at 308~315 nm and emission at 400~430 nm and it shows the most intense fluorescence when the carboxylic acid moiety is in the ionized form [2]. The fluorescence spectra of LA in methanol / 0.02 M KH_2PO_4 (9/1, v/v, pH 3.0) solution is presented in Figure 2. As can be seen, the excitation λ_{max} at 310 nm and emission λ_{max} at 420 nm are shown in these spectra. To understand the effect of pH on the fluorescence intensity, LA standard solution was dissolved in the phosphate buffer solutions with pH 2.5, 3.0, 3.5, 4.0 and 4.5, and the fluorescence intensity was detected at excitation 310 nm and emission 420 nm. The fluorescence intensity at pH 4.5 was 1.5 times higher than that at pH 3.0 as shown in Figure 3. The pH of 0.02M KH_2PO_4 buffer solution, which was used as a mobile phase in this study, was 4.5. It was reported that increasing the pH of the mobile phase decreased the capacity factor, k', and led to a decrease of LA retention time [8]. To improve the HPLC resolution, it was therefore necessary to adjust the mobile phase composition. LA was able to be separated from other components on HPLC using a mobile phase of methanol / 0.02M KH_2PO_4 (84/16, v/v) according to our study. Figure 4 shows the HPLC chromatograms of the LA standard and LA in tissue samples. The LA peak is well separated from other peaks and its k' is 4.3.

II. *Sample Preparation*

(I) *Solvents for Extraction*

Three solvent systems, methanol [13], acetonitrile [1, 3, 6, 8, 18], and ethyl acetate [12, 16] were evaluated as extraction media. Five grams each of chicken muscle and chicken liver was spiked with 0.2 ppm LA and then extracted with 30 ml of methanol, acetonitrile, or ethyl acetate three times. After filtration, the methanol and acetonitrile extraction solution was partitioned with hexane and the lower layer was collected and evaporated to dryness. The ethyl acetate extraction solution was directly evaporated to dryness without hexane partition processing. The residues were then dissolved in 5 ml of methanol and filtered through a 0.45 μm membrane prior to HPLC analysis. The LA recovery of this test is shown in Table 1. Samples extracted with methanol had lower recoveries (86.58 and 83.82%), much higher levels of interference in HPLC analysis (data not shown), and were also difficult to evaporate.

Much higher recovery (97.56%) was obtained when the chicken muscle was extracted with ethyl acetate. However, when chicken liver was extracted with ethyl acetate, recovery was only 81.60%. Using acetonitrile as an extraction solvent, the recovery was up to 95.47% on chicken muscle and 93.09% on chicken liver and there was much less interference in the HPLC analysis. Therefore, acetonitrile was selected as an extraction solvent in this study.

(II) *Cleanup Operation*

The cleanup operation for removing the impurities can be achieved by partitioning with hexane followed by passing the extracts through a silica cartridge. Alumina is able to remove the impurities from samples but it is not suitable for sample cleanup operation because it strongly absorbs LA, resulting in low LA recovery [11]. The silica cartridge was widely used for cleanup operations [1,3,6,8]. Chloroform is usually used as a dissolving solvent as well as a washing solvent that removes the impurities of extracts in the cartridge while keeping LA in the cartridge [3], but because of chloroform's carcinogenicity, a low toxic solvent system was used instead. Preliminary study showed that hexane was able to completely dissolve the LA in extracts. Chloroform was still considered as an ideal washing solvent for the silica cartridge itself, however, and cleanup was achieved by washing the silica cartridge with 5 ml of chloroform twice. The washed impurities were yellow in color and their spectrophotometric spectrum (λ_{max} = 465 nm) was very similar to the spectrum of carotenoid.

The eluting solvent systems, chloroform / methanol (9/1, v/v) [8], ethyl acetate [3], acetonitrile and methanol were tested in this study. Since the chemicals found in liver, in general, are much more complex than those in other animal tissues, sample of chicken liver was, therefore, selected to test the cleanup operation and select the eluting solvent. Five grams of chicken liver was weighed out and prepared as described in Methods and 100 μl of LA standard solution (10 μg/ml) was introduced to the acetonitrile phase prior to vacuum evaporation. After evaporation to dryness, the residue was dissolved in hexane, applied to the silica cartridge, and then washed with chloroform, followed by an elution of (1) 10 ml of chloroform / methanol (9/1, v/v), (2) 10 ml of ethyl acetate, or (3) 10 ml of acetonitrile and 10 ml of methanol. After evaporation to dryness, the residue was dissolved in 2 ml of methanol and analyzed by HPLC. Both chloroform / methanol and acetonitrile and methanol gave ideal recoveries of 95.77% and 96.03%, respectively (Table 2). The 10 ml acetonitrile and 10 ml methanol were therefore selected as eluting solvents because of their low toxicity compared to chloroform. The complete LA analytical procedure was thus established and this is presented schematically in Figure 5. The whole procedure takes about 3 hours.

III. *Recovery and Detection Limit Test*

The LA recoveries of four tissue samples were in the range of 90.42~97.70% as shown in Table 3. The coefficient of variation was within 5%. The detection limit for these samples was taken to be 0.005 ppm. This detection limit is similar to that reported by Ishikuro [8], but it is still higher than for the method reported by Tarbin *et al.* [6], who developed an HPLC method using a polymeric and porous graphitic carbon column that gave a detection limit of 0.002 ppm on chicken muscle.

IV. *Investigation of LA Residue in Commercial Chicken Muscle, Chicken Liver, Bovine Muscle, and Bovine Liver*

No LA residue was found in any of the 20 tested samples. However, the purpose of this study was mainly to set up an analytical method for analysis of LA residue in meat products. Hence, sample size and sampling location were not emphasized in this study. A thorough investigation of all commodities is necessary to determine the status of LA residue in commercial products in Taiwan.

CONCLUSIONS

This study presented an HPLC method for the analysis of LA residue in chicken muscle, chicken liver, bovine muscle, and bovine liver. LA in tissue samples was extracted with acetonitrile, partitioned with hexane to remove impurities, cleaned up with a silica cartridge and finally analyzed with HPLC-fluorometric detector. LA recoveries for four sampled tissues were in the range of 90.42~97.70%. The detection limit was 0.005 ppm. Analysis time for one run was about 3 hours.

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禽畜產品中拉薩羅（Lasalocid）檢驗方法之探討

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摘 要

利用高效液相層析法建立鷄肉、鷄肝、牛肉及牛肝中拉薩羅(lasalocid)殘留量之分析方法。拉薩羅以乙腈自檢體中萃取，再加入正己烷去雜質及濃縮後，經矽膠層析匣(silica Sep-Pak cartridge)淨化，最後利用高效液相層析儀以螢光檢測分析。層析管爲Cosmosil 5C18-AR-II (5 μm, 4.6 x 150 mm)，移動相爲甲醇/0.02M KH_2PO_4 (84/16)，於激發波長310 nm及放射波長420 nm偵測。檢體中添加拉薩羅0.05、0.1及0.2 ppm時，其回收率分別爲鷄肉93.17～95.61%，鷄肝91.53～93.59%，牛肉96.93～97.70%及牛肝90.42～95.92%，變異係數皆小於5%。本方法在四種檢體中之最低檢出限量均爲0.005 ppm。利用此方法分析市售鷄肉、鷄肝、牛肉及牛肝檢體共20件，結果均未檢出。目前我國規定肉品中不得殘留拉薩羅。

關鍵詞：拉薩羅，抗球蟲劑，禽畜產品，高效液相層析法。