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Short Communication

Improved high-performance liquid chromatographic procedure for the determination of lasalocid in chicken tissues and egg using polymeric and porous graphitic carbon columns

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ABSTRACT

A high-performance liquid chromatographic (HPLC) method for the determination of the ionophore coccidiostat lasalocid in poultry muscle and eggs was developed. The drug was extracted from tissue with acetonitrile. The extract was partitioned between saturated salt and carbon tetrachloride and the organic layer evaporated to dryness. Clean-up was by solid-phase extraction on a silica column. HPLC analysis was carried out on either a polymeric PLRP-S or a porous graphitic carbon Hypercarb column with a basic mobile phase and fluorescence detection with excitation at 310 nm and emission at 420–430 nm. Average recoveries from poultry muscle at the 0.002, 0.010 and 0.050 mg kg^{-1} levels were 65.7, 72.0 and 77.9%, respectively. Average recoveries from egg at the 0.010 and 0.100 mg kg^{-1} levels were 76.2 and 76.4%, respectively.

INTRODUCTION

Lasalocid (Fig. 1) is a polyether ionophore antibiotic used prophylactically in the prevention of coccidiosis in chickens and as a growth promoter in cattle [1]. It is added to medicated animal feeds as the sodium salt from 75 to 125 mg kg^{-1} for the treatment of coccidiosis and from 11 to 33 mg kg^{-1} when used as a growth promoter. A study on cattle tissue [2] using radiolabelled lasalocid has shown that the most radioactivity is concentrated in the liver, the most abundant residue found being the parent drug. A feeding trial on seven-day-old broiler chicks [3] established that at feeding levels of 0.1 to 5.0 mg kg^{-1}, residue levels were 0.006–0.008 mg kg^{-1} in muscle, 0.005–0.010 mg kg^{-1} in kidney and 0.006–0.022 mg kg^{-1} in liver. Levels dropped to below 0.005

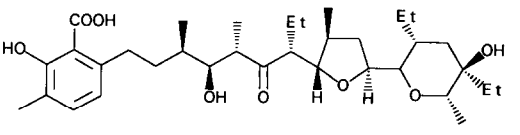

Fig. 1. Structure of lasalocid.

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mg kg^{-1} after one day withdrawal of the drug in all three tissues.

Analytical methods reported for the determination of lasalocid in tissues include normal-phase high-performance liquid chromatography (NP-HPLC) with fluorometric detection [2], reversed-phase high-performance liquid chromatography (RP-HPLC) with fluorometric detection [4,5] and with precolumn derivatization and fluorometric detection [6], pyrolysis gas chromatography–mass spectrometry (GC–MS) [7] and thermospray liquid chromatography–tandem mass spectrometry (LC–MS–MS) [8]. Three methods relate to the determination of lasalocid in beef liver tissue and three in chicken tissue. Detection limits claimed ranged from 0.005 mg kg^{-1} [4] to 0.150 mg kg^{-1} [6]. The GC–MS and LC–MS–MS methods were used as confirmatory procedures. More recently, the results of small-scale interlaboratory studies on the use of the NP-HPLC and the GC–MS confirmatory methods for the determination of lasalocid in beef liver and chicken skin have been published [9,10].

This paper describes a simple, rapid extraction procedure coupled with the fluorometric determination of underivatized lasalocid by RP-HPLC on polymeric and porous graphitic carbon (PGC) columns.

EXPERIMENTAL

Reagents

Lasalocid sodium salt was purchased from Sigma (Poole, UK). 1,1,3,3-Tetramethylguanidine was purchased from Aldrich (Gillingham, UK) and redistilled before use. Bond-Elut Si (500 mg/2.8 ml) cartridges (Analytichem International) were obtained from Jones Chromatography (Hengoed, UK) and were treated with 5 ml of hexane immediately prior to use.

Apparatus

Phase separation paper (Whatman PS-1), Büchi Rotavapor and a nitrogen blowdown apparatus with heating block were used.

High-performance liquid chromatography

Polymeric column. Samples (10 μl) were injected onto a PLRP-S reversed-phase (styrene–divinylbenzene copolymer) (Polymer Labs., Church Stretton, UK) 5-μm column (250 mm × 4.6 mm I.D.). The column was protected with a guard column (5.0 mm × 3.0 mm I.D.) packed with PLRP-S reversed-phase material. The mobile phase was 0.01 *M* disodium tetraborate (borax) pH 10.0–acetonitrile (40:60, v/v). The mobile phase was maintained at a constant flow-rate of 1.0 ml min^{-1} (Waters 6000 A solvent delivery system). Detection was by fluorescence (Perkin-Elmer LS-4) with excitation at 310 nm (slit 15) and emission at 430 nm (slit 20). Quantitation was by reference to replicate injections of blank extract made up with standard in mobile phase.

PGC column. Samples (25 μl) were injected by an autosampler (Waters 712 WISP) onto a Hypercarb (Shandon Scientific, Runcorn, UK) 7-μm column (100 mm × 4.6 mm I.D.), The mobile phase was 5% 1,1,3,3-tetramethylguanidine in acetonitrile. The mobile phase was maintained at a constant flow-rate of 0.5 ml min^{-1} (Waters 590 pump). Detection was by fluorescence (Philips PU4027) with excitation at 310 nm and emission at 420 nm. Quantitation for egg was by reference to replicate injections of standard in mobile phase. Quantitation for muscle tissue was by reference to replicate injections of blank extract made up with standard in mobile phase.

Tissue

Untreated chickens were supplied by MAFF, Gleadthorpe Experimental Farm. The chickens were plucked, eviscerated and stored at −20°C until required. Breast and thigh meat were removed, finely minced together and stored at −20°C until taken for analysis. Eggs were purchased locally, homogenised in batches of six and stored at −20°C until taken for analysis.

Extraction and clean-up procedure for analysis on polymeric column

Finely minced poultry muscle tissue (10 g) and acetonitrile (50 ml) were homogenised for 2 min, placed in an ultrasonic bath for 5 min and centrifuged for 5 min at 1860 *g*. The supernatant was transferred to a separating funnel and extraction of the solid residue repeated with a further 50 ml

of acetonitrile. The combined supernatants were shaken for 1 min with carbon tetrachloride (30 ml) and saturated aqueous sodium chloride (20 ml). The organic layer was filtered through anhydrous sodium sulphate and phase separation paper and evaporated to dryness. The residue was transferred to a Bond-Elut Si cartridge with hexane (3 × 2 ml). The cartridge was washed with chloroform (5 ml) and eluted with chloroform–methanol (95:5, v/v) (10 ml). The eluate was evaporated to dryness and the residue transferred to a small vial with hexane (3 × 2 ml). The hexane was removed under a stream of nitrogen and the residue redissolved in the appropriate mobile phase (0.5 ml).

Extraction and clean-up procedure for analysis on PGC column

Finely minced poultry muscle tissue or homogenised egg (2 g) and acetonitrile (25 ml) were homogenised for 2 min, placed in an ultrasonic bath for 5 min and centrifuged for 5 min at 1860 *g*. The supernatant was transferred to a separating funnel and extraction of the solid residue repeated with a further 25 ml of acetonitrile. The combined supernatants were shaken for 1 min with carbon tetrachloride (50 ml) and saturated aqueous sodium chloride (20 ml). The organic layer was filtered through anhydrous sodium sulphate and phase separation paper and evaporated to dryness. The residue was transferred to a Bond-Elut Si cartridge with hexane (3 × 2 ml). The cartridge was washed with chloroform (5 ml) and eluted with chloroform–methanol (95:5, v/v) (10 ml). The eluate was evaporated to dryness and the residue transferred to a small vial with hexane (3 × 2 ml). The hexane was removed under a stream of nitrogen and the residue redissolved in the appropriate mobile phase (0.5 ml).

Protocol

Samples were analysed in batches of eight. During method validation each batch contained four to six spikes, a tissue blank and a tissue blank for use as a standard (muscle tissue only).

RESULTS AND DISCUSSION

Lasalocid possesses a native fluorescence that has been utilised for the determination of residues in chicken tissues by RP-HPLC using an acidic or neutral mobile phase [4,5]. However lasalocid is most strongly fluorescent when the compound is in the ionised form, *i.e.* under alkaline conditions [2]. This property was utilised in the NP-HPLC procedure for the analysis of lasalocid residues in beef liver tissue [2]. Determination was on a silica column, using a three-component mobile phase containing ammonia. The basic nature of such a mobile phase has a corrosive effect on silica-based columns. In the NP-HPLC procedure this problem was circumvented by using a silica column as a presaturation column prior to the injector. However, the unstable nature of the mobile phase coupled with its corrosive action on silica columns indicated the advisability of developing an alternative HPLC procedure. Polymeric reversed-phase and PGC columns were chosen because of their wide pH stability (pH 1–13).

Using the polymer column, a mobile phase consisting of 0.01 *M* disodium tetraborate pH 10 and acetonitrile in the ratio 40:60 and a flow-rate of 1.0 ml min^{-1} were found to be suitable for the determination of lasalocid in muscle tissues.

TABLE I

RECOVERIES OF LASALOCID FROM TISSUE SPIKED AT 0.002 AND 0.010 mg kg^{-1} ON A PLRP-S COLUMN

Day	Recovery (mean ± S.D.) (%)	C.V. (%)	*n*
0.002 μg kg^{-1}			
1	59.9 ± 8.3	13.9	4
2	68.2 ± 8.8	12.9	4
3	68.2 ± 3.1	4.5	5
Overall	65.7 ± 7.5	11.4	13
0.010 mg kg^{-1}			
1	69.0 ± 2.4	3.5	5
2	74.3 ± 5.0	6.7	5
3	72.8 ± 3.5	4.8	5
Overall	72.0 ± 4.2	5.8	15

However, when the same procedure was used for eggs, an interfering peak was found on the chromatogram.

Separation on a PGC column is thought to occur by a different mechanism from a silica-based reversed-phase column. More organic modifier is usually required to chromatograph the same compound than on a silica-based C_{18} column. In this case 100% acetonitrile with a base modifier was needed. Lasalocid gave poor chromatography when triethylamine was used as base modifier. However, the use of 1,1,3,3-tetramethylguanidine gave good peak shape. Moreover, the interfering peak in the egg extracts was no longer present under these conditions. Muscle extracts also gave good separation under these condi-

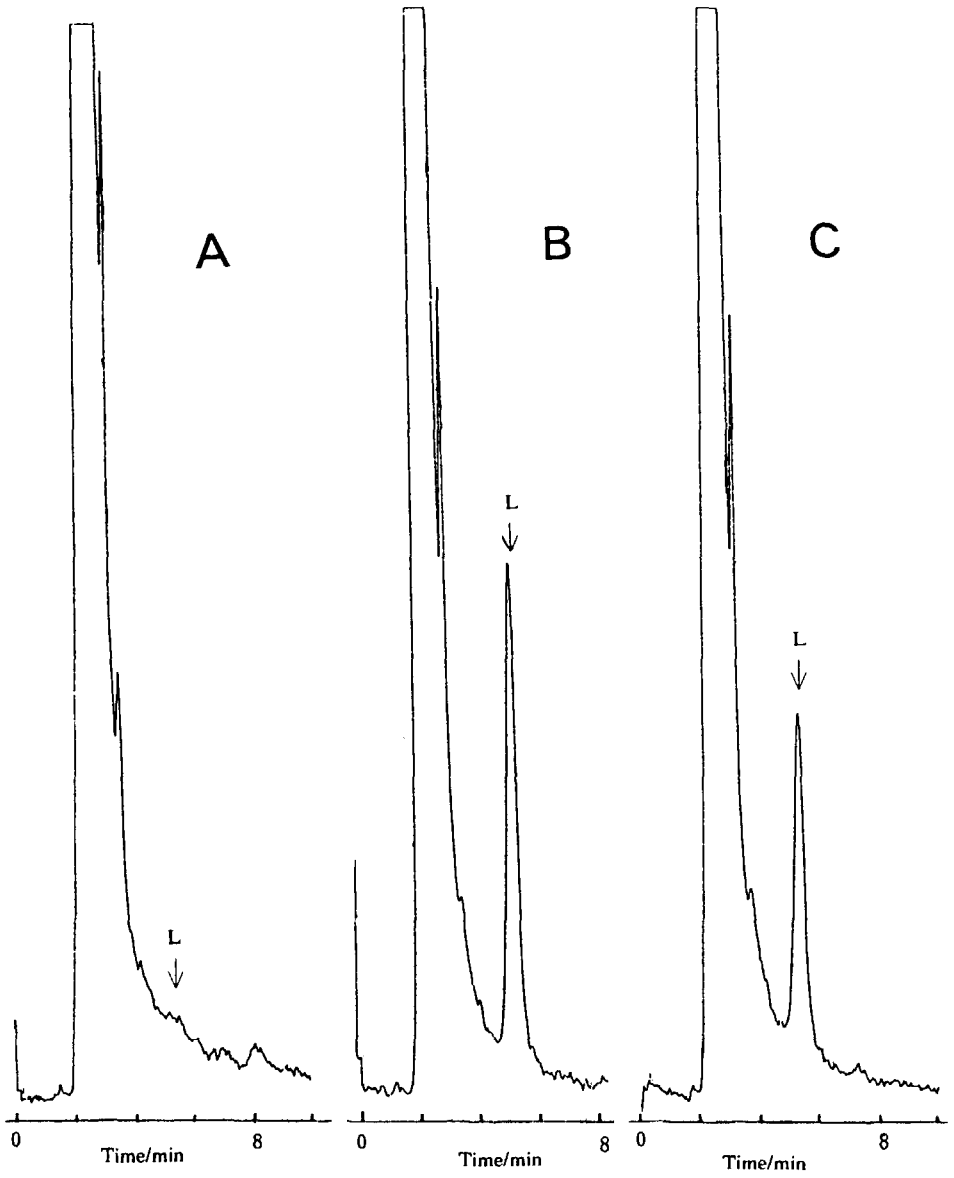

Fig. 2. Chromatograms of (A) blank poultry muscle extract, (B) blank poultry muscle extract plus lasalocid standard (0.010 mg kg^{-1}) and (C) spiked poultry muscle extract (0.010 mg kg^{-1}) using a PLRP-S 250 mm × 4.6 mm I.D. column. L = position of lasalocid peak.

tions. The standard curve for lasalocid was linear from 0 to 10.00 $\mu g\ ml^{-1}$ with a correlation coefficient of 1.0000.

Extraction and clean-up were modified from the procedure of Okada *et al.* [11]. The extraction solvent was changed to acetonitrile and prepacked Bond-Elut Si cartridges used for clean-up of the extract. Blank muscle tissue extract made up with standard lasalocid in mobile phase gave peak heights of 88.8% [coefficient of variation (C.V.) 2.8%] of those of standard lasalocid in mobile phase. Hence quantitation for muscle tissue was carried out by reference to blank extract made up with standard in mobile phase.

The limit of determination for muscle tissue was taken to be 0.002 mg kg^{-1}. Overall recoveries were 65.7% (C.V. 11.4%) at 0.002 mg kg^{-1}, 72.0% (C.V. 5.8%) at 0.010 mg kg^{-1} and 77.9% (C.V. 4.3%) at 0.050 mg kg^{-1}. Repeatability and reproducibility at the 0.002 and 0.010 mg kg^{-1} levels after chromatography on the polymer column are summarised in Table I. Representative chromatograms on the polymer column of blank tissue extract, blank tissue extract with added la-

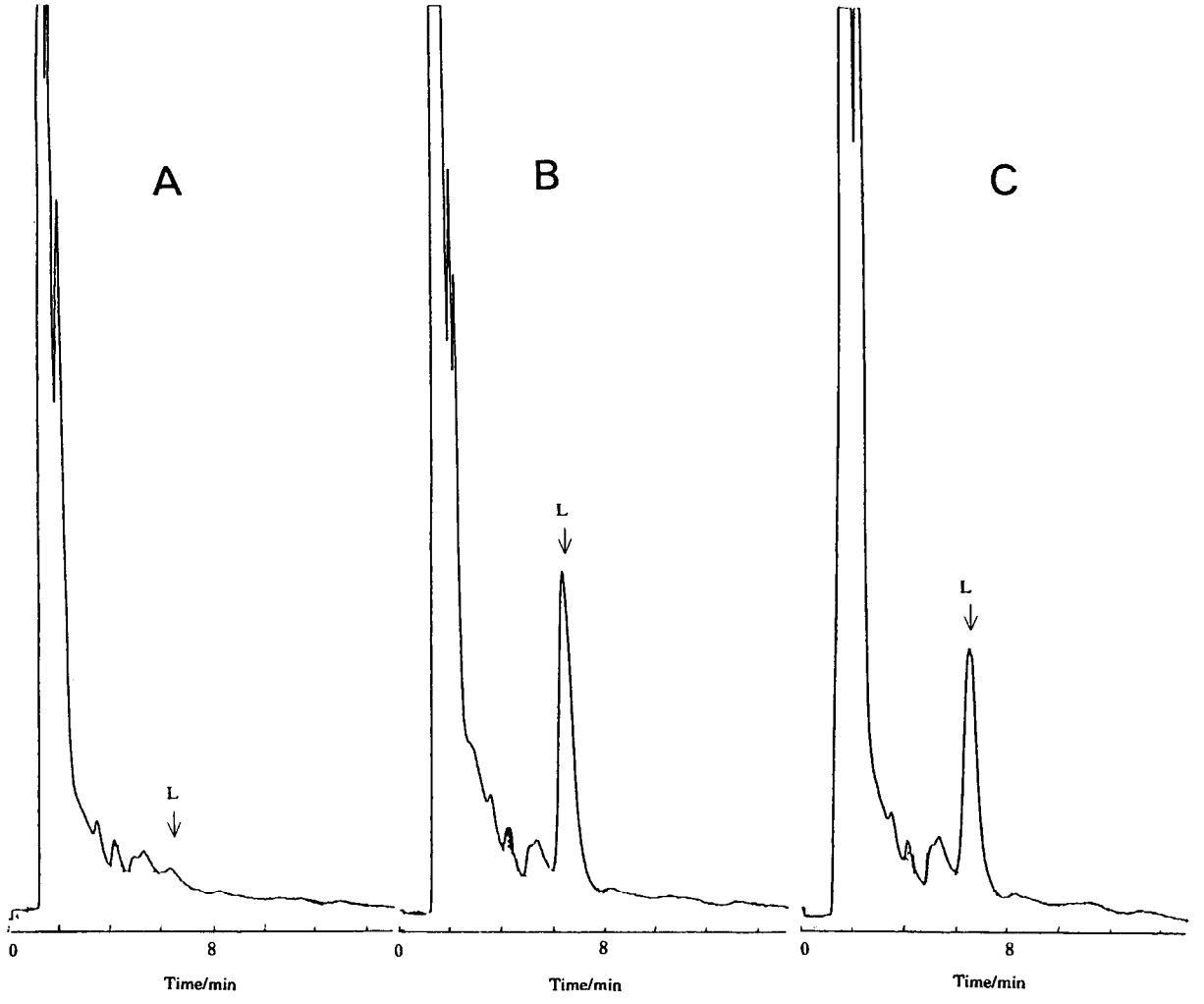

Fig. 3. Chromatograms of (A) blank poultry muscle extract, (B) blank poultry muscle extract plus lasalocid standard (0.010 mg kg^{-1}) and (C) spiked poultry muscle extract (0.010 mg kg^{-1}) using a Hypercarb 100 mm × 4.6 mm I.D. column. L = position of lasalocid peak.

TABLE II

RECOVERIES OF LASALOCID FROM EGG SPIKED AT 0.010 AND 0.100 mg kg^{-1} ON A HYPERCARB COLUMN

Day	Recovery (mean ± S.D.) (%)	C.V. (%)	*n*
0.010 mg kg^{-1}			
1	71.8 ± 4.9	6.9	5
2	88.1 ± 3.0	3.4	4
3	69.6 ± 10.1	14.6	4
Overall	76.2 ± 10.4	13.7	13
0.100 mg kg^{-1}			
1	80.9 ± 4.6	5.7	5
2	77.2 ± 4.8	6.2	4
3	71.3 + 9.4	13.1	5
Overall	76.4 ± 7.9	10.3	14

salocid standard at a concentration equivalent to 0.010 mg kg^{-1} and extract from tissue spiked at 0.010 mg kg^{-1} are shown in Fig. 2. Representative chromatograms on the PGC column of blank tissue extract, blank tissue extract with added lasalocid standard at a concentration equivalent to 0.010 mg kg^{-1} and extract from tissue spiked at 0.010 mg kg^{-1} are shown in Fig. 3.

The method was validated to the 0.010 mg kg^{-1} level for egg tissue incorporating HPLC on the PGC column. Overall recoveries were 76.2% (C.V. 13.7%) at 0.010 mg kg^{-1} and 76.4% (C.V. 10.3%) at 0.100 mg kg^{-1} (Table II). Representative chromatograms of blank egg extract, standard lasalocid at 0.010 mg kg^{-1} and extract from

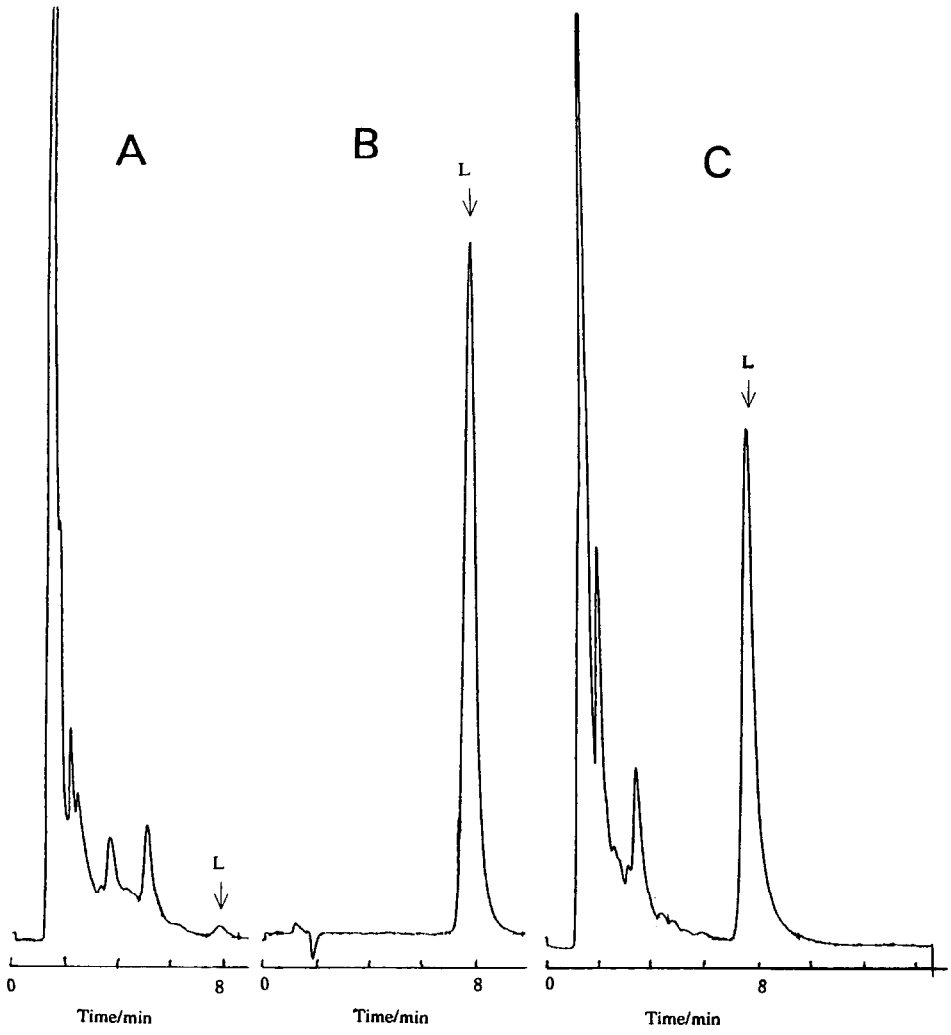

Fig. 4. Chromatograms of (A) blank egg extract, (B) lasalocid standard (0.010 mg kg^{-1}) and (C) spiked egg extract (0.010 mg kg^{-1}) using a Hypercarb 100 mm × 4.6 mm I.D. column. L = position of lasalocid peak.

blank egg spiked at 0.010 mg kg^{-1} are shown in Fig. 4.

CONCLUSION

A method for the analysis of the ionophore antibiotic lasalocid by RP-HPLC on a polymeric column has been developed. It is simple, rapid (eight samples may be extracted and analysed per batch) and has an improved limit of determination of 0.002 mg kg^{-1} for muscle tissue and 0.010 mg kg^{-1} for egg.

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