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Iso- and epi-iso-chlortetracycline are the principal metabolites of chlortetracycline in the hen's egg

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Abstract

Chlortetracycline (CTC) is a broad spectrum antibiotic, licensed for use without any withdrawal period, in chickens laying eggs intended for human consumption. In the European Union, a maximum residue limit (MRL) in eggs of 200 μg/kg for the sum of the concentrations of CTC and its 4-epimer (4-epi-CTC) has been established. Two major CTC metabolites have been identified in eggs. These compounds, iso-CTC and 4-epi-iso-CTC, have never previously been shown to be significant products of CTC metabolism in poultry or in any other species. The total amount of CTC present in eggs, as all of the chemical forms measured, can exceed the MRL by anything up to a factor of four (170–820 μg/kg).

Keywords: Eggs; Food analysis; Chlortetracycline; Tetracyclines; Antibiotics

1. Introduction

Chlortetracycline (CTC) is the oldest member of the tetracycline group of antibiotics. It is obtained by anaerobic fermentation of *Streptomyces aureofaciens* and is prepared industrially by bulk fermentation. CTC is active against a wide range of gram-positive and -negative organisms. CTC acts by inhibiting the attachment of aminoacyl-tRNA to the 30S ribosome, thus preventing protein synthesis. CTC is widely used in the UK as an aid in the treatment of respiratory and systemic infections in pigs, poultry, etc.

Under mildly acidic conditions, CTC can reversibly epimerise to form 4-epi-CTC (Fig. 1). The existence of 4-epi-CTC has been recognised in the European Union's legislation on the control of veterinary drug residues [1]. The maximum residue limit (MRL) for CTC in edible animal tissues has been defined as the sum of the concentration of CTC and 4-epi-CTC. In eggs, the European Union has established an MRL for CTC plus 4-epi-CTC of 200 μg/kg [2]. All regulatory laboratories will need to evaluate the suitability of their routine methods for the detection of CTC in eggs. The use of veterinary medicines is subject to strict withdrawal periods to ensure that drug residue concentrations in edible tissues are less than the MRL. Few drugs are licensed for use in birds laying eggs intended for human consumption. CTC is, however, currently licensed for use in egg-laying chickens, at a concentration in feed of 300 mg/kg, without any withdrawal period. This follows a number of studies that have shown that CTC does not transfer into eggs to any significant extent [3–6]. Eggs collected from birds fed CTC at 200–600 mg/kg contained CTC at concentrations ranging from 0–190 μg/kg, following zero withdrawal.

The majority of chemical methods published recently for the determination of CTC residues in

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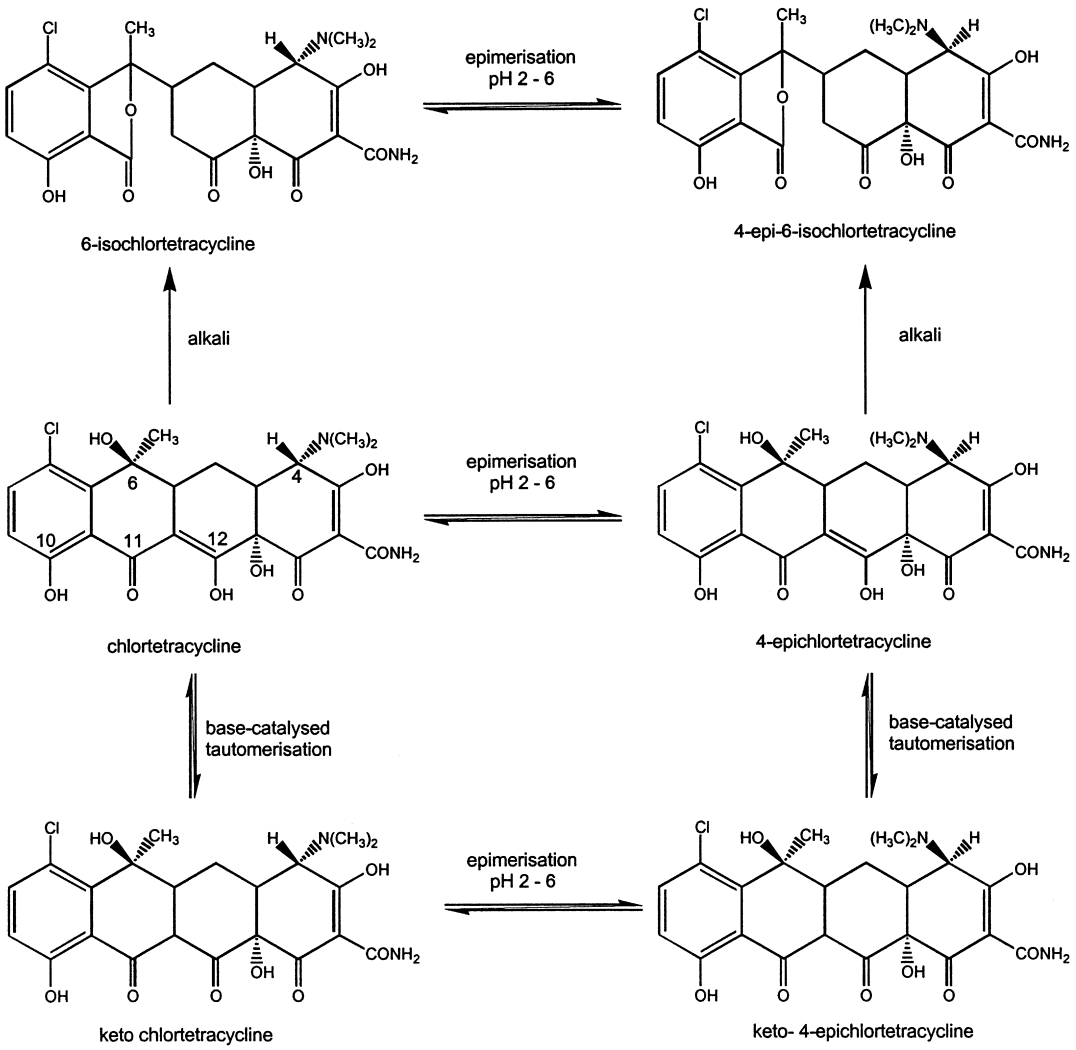

Fig. 1. Structure of CTC and related compounds.

meat have involved the use of either high-performance liquid chromatography (HPLC) or liquid chromatography–mass spectrometry (LC–MS). This area was the subject of a comprehensive review by Oka and Patterson [7]. The HPLC assay described by Oka et al. [8] or variants thereof, is perhaps the most widely used procedure. CTC is extracted from tissues in an acidic buffer, and is cleaned up using C_{18} solid-phase extraction. The UV absorption of CTC (350 nm) is measured at pH 2.0 following HPLC separation with a LiChrosorb RP-8 column. Farrington et al. [9] developed an improved clean-up procedure that involved clean-up of CTC from tissue homogenates using a copper chelate affinity column. A similar detection procedure was used.

The metal binding ability of the tetracyclines has been exploited in a number of HPLC assays for CTC. Following complexation with divalent or trivalent cations, CTC fluoresces strongly. Complexes between CTC and calcium [10], zirconium [11] and aluminium [12] have all been used in the HPLC determination of this drug.

Treatment of CTC and 4-epi-CTC with alkali results in the formation of iso-CTC and 4-epi-iso-CTC, respectively. The strong fluorescence of these species has been exploited in a number of published

HPLC methods for the determination of CTC residues [13–15].

CTC, like the other tetracyclines is non-volatile. Consequently, LC–MS is the only practical technique for the mass spectrometric determination of CTC residues. Methods employing a range of ionisation techniques including particle beam MS [16], frit fast atom bombardment (FAB) MS [17], electrospray MS–MS [18] and atmospheric pressure chemical ionization (APCI) MS [19] have been described recently. The last three methods produce the same fragment ions, namely: *m/z* 479, 462 and 444, corresponding to $[M+H]^+$, $[M+H-NH_3]^+$ and $[M+H-NH_3-H_2O]^+$, respectively.

In this laboratory, three validated methods for the analysis of CTC are in regular use. These methods are: HPLC with fluorescence detection of iso-CTC [13], HPLC with fluorescence detection of an Al^{3+}–CTC complex [12], and HPLC–APCI-MS [19]; and have been used to control residues of CTC and other tetracyclines in red meat produced in Northern Ireland for many years. European Union legislation on drug residues has now been extended to include poultry, and will be extended further in 1999 to include eggs. This study arose out of attempts to validate our existing methods for the determination of CTC residues in incurred egg samples.

2. Experimental

2.1. Animal study

Six layer chickens in mid-lay (approximately 30 weeks of age) were purchased from a local farm. The birds were housed in individual wire-floored cages and were allowed ad libitum access to fresh water at all times. The birds were fed 120 g, once daily, of a commercially available drug-free ration for two weeks. Eggs were collected to act as controls, were homogenised and stored at −20°C prior to analysis. The birds were then fed a similar diet containing 300 mg CTC per kg for five days. The CTC preparation used was obtained from Sigma (Poole, UK) and contained no iso-CTC. On the fifth day, the eggs were collected and treated as described above. All analyses were completed within one week of collection.

2.2. Iso-CTC assay

This assay was performed exactly as described by Blanchflower et al. [13]. Briefly, CTC residues were extracted from homogenised tissues into 0.1 mol/l glycine in 1.0 mol/l HCl. Samples were concentrated and cleaned up using Bond-Elut 3-ml cyclohexyl cartridges. CTC residues were converted to iso-CTC at pH 12.0. A reversed-phase polymeric column (PLRP-S 100 Å, 5 μm, 150×4.6 mm I.D., Polymer Labs., UK) was used with a mobile phase that consisted of 0.1 mol/l glycine, pH 12.0–acetonitrile (87.5:12.5, v/v). Fluorescence was measured at 420 nm following excitation at 340 nm.

2.3. Al^{3+} derivatisation assay

This method was performed exactly as described by McCracken et al. [12]. Briefly, CTC residues were extracted from tissues and subjected to a clean-up step in a procedure similar to that described above. A gradient, based on 0.02 mol/l oxalic acid–acetonitrile was employed to separate CTC from oxytetracycline and tetracycline. The column used was a Chromsep (Chrompack, UK) glass column containing Chromspher C_8 (5 μm). The Al^{3+} derivative of CTC was formed by post-column derivatisation with 0.75 mol/l $AlCl_3$. A wavelength of 390 nm was used for excitation, with emission at 490 being monitored.

2.4. LC–APCI-MS assay

This method was performed essentially as described by Blanchflower et al. [19]. Briefly, the extraction procedure developed for the Al^{3+} method was used. A gradient system was used, employing a mobile phase that consisted of acetonitrile, 10 mmol/l oxalic acid and 10 μmol/l EDTA. The LC–MS system consisted of a VG Platform II (Micromass, UK), fitted with an APCI probe. The instrument was operated in the positive ion mode. Full scan data were collected in order to obtain spectra from standards and single ion data (dwell time 0.5 s) were collected when analysing samples. The source of the instrument was maintained at 150°C. The flow-rates of the drying and sheath gases were 300 and 50 l/h, respectively. The temperature of the APCI probe was

500°C. The source was cleaned after about 18 h operating time, a simple procedure that took about 0.5 h. The instrument was calibrated weekly using a polyethylene glycol mixture. The LC–MS was set to collect multiple single ion data for ions at m/z 479, 462 and 444. These corresponded to $[M+H]^+$, $[M+H-NH_3]^+$ and $[M+H-NH_3-H_2O]^+$, respectively.

3. Results and discussion

3.1. CTC content of eggs measured using the iso-CTC assay

Fig. 2 shows a typical HPLC trace obtained (egg 6). This egg contained substantial concentrations of both CTC and 4-epi-CTC, as judged by comparison with the CTC and 4-epi-CTC standards (each equivalent to 400 μg/kg). The mean±S.E.M. ($n=6$) concentrations of CTC and 4-epi-CTC in six eggs from therapeutically treated layer chickens were 320±50 and 160±30 μg/kg, respectively. The MRL for CTC in eggs is 200 μg/kg. Therefore these eggs, taken from birds that had been treated in accordance with the product license for CTC, apparently had a CTC content of up to almost three-times the legally binding MRL. Since this was clearly a most unexpected result, the same eggs were analysed using the Al^{3+} derivatisation HPLC assay.

3.2. CTC content of eggs measured using the Al^{3+} derivatisation assay

Fig. 3 shows a typical HPLC trace obtained (egg 6). In stark contrast to Fig. 2, virtually no CTC or 4-epi-CTC was present in this egg, as judged by comparison with the CTC and 4-epi-CTC standards (equivalent to 500 μg/kg). The mean±S.E.M. ($n=6$) concentrations of CTC and 4-epi-CTC in six eggs from therapeutically treated layer chickens were 80±10 and 40±10 μg/kg, respectively. However, these results were much more in accord with the concentration that we expected to encounter. In an attempt to resolve the gross disparity between the two sets of results, the eggs were analysed using LC–APCI-MS.

3.3. CTC content of eggs measured using LC–APCI-MS

Fig. 4 shows a typical LC–MS trace obtained (egg 6). Under the conditions chosen, both CTC and 4-epi-CTC give rise to three ions at m/z 479, 462 and 444, arising from the successive loss of am-

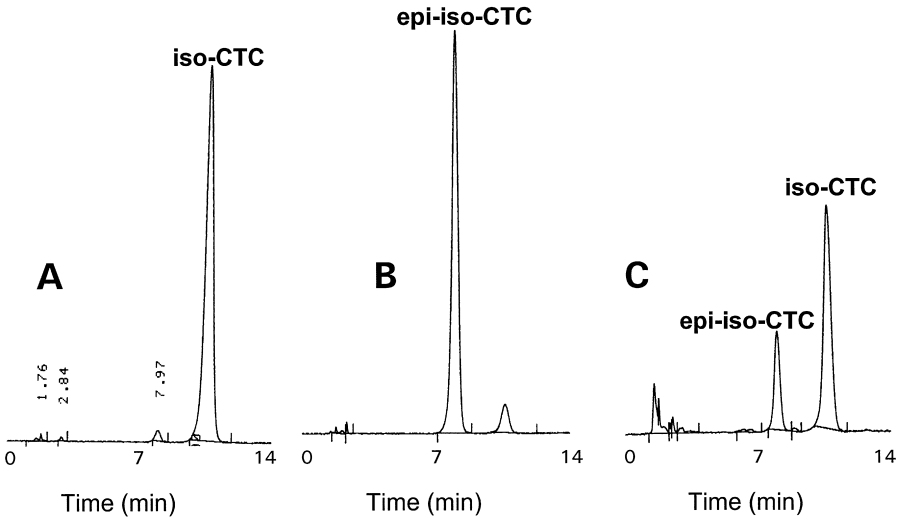

Fig. 2. Traces obtained using HPLC with fluorescence detection of (A) CTC standard (400 μg/kg), (B) 4-epi-CTC standard (400 μg/kg), and (C) egg 6; following analysis using the iso-CTC method [13].

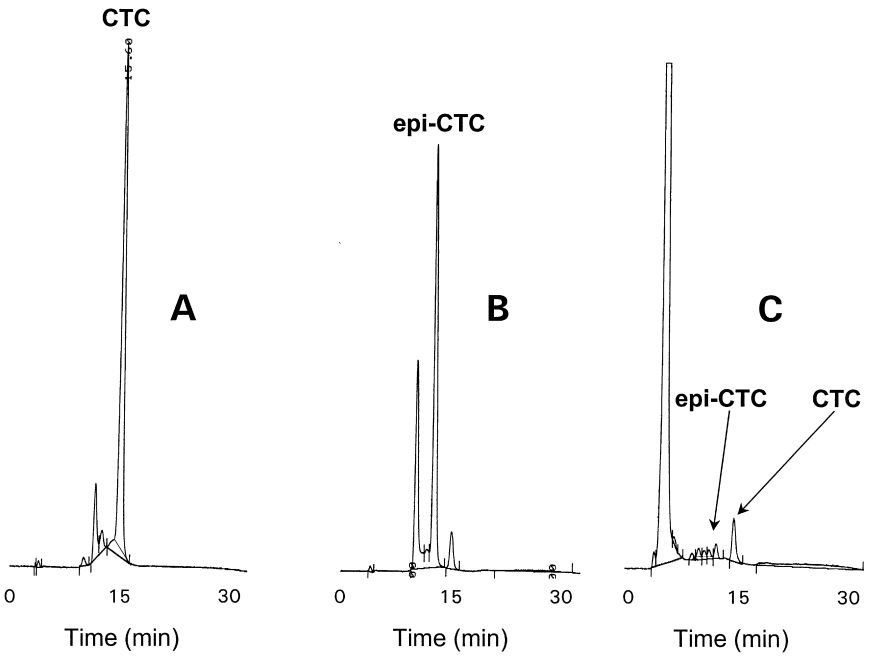

Fig. 3. Traces obtained using HPLC with fluorescence detection of (A) CTC standard (500 μg/kg), (B) 4-epi-CTC standard (500 μg/kg), and (C) egg 6; following analysis using the Al^{3+} derivatisation method [12].

monia and water from the $[M+H]^+$ ion. Minor peaks containing all three of these ions were present in some eggs at the retention times corresponding to CTC and its 4-epimer. These peaks co-eluted with standard solutions, and were identified as CTC and 4-epi-CTC. The mean±S.E.M. ($n=6$) concentrations of CTC and 4-epi-CTC in eggs from therapeutically treated layer chickens were 90±10 and 30±10 μg/kg, respectively. These results were very similar to those obtained using the Al^{3+} derivatisation assay; but differed significantly from those recorded using the iso-CTC assay. However, in addition to the minor peaks corresponding to CTC and 4-epi-CTC, two major peaks were observed.

We have recently reported the presence of keto isomers of CTC and epi-CTC in pig tissues [19]. Our original hypothesis was that the unidentified peaks in the incurred eggs corresponded to keto-CTC and keto-4-epi-CTC (Fig. 1). However, this was not the case for two reasons. Firstly, the unidentified peaks did not show the same retention time as either keto-CTC or keto-4-epi-CTC (Fig. 4). Secondly, the keto isomers of CTC both produce the same fragments as CTC itself, at m/z 479, 462 and 444 [19]. The two unidentified peaks lacked the fragment ion at m/z 444 (Fig. 4).

3.4. Identification of unknown peaks

Subsequent investigations suggested that the unidentified peaks on the APCI trace might be iso-CTC and 4-epi-iso-CTC. We were able to obtain an iso-CTC standard, but were unable to obtain pure 4-epi-iso-CTC. However, pure iso-CTC standard readily epimerises to form a mixture of iso-CTC and 4-epi-iso-CTC. Under the APCI conditions used, iso-CTC and 4-epi-iso-CTC form an $[M+H]^+$ ion at m/z 479 and lose ammonia to form an ion at m/z 462. However, neither compound can lose water to form a fragment at m/z 444 (Fig. 5). Their fragmentation pattern is therefore similar to that of the unidentified peaks in the incurred egg samples. The co-elution of the unidentified peaks with iso-CTC and 4-epi-iso-CTC provided further proof of the identity of these compounds (Fig. 6). It is not known at this stage if these metabolites are formed as a result of an

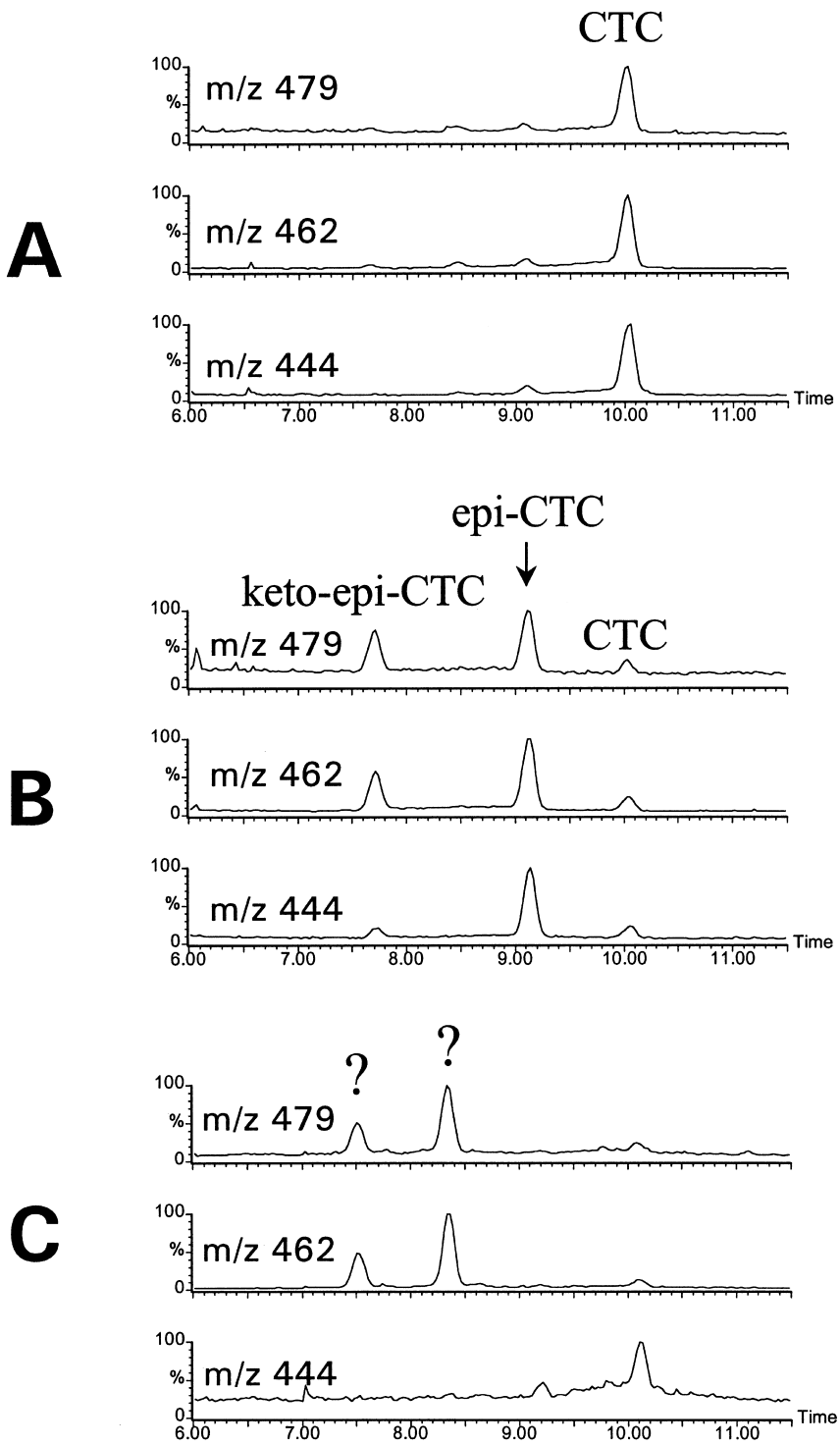

Fig. 4. Selected ion monitoring traces (m/z 479, 462 and 444) of (A) CTC standard (500 μg/kg), (B) 4-epi-CTC standard (500 μg/kg), and (C) egg 6; following analysis using the LC–APCI-MS method [19]. Time in min.

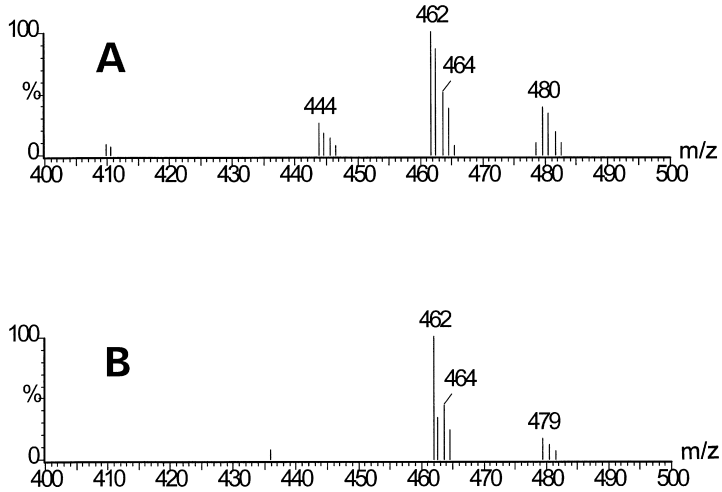

Fig. 5. Positive ion APCI scans of (A) CTC and (B) iso-CTC standards.

enzyme-catalysed reaction, or as a result of pH conditions in either the chicken or the egg. Further studies are in progress that may provide answers to this question.

There are three main classes of HPLC methods for the determination of CTC residues. These are based: (a) on the introduction of a chromophore following derivatisation with metal ions; (b) on the UV absorbance of the molecule in an acidic mobile phase; and (c) on the pre-column formation and quantification of iso-CTC. Can iso-CTC and 4-epi-iso-CTC be detected using these assays?

3.5. Detection of iso-CTC and 4-epi-iso-CTC by assays employing metal ion derivatisation

A number of methods have been described that involve derivatisation of the tetracyclines with a metal ion to form fluorescent derivatives. Metal ions such as aluminium [12], calcium [10] and zirconium [11] have been employed in such assays. One of these methods, developed in this laboratory, based on post-column derivatisation with Al^{3+} followed by fluorescence detection [12], clearly does not detect either iso-CTC or 4-epi-iso-CTC (Fig. 3). All of the metal-binding assays require the integrity of the metal ion-binding site on the tetracycline molecule. There is considerable controversy in the literature concerning the assignment of the site of metal ion co-ordination in the tetracycline molecule. However, a number of studies [20,21] have suggested that chelation occurs via the oxygen atoms at C11 and C12. The chemistry of this region is disrupted by the base-catalysed formation of the phthalide iso-CTC. The iso derivatives of tetracyclines have been shown to have no metal binding activity at C11 and C12 [22]. Therefore, it is probable that none of the CTC assays based on the formation of metal ion derivatives will be capable of detection of the iso derivatives of CTC.

3.6. Detection of iso-CTC and 4-epi-iso-CTC in assays employing UV detection in an acidic mobile phase

The most widely used assays for CTC employ HPLC with UV detection (350–370 nm) in an acidic (pH 2.0) mobile phase. Many of these assays are based on the method originally developed by Oka et al. [8]. Another widely used method, described by Farrington et al. [9] employed metal chelate affinity chromatography clean-up followed by UV detection at 350 nm in a similar acidic mobile phase. However, even if the copper-based affinity column retained iso-CTC, neither of these methods could detect iso-

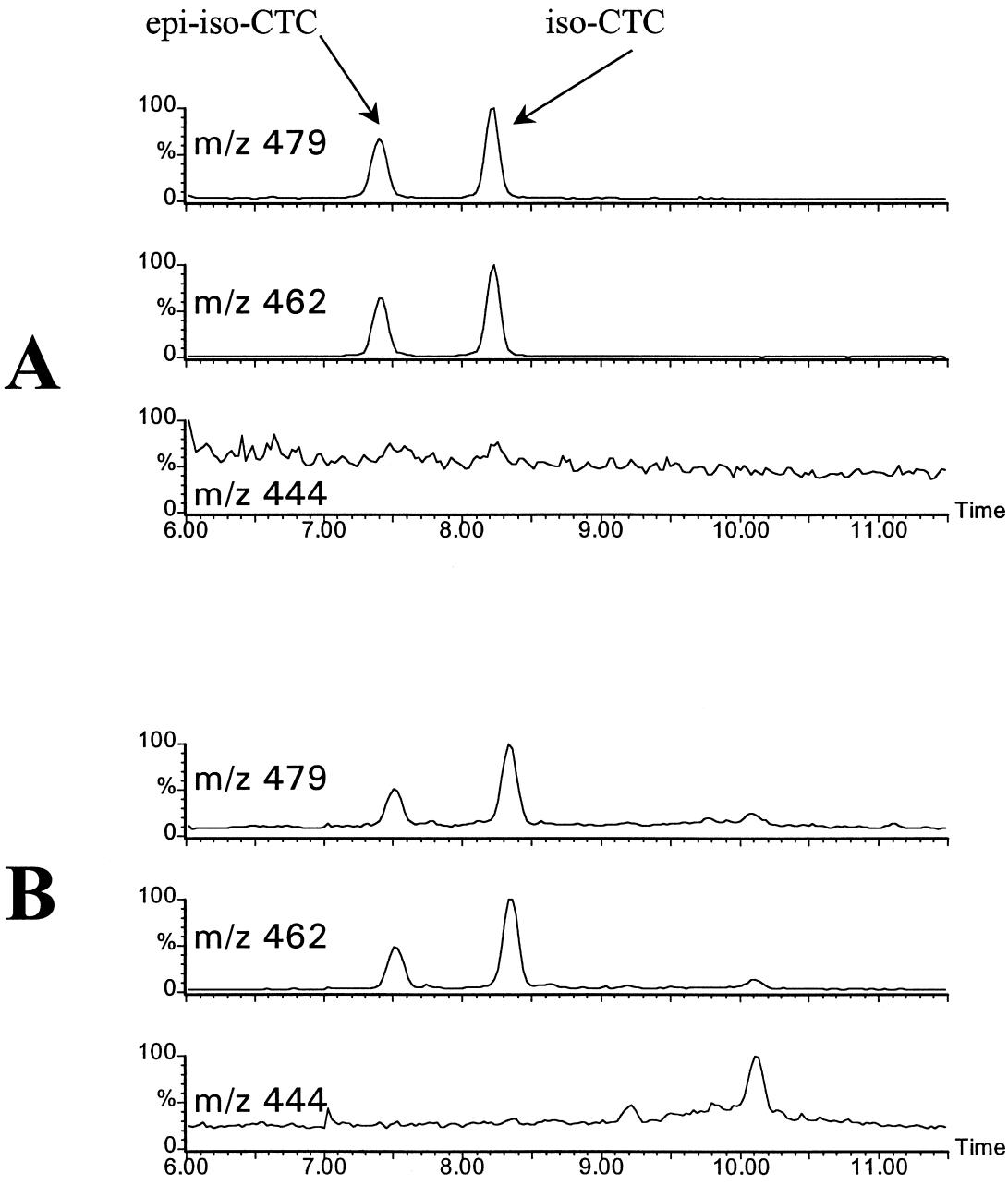

Fig. 6. Selected ion monitoring traces (m/z 479, 462 and 444) of (A) iso-CTC standard (500 μg/kg) and (B) egg 6; following analysis using the LC–APCI-MS method [19]. Time in min.

CTC. The UV chromophore of iso-CTC is highly pH dependent. At pH 12, there is strong UV absorbance around 350 nm. However, at pH 2.0, this is completely abolished (Fig. 7). Virtually all of the UV absorbance based assays for CTC measure the 350 nm chromophore. None of these assays will therefore detect the iso derivatives of CTC in acidic mobile phases.

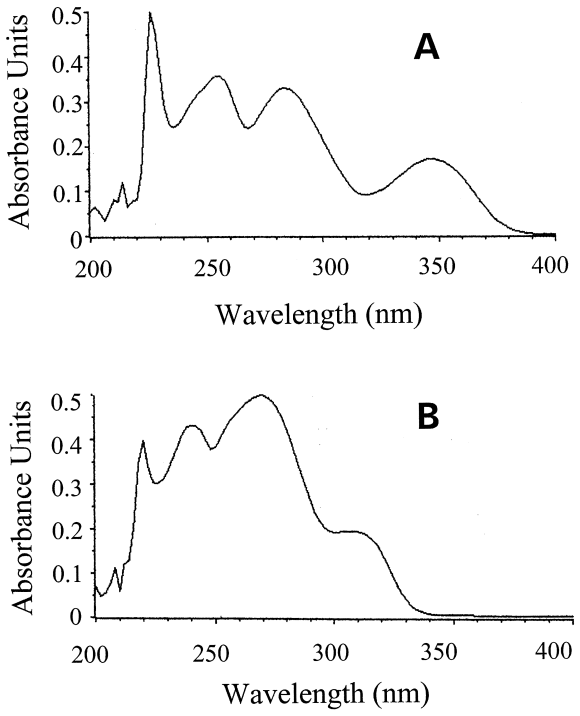

Fig. 7. UV absorbance of iso-CTC at (A) pH 12.0 and (B) pH 2.0.

3.7. Detection of iso-CTC and 4-epi-iso-CTC by assays employing fluorescence detection following base-catalysed formation of iso- derivatives

This method has been in routine use in this laboratory [13] and others [14,15] for many years for the confirmation of CTC residues in red meat. CTC and 4-epi-CTC are detected as their corresponding fluorescent iso derivatives. Obviously, any iso derivatives that are present in a sample will also be detected and quantified. Currently, the MRL for CTC is the sum of CTC and 4-epi-CTC. Iso-CTC and 4-epi-iso-CTC are not included in the total. However, iso-CTC is not a significant mammalian CTC metabolite. Eisner and Wulf [23] identified it as a minor CTC component in canine urine, in early studies. It does not, however, contribute significantly to tissue residue levels in red meat (unpublished observations). Consequently, the performance of our normal assay in the analysis of red meat is unaffected. However, substantial amounts of iso-CTC are clearly present in eggs (Figs. 2 and 4).

3.8. Inter-assay differences in the measured CTC concentrations

The validated methods available in this laboratory for the analysis of CTC utilise very different chemical principles. They also, apparently, give very different results when applied to the analysis of CTC in eggs. However, given that the vast majority of CTC/4-epi-CTC present in eggs is present as the corresponding iso derivative, the degree of agreement between the three assays is remarkable (Table 1). The amount of CTC and 4-epi-CTC measured using the Al^{3+} assay (columns 4 and 5, respectively) in the eggs was very similar to that measured using LC–APCI-MS (columns 7 and 9, respectively). In addition, the amount of CTC (being the sum of CTC and iso-CTC) and 4-epi-CTC (being the sum of 4-epi-CTC and 4-epi-iso-CTC) measured using the iso-CTC HPLC assay (columns 1 and 2, respectively) was very similar to that measured using LC–APCI-MS (columns 12 and 13, respectively).

The MRL for CTC in eggs is 200 μg/kg, and is expressed as the sum of the tissue concentrations of CTC and 4-epi-CTC. The existence of the iso derivatives of CTC as the major metabolite in any human food has never been previously demonstrated. Taken together, the total amount of CTC present in these eggs, in all of the chemical forms measured, can exceed the MRL by anything up to a factor of four (170–820 μg/kg, Table 1).

3.9. Implications of this work

Few veterinary medicines are licensed, in the UK, for use in layer chickens without any withdrawal period. CTC is one such compound. Previous work, using microbiological assays, has shown that very little CTC accumulates in eggs [3–6]. However, the finding, reported here, that substantial quantities of iso-CTC/4-epi-iso-CTC are formed in eggs merits extensive further study. Iso-CTC shows no activity in a microbiological growth inhibition test – the four plate test (unpublished observations). However, its toxicological properties are unknown. Regulatory authorities throughout the world must be made aware of the fact that the vast majority of assays for CTC will fail to detect this CTC metabolite in eggs. Studies on the pharmacokinetics of iso-CTC forma-

Table 1
CTC/metabolite concentrations in six eggs measured using three validated methods for CTC analysis

Egg	Iso-CTC HPLC assay			Al^{3+} derivatisation HPLC assay			HPLC–APCI-MS assay						
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)
	CTC[a]	4-epi-CTC[b]	Total (1+2)	CTC	4-epi-CTC	Total (4+5)	CTC	iso-CTC	4-epi-CTC	4-epi-iso-CTC	Total (7–10)	CTC+iso-CTC (7+8)	4-epi-CTC+ 4-epi-iso-CTC (9+10)
1	110	50	160	20	10	30	30	80	10	50	170	110	60
2	420	230	650	120	50	170	140	390	50	240	820	530	290
3	290	170	460	70	50	120	90	250	30	140	510	340	170
4	290	160	450	70	30	100	80	270	30	150	530	350	180
5	450	210	660	90	50	140	100	260	40	190	590	360	230
6	350	150	500	80	20	100	80	240	0	150	470	320	150

All values have been corrected for analytical recovery, and are reported as μg/kg. The MRL for CTC in eggs is 200 μg/kg of CTC and 4-epi-CTC combined.
[a] Assay measures the sum of endogenous iso-CTC+CTC.
[b] Assay measures the sum of endogenous 4-epi-CTC and 4-epi-iso-CTC.

tion in eggs, chickens and red meat species, the incidence of CTC residues in eggs, and the effect of low-level feed contamination with CTC on egg CTC residues are under way in this laboratory and will be reported elsewhere. Further studies on the toxicological properties of iso-CTC are also urgently needed to facilitate a re-evaluation of the permissible use of CTC in layer chickens.

In conclusion, the previously accepted position, that CTC residues do not occur in eggs, has been shown to be false. Substantial quantities of iso-CTC and 4-epi-iso-CTC accumulate in the eggs of birds that had been treated exactly in accordance with the manufacturer's instructions. The total amount of these residues is the equivalent of up to four-times the MRL for CTC in eggs. Although microbiologically inactive, the toxicological properties of iso-CTC and 4-epi-iso-CTC are unknown.

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