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Automated residue analysis of tetracyclines and their metabolites in whole egg, egg white, egg yolk and hen's plasma utilizing a modified ASTED system

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Abstract

An automated analytical method is described allowing simultaneous determination of all tetracyclines and metabolites in whole egg, egg yolk, egg white and blood plasma of the hens. Sample pretreatment is restricted to homogenization and a dilution step. Clean-up is by on-line dialysis and on-line solid-phase extraction utilizing an extended ASTED system, followed by liquid chromatography with UV or fluorescence detection with post-column pH adjustment and confirmational analysis by LC–MS–MS. After feeding oxytetracycline (OTC), tetracycline (TC) and chlortetracycline (CTC) to laying hens, not only residual parent compounds could be found in the eggs but also in vivo formed 4-epimers, isochlortetracycline (ICTC) and tentatively identified *N*-desmethyl metabolites of OTC, TC and ICTC.

Keywords: Tetracyclines

1. Introduction

In 1998 the European Federation of Animal Health (FEDESA) conducted a survey on the veterinary use of antibiotics in the 15 member states of the European Union and Switzerland (cited in Ref. [1]). Therapeutic and prophylactic use in 1998 of tetracyclines with 2294 t ranked far ahead of macrolides 424 t, penicillins 322 t, aminoglycosides 154 t, sulfonamides/trimethoprim 75 t, fluoroquinolones 43 t, others 182 t, and of the sum of antibiotics used for growth promotion with 1599 t. Tetracyclines (TCs) are approved for treating laying hens with a maximum residue limit of 200 μg/kg for oxytetracycline (OTC), tetracycline and chlortetracycline (CTC), respectively, expressed as the sum of parent compound and its 4-epimer. According to EU Commission decisions 93/256 and 93/257, reference materials should be used to verify or validate methods for residue control [2,3]. Currently no reference materials are available with incurred residues of tetracyclines nor exist analyte stability studies with incurred residues of tetracyclines in food matrices. Having gained experience in an analyte stability study of incurred nitrofuran residues in whole eggs [4] with the ASTED system, we decided to perform an analyte stability study of tetracyclines in eggs as a first step of obtaining the respective reference materials. The ASTED system seemed excellently suited for analyzing large numbers of samples, as it provides the means of uninspected automated residue analysis with sample pretreatment restricted to homogenizing and diluting the samples. Furthermore, the consumption of organic solvents is minimal.

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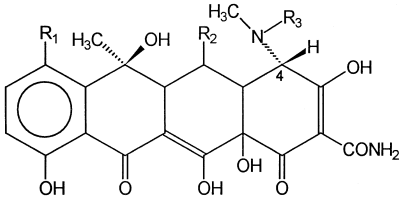
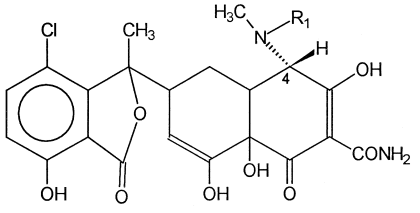
The first feeding study, however, revealed surprising results. Residues were not restricted to the parent compounds. We observed substantial amounts of in vivo formed 4-epimers and other metabolites [5], Fig. 1. This led to the decision to investigate the residue behavior, depletion characteristics and distribution patterns of OTC, TC and CTC by analyzing not only whole egg, but also egg white, egg yolk, plasma and the feed. For this purpose the ASTED system, as it has been used by us for nitrofurans [4] or by Agasøster for the analysis of OTC [6], had to be modified and instrumentally extended. Provisions for extra cleansing steps had to be incorporated. This was necessary due to deterioration of the dialysis and enrichment procedure during continuous several month's use of the ASTED system, especially when analyzing egg yolk. A post-column derivatization procedure was installed to allow the detection of the iso-derivatives of CTC. These have been identified by Kennedy et al. to be the dominating residues in eggs after feeding CTC [7]. This paper describes the instrumental set-up and analytical procedure which could be used for continuous automated analysis of over 2000 egg samples within a period of two years without special maintainance for the system.



Compound		R_1	R_2	R_3
Tetracycline	TC	H-	H-	CH_3-
Oxytetracycline	OTC	H-	HO-	CH_3-
Chlortetracycline	CTC	Cl-	H-	CH_3-
N-Desmethyltetracycline*	DM-TC	H-	H-	H-
N-Desmethyloxytetracycline*	DM-OTC	H-	HO-	H-

* tentatively identified



Compound		R_1
Isochlortetracycline	ICTC	CH_3-
4-N-Desmethylisochlortetracycline*	DM-ICTC	H-

* tentatively identified

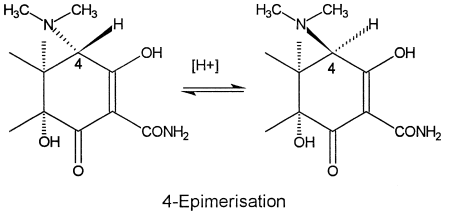


Fig. 1. Chemical structures of tetracyclines and their metabolites found as incurred residues in eggs and hen's plasma.

2. Experimental

2.1. Chemicals and reagents

TC, OTC, CTC (all as hydrochloride salts) were purchased from Sigma (Deisenhofen, Germany). Epi-tetracycline (e-TC), epi-oxytetracycline (e-OTC), epi-chlortetracycline (e-CTC) and isochlortetracycline (ICTC), α- and β-apo-oxytetracycline, anhydro- and epi-anhydro-tetracycline and anhydro- and epi-anhydro-chlortetracycline were obtained from Acros (Fisher Scientific, Schwerte, Germany). Analytical grade citric acid, oxalic acid, potassium dihydrogenphosphate, disodium hydrogenphosphate, sodium hydroxide and formic acid were purchased from Merck (Darmstadt, Germany), Triton X-100 and sodium glycine were from Sigma. Technical grade acetonitrile was distilled over K_2CO_3 and water was distilled to high-performance liquid chromatography (HPLC) quality.

2.2. Equipment

For sample pretreatment homogenization with an Ultra-Turrax from IKA Labortechnik (Staufen, Germany) was used. The sample preparation system was based on an ASTED unit from Gilson Medical Electronics (Abimed, Langenfeld, Germany) consisting of a Model 231 auto-sampling injector with a rack for 36 sample vials (4000 μl), two Model 401 dilutors equipped with 5-ml syringes, two flat-bed dialyser blocks coupled in series, with a total donor channel volume of 740 μl and a total recipient

channel volume of 1300 μl. The cuprophane dialysis membranes (Gilson) had a molecular mass cut-off of 15 000. This instrumentation (Fig. 2) was extended by the six-port valve A Model ELV-7000 from Kranich (Göttingen, Germany) and a low-pressure valve C Model LMV 870 from Kontron (Berlin, Germany). All valves were electronically controlled by the autosampler program for automatic function. A 5-ml loop from Rheodyne (Berkley, CA, USA) was installed on the additional six-port valve A. The on-line enrichment was accomplished utilizing a 20×4 mm cartridge from Chromatographie-Service (CS) (Langerwehe, Germany), packed with PLRP-S 100 Å, 70 μm particles, from Polymer Labs. (Shropshire, UK) as trace enrichment column (TEC). The autosampler rack was a specially designed aluminium block which could be thermostated and was held at 6°C (Model RM6 cryostat from Lauda, Königshofen, Germany). All the other instrumentation was operated at ambient temperature.

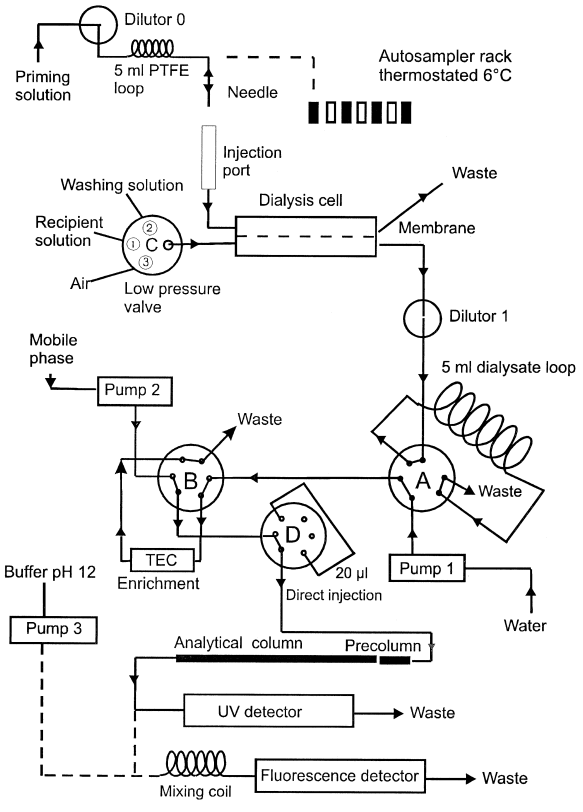


Fig. 2. Configuration of the extended ASTED system.

The analytical column was a Supersphere RP-8 column from Merck (4 μm, 250×3 mm I.D.). Acetonitrile–0.01 *M* aqueous oxalic acid (50:50, v/v) was used as mobile phase 1 at a flow-rate of 0.35 ml/min. Acetonitrile–0.005 *M* aqueous oxalic acid (50:50, v/v) had to be used as mobile phase 2 to separate OTC from e-OTC and DM-OTC from its 4-epimer.

The UV detector (Model L-4000, Merck–Hitachi, Darmstadt, Germany) was in general operated at 360 nm or at 310 nm when analyzing ICTC and DM-ICTC with the corresponding epimers. The fluorescence detector (Model F1050, Merck–Hitachi) was operated with high sensitivity at an excitation wavelength of 350 nm and an emission wavelength of 420 nm for monitoring the iso-derivates of CTC after post-column pH adjustment to pH 12 with a 0.4 *M* glycine buffer, pH 12. For this purpose an additional HPLC pump Model 510 from Waters (Milford, MA, USA) with a flow-rate of 0.1 ml/min was used with a mixing T-piece from CS and a knitted PTFE mixing coil, 5 m×0.3 mm I.D. from ICT Handels (Bad Homburg, Germany). All data from the two detectors were acquired and integrated with the APEX system from Autochrom (Milford, MA, USA). To allow direct manual injections onto the analytical column an additional six-port valve Type 7125 (valve D) with a 20-μl sample loop from Rheodyne was installed between valve B and the analytical column (see Fig. 2).

For the analyses with tandem mass spectrometry (MS–MS) the ASTED clean-up procedure was used up to the point of eluting the analytes with 350 μl mobile phase 1 from the TEC into a vial. An aliquot of 100 μl was injected into the LC–MS–MS system employing a mobile phase that consisted of acetonitrile–aqueous 0.5% formic acid (40:60, v/v), flow-rate 0.4 ml/min.

The LC–MS–MS system was a VG Quattro II (Micromass, Manchester, UK) with an electrospray source. The instrument was operated in the positive ion mode.

The electrospray conditions were as follows: temperature of the heated capillary was 80°C, the spray voltage was 3.5 kV and the sheath (base) gas was 350 l/h nitrogen. Cone voltage was 22 V. The nebulizer (drying) gas was nitrogen with 13 l/h, collision induced dissociation was performed using

argon as the collision gas at a pressure of 3.5 bar and a collision energy of 17 eV.

MS data were acquired with a dwell time of 0.5 s and scanwidth of 1 amu. The MS1 was set to transmit only the parent mass of interest and the parent ions were fragmented in the collision cell. A daughter ion was selected and the MS2 was set to transmit that mass only (Table 1).

2.3. Preparation of standard solutions

Stock solutions of OTC, TC, CTC and ICTC (1 mg/ml as dry free base) were prepared in methanol–0.01 mol/l aqueous oxalic acid (50:50, v/v). Spiking solutions (100 μg/ml) were prepared just before use by diluting the stock solution with acetonitrile–0.01 mol/l aqueous oxalic acid (30:70, v/v). Standards for direct analysis (no on-line dialysis) were prepared by diluting the spiking solutions with mobile phase.

2.4. Preparation of fortified samples for calibration

Whole egg or egg white was homogenized with an Ultra-Turrax and divided in portions of 150 g. The portions except one for blank controls were spiked to contain 50 μg/kg (only with whole egg), 100 μg/kg, 200 μg/kg, 300 μg/kg, 400 μg/kg and 600 μg/kg of the corresponding tetracycline(s) and left for 1 h on a laboratory shaker. Egg yolk was isolated from intact egg yolks with a syringe and divided in portions of 25 g. The portions except one for blank controls were spiked to concentrations of 100, 200, 400 and 600 μg/kg and left for 1 h at room temperature. Then 75 g of sample dilution buffer (0.3 *M* citrate buffer, pH 5.75) was added. The mixture was agitated for 1 h on a laboratory shaker. Then the materials were filled into 50-ml polyethylene tubes from Greiner (Solingen, Germany), capped and kept frozen at −18°C until analysis.

Plasma was obtained by centrifuging a mixture of whole blood from untreated hens with a solution of sodium heparin for 10 min at 1920 *g*. The supernatant was divided in portions of 5 g and spiked with a diluted spiking standard (1 μg/ml) to levels of 200, 300 and 600 μg/kg and kept at −18°C in 10-ml polyethylene tubes (Greiner) until analysis.

2.5. Sample pretreatment

All samples were diluted with sample dilution buffer (0.3 *M* sodium citrate buffer, pH 5.75). A 10-g amount of homogenized (Ultra-Turrax 10 s) whole egg or egg white was diluted with 20 g of

Table 1
Parent and daughter ions of tetracyclines and their metabolites[a]

Analyte	MS1 *m/z* parent $[M+H]^+$	MS2 *m/z* daughter ions $[M+H\text{-}NH_3]^+$ and $[M+H\text{-}NH_3\text{-}H_2O]^+$
N-DM-OTC	447	412 430
OTC	461	426 443
N-DM-TC	431	396 414
TC	445	410 428
CTC	479	444 462
N-DM-ICTC	465	448
ICTC	479	462

[a] The ions *m/z* corresponded to $[M+H]^+$, $[M+H\text{-}NH_3]^+$ and $[M+H\text{-}NH_3\text{-}H_2O]^+$, respectively. The iso-derivates of chlortetracycline do not form the $[M+H\text{-}NH_3\text{-}H_2O]^+$ ion. Cone voltage and collision energy of OTC, TC, CTC and ICTC were tuned for maximum response from the daughter ions.

sample dilution buffer and mixed well. A 30-g amount of sample dilution buffer was added to 10 g of egg yolk and the mixture was intensively shaken for 5 min. To 2 g of lyophylized whole egg 6 g of water was added. After shaking, the mixture was allowed to stand for 2 h at 6°C. Additional shaking provided a homogeneous suspension, 5 g of which was mixed with 10 g sample dilution buffer.

A 2-g amount of plasma were diluted with 4 g of sample dilution buffer. These dilutions were ready for analysis by the ASTED system.

A 10-g amount of hen's dry feed was weighed into a 1-l volumetric flask and intensively shaken for 1.5 h with 650 ml of 0.01 *M* aqueous oxalic acid, containing 0.5% methanol. The same solvent was used to fill up to the mark. After mixing again, the suspension was filtrated through a paper filter from Schleicher and Schüll (Düren, Germany). A 5.0-ml volume of the clear filtrate was diluted to 50 ml with mobile phase. This solution was ready for analysis by manual direct injection via a 20-μl sample loop.

2.6. Experimental set-up

2.6.1. Settings, solutions and checks

Before starting the automated procedure, the two valves A and B were set into the inject position as illustrated in Fig. 2. Valve C is switched to position 1. The TEC loading and washing pump 1 was set at a flow-rate of 1.0 ml/min. Pump 2 was set at a flow-rate of 0.35 ml/min. Only for the analysis of the iso-derivates of CTC the post-column pump 3 was used at a flow-rate of 0.1 ml/min using indicator paper to check that the mixing coil effluent was pH 12. The tubings supplying the dialysis cell with the different solutions were checked to be free of air bubbles. A chromatography test was regularly performed by direct analysis of a standard solution via the manual injection valve D. The priming solution for the donor side of the dialysis cell was 0.05% (v/v) Triton X-100 in 0.06 *M* phosphate buffer, pH 6.0. The recipient solution was 0.06 *M* citrate buffer, pH 5.8, the dialysis cell washing solution was 1.3% (v/v) Triton X-100 in 0.3 *M* phosphate buffer, pH 5.8 and the washing solution for the TEC was HPLC-grade water.

Vials containing samples and vials containing 0.3 *M* phosphate buffer with Triton X-100, pH 6.0 as washing solution for the donor part of the dialysis cell were placed intermittently in the autosampler rack (thermostated to 6°C) with the washing solution in the first place.

2.6.2. System program[1]

Sample loading. Before aspiring the sample, 50 μl of air was aspired by the dilutor. Then the dilutor aspired 1500 μl of the diluted sample from the sample vial and injected 1200 μl of the diluted sample via the injection port directly into the donor side of the dialysis cell (740 μl). At the start of dialysis segmentation with an air bubble of 850 μl via valve C (Pos. 3) prevented any longitudinal diffusion of the sample into the transporting solution at the recipient side.

Dialysis, trace enrichment, transfer to analytical column. While the sample was held in the static mode for 15.5 min in the donor channel, dilutor 1 transported the recipient solution (valve C, Pos. 1) through the recipient channel of the dialysis cell in three pulses of 1350 μl and a final fourth pulse of 1450 μl with flow-rates of 0.36 ml/min for aspiring and of 1.5 ml/min for dispensing the dialysates into the 5.0 ml loop. Effective dialysis time was 12.5 min (from loading of the donor channel until transport of the last dialysate). During collection of the dialysate, the TEC was conditioned with HPLC grade water via pump 1. After switching valve A, the dialysate in the 5.0-ml loop was transferred to the TEC by pump 1.

After enrichment of the dialysate, pump 1 washed the TEC with 2.0 ml of water before valve B was switched to the inject position. Now the enriched analytes were back-flushed by pump 2 onto the analytical column by elution of the TEC for 30 min with mobile phase. While valve B was in inject position, pump 1 pumped 30 ml of water through the 5.0 ml loop and valve B to waste. After 30 min of elution of the analytes onto the analytical column, valve B was switched back to the load position and valve A was switched back for next loading of the loop. This situation is depicted in Fig. 2. In this position pump 1 is conditioning the TEC for the next analysis with water, thus removing any mobile phase (containing acetonitrile) from the TEC.

[1]The detailed listing of the ASTED program can be obtained from the corresponding author.

Purging and cleaning of dialysis cell after each sample. With the start of the enrichment (valve A has just switched from inject to load and the TEC was already conditioned with water) dilutor 0 begun to remove the dialysed sample from the donor channel to waste and the injection needle was rinsed with priming solution. After this, both the donor and the recipient channel were simultaneously cleaned with 3.6 ml washing solution containing 1.3% Triton X-100. Dilutor 0 (donor side) aspired the washing solution from the first, third, fifth, ... vial in the autosampler rack. The dilutor 1 of the recipient side aspired the washing solution through valve C switched to position 2. After cleaning the dialysis cell, the washing solution was removed simultaneously from both chambers of the dialysis cell by the two dilutors. For this purpose 25 ml of the priming solution were dispensed with 0.72 ml/min through the donor channel and 15 ml of the recipient solution was aspired with 0.36 ml/min through the recipient channel and through valve C switched to position 1.

Off-line cleansing of the dialyser. The connecting tubing and the donor and the recipient channel of the dialysis cell were washed weekly with 0.1 *M* NaOH. For this procedure the tubing between recipient channel to the dilutor of the recipient side and to the tubing to valve C were disconnected to avoid deleterious effects of alkali to the valves.

3. Results and discussion

3.1. Sample pretreatment

For an automatic 24 h analysis, it is necessary that the egg samples in the autosampler rack remain stable and homogeneous. This was achieved previously by diluting the egg material with a weak alkaline solution of pH 8 containing sodium azide [6,8]. However, CTC is unstable at values above pH 7 being degraded to ICTC (Fig. 3). Diluting egg samples with sodium citrate buffer pH 5.75 at 0.3 *M* or higher molarity resulted in homogeneous and translucent sample solutions of pH 6.0. With this sample dilution buffer formation of ICTC was not observed and only negligible amounts (≤3%) of the parent tetracyclines were transformed to the corresponding 4-epimers and vice versa within 24 h, if the

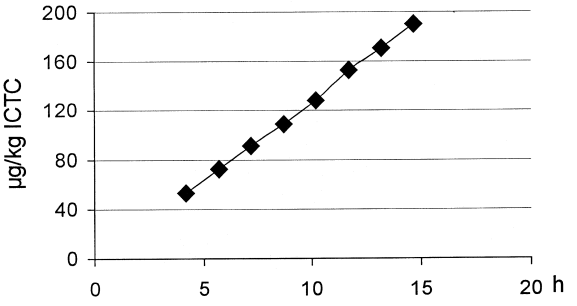


Fig. 3. ICTC formation in an egg sample fortified with CTC at 400 µg/kg and diluted with sodium azide.

autosampler rack was thermostated at 6°C. At higher temperatures epimerization increased significantly. Buffer pH 5.75 (resulting in sample pH of 6.0) was an optimum for keeping egg proteins dissolved. When using a buffer of pH 5.0 or 6.3 instead, proteins deposited slowly and either clogged the injection needle of the autosampler or fouled the tubings and dialysis membrane. Best results were obtained when whole egg, egg white and plasma samples were diluted 1:2 with dilution buffer and 1:3 for egg yolk. The higher dilution for yolk was necessary to achieve a high relative recovery of tetracyclines and to avoid more intense cleaning steps for the system. In contrast, the amprolium assay of egg yolk with an on-line dialysis and on-line SPE system similar to the ASTED [9] needed off-line sample clean-up. We did not find differences in the results for the tetracyclines in egg samples either protected from diffuse daylight or not. Thus, the only precaution taken was to avoid direct sunlight to the system. If not analyzed immediately, homogenized whole egg, egg white and plasma samples were stored undiluted (to avoid even minor epimerization) at −18°C. Egg yolk samples had to be diluted with buffer to avoid the well-known irreversible and substantial viscosity increase when yolk is stored at temperatures below −6°C [10].

3.2. Clean-up

The clean-up for egg samples with residues of tetracyclines was performed fully automatically with the ASTED system and involved on-line dialysis and on-line solid-phase extraction to trap the analytes from the dialysate using a column packed with a

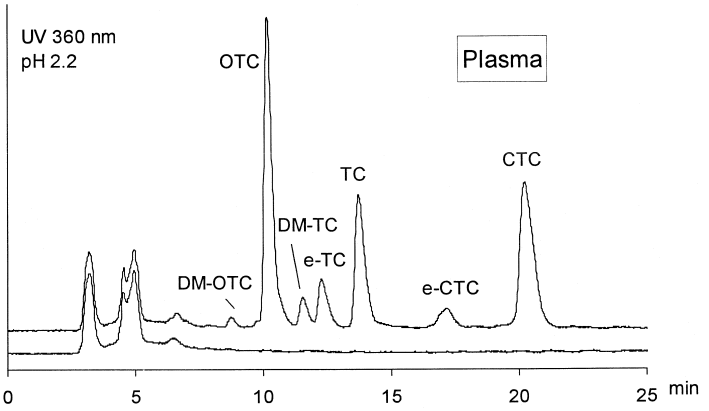


Fig. 4. Plasma with incurred residues of OTC, TC and CTC and their metabolites (UV 360 nm), lower chromatogram: plasma blank.

polystyrene–divinylbenzene copolymer (PLRP-S). As can be seen from the chromatograms (Figs. 4–7), this clean-up in combination with UV and fluorescence detection provides analyte signals without interference from the matrix.

The pH of the recipient solution was selected to be similar to that of the diluted sample in the donor cell. This avoided fouling of the membrane surface by matrix components. As mentioned in the literature, dialysis of egg samples can cause clogging of the dialysis membrane [6]. After experiencing this effect, we extended the ASTED system by a low-pressure valve C (Fig. 2) allowing a cleaning procedure for both, the donor and recipient channel, with a buffer containing 1.3% Triton X-100. The high concentration of this tenside was needed to solubilize egg matrix. However, despite this cleaning step, it was necessary after one week of continuous use to remove matrix components from the dialysis membrane with 0.1 *M* sodium hydroxide solution as recommended in the ASTED user's manual. This also inhibited growth of bacteria and mold in the dialysis cell.

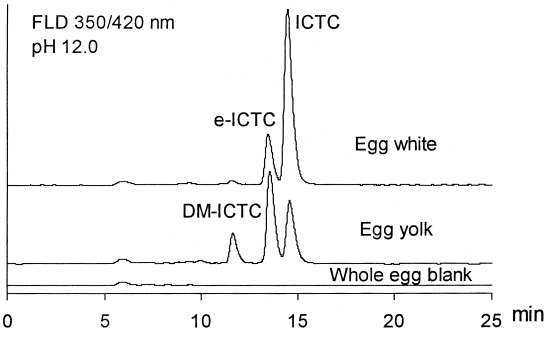


Fig. 5. Chromatograms with incurred residues of isochlortetracycline (ICTC), epi-isochlortetracycline (e-ICTC) and tentatively identified *N*-desmethyl-isochlortetracycline (DM-ICTC) in egg matrices after feeding hens with CTC, as detected by fluorescence after post-column pH adjustment. Lower chromatogram: whole egg blank.

The dialysis process depends on different parameters, e.g., static/dynamic modes, flow-rates, buffer composition and pH, temperature. An extensive review about the influence of these parameters on the result of the dialysis is given by van de Merbel et al. [11].

Our operation parameters for the dialysis were similar to those optimized by Agasøster [6], with the donor channel held in static mode and with four dynamic pulses of the recipient buffer at a flow-rate of 0.36 ml/min. Although higher flow-rates for the recipient can improve absolute recoveries, 0.36 ml/min was the maximum flow-rate without exerting a too negative pressure on the recipient side which we observed could disrupt the membrane. We added to the original ASTED system pump 1, and valve A (Fig. 2), with a 5.0-ml loop as a reservoir to collect the dialysate, which was then transferred in a single step to the TEC. With this arrangement high back-pressure conditions could be applied allowing the use of fine particle materials for on-line SPE, longer SPE columns and also a washing step with 20 ml of HPLC-grade water. This was necessary to remove the citrate contained in the recipient solution and some low molecular matrix components from the TEC before transferring the analytes to the HPLC

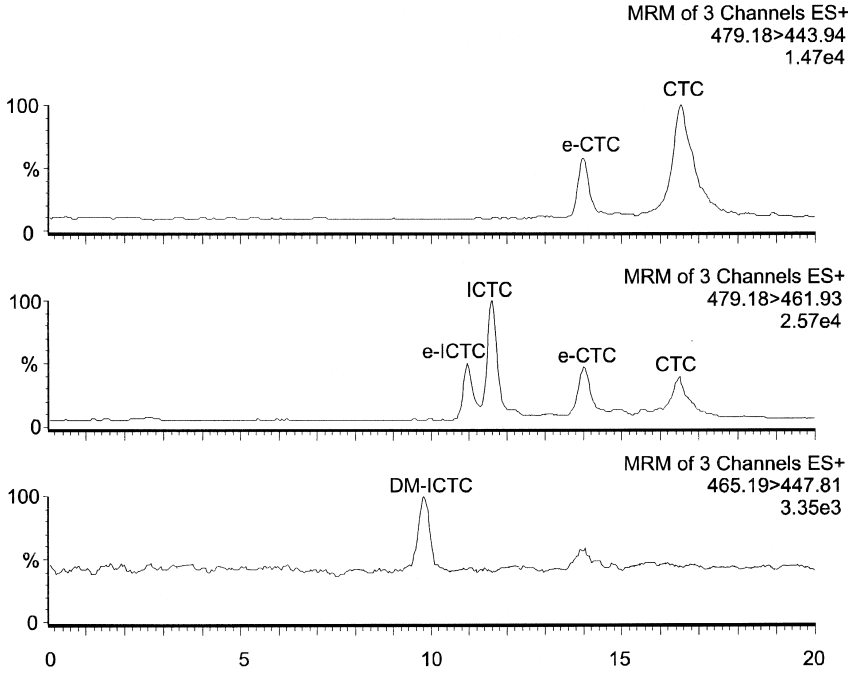


Fig. 6. Whole egg with incurred residues of CTC, as detected by MS–MS.

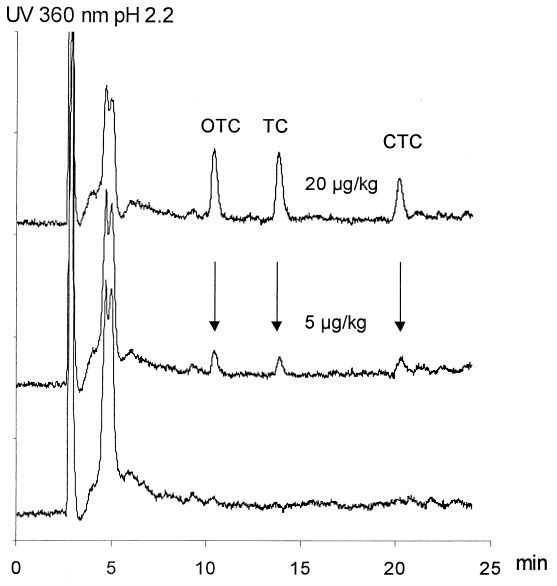


Fig. 7. Chromatograms at the limit of detection (UV detection at 360 nm): whole egg samples fortified with OTC, TC, CTC at concentration levels of 20 μg/kg, 5 μg/kg and a chromatogram of a whole egg blank.

column. Without this rinsing procedure chromatographic separation of the tetracyclines and metabolites became very poor.

The PLRP-S TEC had a sufficient capacity for tetracyclines. Even with samples fortified up to 1800 μg/kg, absolute amount of tetracyclines 240 ng, no breakthrough could be observed.

Despite optimization, on-line dialysis with on-line solid-phase extraction never provides quantitative recovery of analytes, mainly due to incomplete transfer of the analytes through the dialysis membrane. Recovery values for ASTED procedures are typically reported in the literature as relative recoveries by comparing the peak areas in chromatograms from spiked matrix material with peak areas in chromatograms from aqueous standard solutions, both carried through the same sample preparation, dialysis and chromatography procedure. With all matrices investigated in this study the relative recoveries were between 90% and 99% (Table 2). The difference to 100% is a matrix-related loss (e.g., adsorption) of analyte. Relative recoveries for nitrofuran residues in whole egg were reported to be 75%

Table 2
Characteristics of the calibration curves obtained from fortified matrices via the peak areas[a]

Matrix	Analyte	Time range (months)	Concentration range (μg/kg)	Mean slope	R.S.D of slope (%)	Relative recovery (%)	No. of calibration sets
Aqueous solutions	OTC	1	100–600	375	1.6		4
	TC	1		484	1.7		4
	CTC	1		287	2.2		4
	ICTC	1		61 501	3.2		4
Whole egg	OTC	12	50–600	365.4	4.5	97	9
	TC	12		464	5.0	96	9
	CTC	12		262.2	5.8	91	9
	ICTC	6		60 334	5.6	98	6
Egg white	OTC	1	100–600	370	2.7	99	4
	TC	1		465	3.0	96	4
	CTC	1		272	4.2	95	4
	ICTC	1		60 844	3.8	99	4
Egg yolk[b]	OTC	1	100–600	264	2.9	94	4
	TC	1		341	3.3	94	4
	CTC	1		195	5.1	90	4
	ICTC	1		43 800	5.2	95	4
Plasma	OTC	5	200–600	356	2.6	95	6
	TC	5		466	2.9	96	6
	CTC	5		266	4.4	93	6
	ICTC	5	100–300	59 817	4.3	97	6

[a] OTC, TC and CTC were determined by UV detection at 360 nm, ICTC was detected by fluorescence detection at 350/420 nm. Relative recovery is defined as the ratio of the calibration curve slope from aqueous standard solutions and from fortified matrices. Each calibration set consists of 12 analyses (two for each concentration level) for whole egg, eight analyses for aqueous solutions, egg white and egg yolk, and six analyses for plasma.

[b] The lower mean slope values for egg yolk are due to the dilution of 1:3 with buffer instead of 1:2.

to 85% [8]. Relative recoveries, however, give no information about the amount of analyte which actually passes the dialysis membrane and is trapped on the TEC, retained during washing and transferred to the analytical column.

Absolute recoveries are calculated from peak areas in chromatograms from spiked material carried through the complete analytical procedure compared to peak areas in chromatograms of aqueous tetracycline solutions of known concentration injected manually through valve D directly on the analytical column. We obtained absolute recoveries of 50% to 56% with all egg matrices and plasma. For amprolium in egg yolk 18% was reported [9]. Agasøster [6] investigated the loss of OTC due to the dialysis step but did not calculate the recovery of the whole procedure. He reports a ''recovery'' of 60%.

3.3. Chromatography and detection

With the use of a 4 μm Supersphere RP-8 column [12] and acetonitrile–0.01 *M* oxalic acid (50:50, v/v) as mobile phase, sharp and symmetric peaks for the tetracyclines could be obtained as well as a good separation of all interesting compounds under isocratic conditions (Table 3) with the exception of two pairs OTC/e-OTC and DMTC/e-TC. Anhydrotetracyclines and apo-OTC were not observed in the samples with incurred residues. CTC was the last compound to elute at 19.0 min before a new chromatography could start. Total analysis time of ca. 1.5 h (16 samples a day) was not limited by the chromatographic run time but by all the cleaning and rinsing steps. The mobile phase also served to elute the trapped tetracyclines and metabolites from the solid-

Table 3
Retention times of tetracyclines and their metabolites using a mobile phase consisting of acetonitrile–0.01 *M* aqueous oxalic acid (50:50, v/v)

Analyte	Retention time (min)
N-DM-OTC	8.9
α-apo-OTC	9.4
OTC	10.3
β-apo-OTC	10.3
N-DM-ICTC	10.4/12.0[a]
e-OTC	10.8
e-N-DM-TC	10.8/11.4[b]
N-DM-TC	
e-TC	11.8
e-ICTC	12.4/13.9[a]
TC	13.3
ICTC	13.5/15.0[a]
e-CTC	15.9
CTC	19.0
e-Anhydro-TC	27.5
Anhydro-TC	34.3
e-Anhydro-CTC	42.5
Anhydro-CTC	54.7

[a] Time delay due to mixing coil for post-column pH adjustment.
[b] Different retention times for the postulated two epimers.

phase extraction column onto the analytical column. To separate OTC from e-OTC and DM-TC from e-TC the oxalic acid molarity in the mobile phase had to be reduced to 0.005 *M*. This allowed the simultaneous analysis of the two critical pairs but was not used in general, because of the much longer overall run time. It was interesting to notice that under the chromatographic conditions described here, the elution sequence of parent tetracyclines and corresponding 4-epimers was not uniform (Table 3). The elution of e-OTC after OTC under reversed-phase HPLC conditions had also been described by Fedeniuk et al. [13], while Bergner-Lang and Mikisch [14] reported a chromatographic system in which the 4-epimers preceded the parent compounds. We could not attribute distinct retention times to the two postulated epimers of each of the tentatively identified *N*-desmethyl metabolites of OTC, TC and ICTC because standards were not available. Probably due to the loss of a methyl group in the amino function, the epimerization is faster for the desmethyl metabolites than for the parent tetracyclines. While those remained stable when kept in the sample rack at 6°C for 24 h, significant differences were obtained for signal intensity at the two retention times for the desmethyl metabolites of OTC and TC when the samples with incurred residues were analyzed at the beginning or at the end of an analytical series. For the desmethyl metabolite of ICTC we observed only one peak.

Naidong et al. [15] achieved the separation of keto-enol tautomers of CTC in a pharmaceutical preparation using a polystyrene column at 10°C. When analyzing CTC standards on a RP-8 column at ambient temperature we did not observe such a separation of CTC tautomers. However, the CTC standards showed two major impurities. One could be assigned by diode array UV spectra and retention time to be tetracycline and the other was presumably 2-acetyl-2-decarboxy-amido-chlortetracycline [16,17].

As can be seen from the chromatograms of blanks and of matrices with incurred or fortified residues (Figs. 4 and 7), UV detection at 360 nm in combination with the preceding ASTED clean-up provided the required specificity to allow the determination of OTC, TC, CTC, DM-OTC, DM-TC and their 4-epimers without interference from matrix compounds. Iso-derivatives of CTC, however, have only poor UV absorptivity at 360 nm and pH 2.2 for the mobile phase. The shift to lower wavelengths with its increase in absorptivity for these compounds led to the appearance of interfering matrix components. Detection at 310 nm, pH 2.2 served as a compromise to allow simultaneous quantification of all CTC-derived products at concentrations above 100 μg/kg.

For lower concentrations and more reliable results, the determination of ICTC, e-ICTC and DM-ICTC was performed by fluorescence detection (λ_{ex} 350 nm, λ_{em} 420 nm) after post-column pH adjustment to pH 12. The change to pH 12 was necessary as the iso-derivatives show intense fluorescence only in highly alkaline media [18].

An additional pump 3 for the glycine buffer, pH 12, a T-piece for joining buffer and HPLC eluate and a knitted PTFE mixing coil kept at ambient temperature were added to the ASTED system (Fig. 2). It is important to stress the difference between this pH adjustment and the assay of Bryan et al. [19], using a heated coil. In our arrangement only preformed, chromatographically separated ICTC and its deriva-

tives are detected by fluorescence, while the higher temperature at pH 12 will also transform CTC with the purpose to detect it more sensitively by fluorescence than by UV absorption [19].

Bryan et al. mentioned that ICTC is rather unstable when using too high temperatures and too long reaction times. Using a relative short mixing time of 1.6 min at ambient temperature no loss of ICTC was found if compared to the UV detection at 310 nm and pH 2.2.

The presence of 4-epimers of tetracyclines and of ICTC in eggs [5,7,18] as in vivo formed metabolites could be confirmed by LC–MS–MS investigations (Fig. 6).

In addition, 4-*N*-desmethyl derivatives with their 4-epimers of OTC, TC and ICTC could be tentatively identified by a loss of 14 units for the molecular ion with otherwise identical mass spectra compared to their parent compounds and by identical UV spectra and shorter retention times in reversed-phase HPLC, indicating the higher polarity of the formed secondary amino function.

Monooxygenases of the cytochrome P 450 system might be responsible for this demethylation of the dimethylamino group [20,21]. Chromatograms of plasma looked similar to chromatograms of egg yolk and ovule (pre-lay eggs) samples with regard to the tetracycline pattern, but not to chromatograms of egg white samples. These results correspond to the place of formation of egg proteins. Proteins of egg yolk are formed in the chicken liver and are transported via plasma into the ovules, while proteins of egg white are formed in the oviducts [22,23].

The additional valve D (Fig. 2) for direct injection onto the analytical column has been implemented into the ASTED system to allow quality checks and adjustments of chromatography and detection without the need of carrying standards through the whole on-line procedure and for determining absolute recoveries (see Section 3.2).

3.4. Quantification, identification, validation and maintenance

For quantitation, calibration curves were prepared by fortifying whole egg, egg white, egg yolk and plasma with the parent tetracyclines and with ICTC according to Section 2.4. Peak area values were plotted against concentration values, showing linear graphs for all matrices and analytes. These calibration curves were prepared repeatedly during the study with highly reproducible results (Table 2).

The data from the calibration curves were also used to calculate the limits of detection and quantitation which were between 11 and 15 μg/kg or 34 and 45 μg/kg, respectively (Table 4).

As can be seen from the data, there are only negligible deviations in the slopes when the calibration curves are compared for the analytes in different matrices. All analytes could be analyzed with relative recoveries between 90 and 99%. Due to the nearly identical UV absorption of the 4-epimers, these were quantified via the corresponding parent compound as it was done accordingly with the other metabolites except for ICTC. With ICTC a separate calibration curve for fluorescence detection was prepared and all other iso-derivatives of CTC were quantified via ICTC.

Residue concentrations from samples of treated hens were calculated using the calibration curves. With each series of sample analyses three whole egg check samples were analyzed. These were fortified with OTC, TC and CTC or ICTC at 200 μg/kg each and were placed at the beginning, the middle and the end of a sample series to be aware of possible trends. The results of the check samples were only used to check the validity of the calibration curve and not for quantification. However, with the check sample data, quality control charts were prepared for quality assurance of system stability (Fig. 8). The identification of incurred residues in the samples from treated

Table 4
Limits of detection (LODs) and limits of quantification (LOQs) for fortified whole egg samples analyzed with the ASTED system with UV detection at 360 nm for OTC, TC and CTC and for ICTC with fluorescence detection at 350/420 nm for ICTC after post-column pH adjustment (determined according to the calibration method of the German Standard Organisation – DIN 32645)

Compound	LOD (μg/kg)	LOQ (μg/kg)
Oxytetracycline	12	36
Tetracycline	11	35
Chlortetracycline	15	45
Isochlortetracycline	11	34

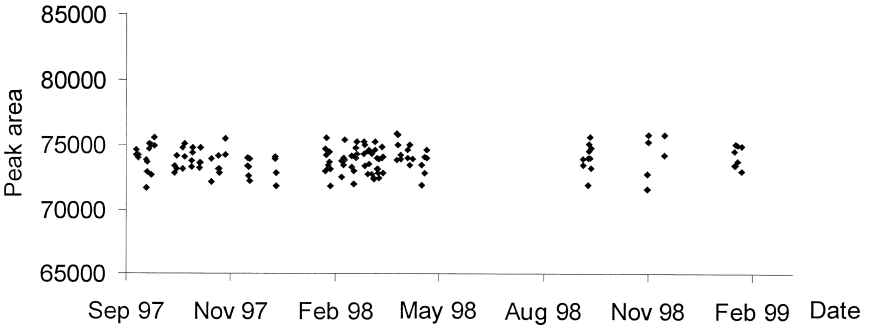


Fig. 8. Quality control chart for the peak areas of OTC from the check samples added to each series of samples with incurred residues over a period of 18 months.

hens were via retention times (Table 3), on-line diode array UV spectra and LC–MS–MS data (Table 1, Fig. 6) in comparison to the samples from the control group of untreated hens. On-line UV spectra were registered at two different pH values (pH 2.2 and 12) leading to characteristic maxima and shifts for the tetracyclines, epimers and other metabolites. Blank samples showed the excellent specificity of the analytical procedures with UV detection at 360 nm (pH 2.2) and fluorescence detection at 350/420 nm (pH 12), as no detectable matrix peaks were eluted in the retention time window of 8.5 to 40.0 min.

In fortified egg or plasma samples spiked with the three parent compounds no formation of 4-epimers or desmethyl metabolites could be observed when analyzed directly after spiking or after prolonged (1 year) storage at −18°C. Also chromatograms of the dry feed showed no significant amount of 4-epimers. From these results we conclude that the 4-epimers in incurred samples are not artefacts but real residues.

Method validation was accomplished by the analysis of blind-fortified samples with very good accuracy and without false-positives or false-negatives.

More than 2000 egg and plasma samples could be analyzed over a period of two years without special maintenance of the system. It was, however, necessary to exchange the trace enrichment column after ca. 200 samples, the dialysis membrane and the precolumn after ca. 600 samples, and the analytical column after ca. 2000 samples, provided that regular checks of the cleanliness of the valves and an off-line cleaning of the dialysis membrane were performed once a week.

4. Conclusions

For analyzing large numbers of samples, automation is advisable to reduce the work load of sample preparation, to improve reproducibility and to allow unattended analyses overnight. This was needed for the investigation of the residues and metabolites obtained in eggs and plasma after feeding oxytetracycline, tetracycline and chlortetracycline to laying hens. The automated analytical method used an hyphenated dialysis with solid-phase extraction for clean-up based on the ASTED system and HPLC with UV, fluorescence or MS–MS detection. In comparison to the performance of the commercially available system ruggedness and precision had to be improved. This was achieved by additional valves and pumps, mainly to allow extensive cleaning of the whole system. The sample preparation was restricted to diluting the samples with a buffer pH 5.75 and avoided the formation of ICTC from CTC, and the formation of 4-epimers as analytical artefacts. More than 2000 egg and plasma samples have been analyzed unattended over a period of two years, demonstrating the ruggedness of the method. The detailed results of the whole egg, egg yolk and egg white and plasma analyses will be published elsewhere.

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