Determination of Tetracycline Antibiotics in Milk Using a Simple Strong Cation-Exchange SPE Cleanup Procedure and LC-MS/MS Analysis

UCT Product Numbers:

CSDAU206: 200mg / 6mL SPE cartridge
SLDA100ID21-3UM: Selectra® DA HPLC column, 100 x 2.1 mm, 3 µm
SLDAGDC21-3UM: Selectra® DA guard cartridge, 10 x 2.0 mm, 3 µm
SLGRDHLDR: Guard cartridge holder

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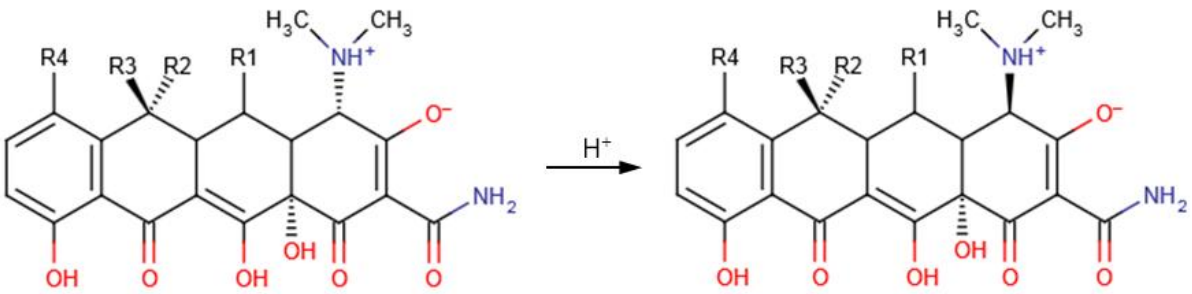
Introduction

Tetracyclines (TC's) are broad spectrum antibiotics that are widely used in animal husbandry for the prevention, control and treatment of bacterial infections [1]. They are amphoteric molecules that always carry a charge and only achieve a "neutral" state as zwitterions. As a result they are highly polar molecules that are only soluble in polar organic solvents (e.g. alcohols) and acidic/basic solutions. TC's are prone to degradation under strongly acidic and alkaline conditions where they form anhydro-, iso- or epi-analogues [2]. Chlortetracycline (CTC), in particular, is vulnerable to alkaline decomposition and forms iso-CTC at high pH. Under mildly acidic aqueous conditions (pH 2–6) the TC's readily undergo epimerization at the C-4 position (amine). In addition to pH instability, TC's are also prone to degradation under certain light and redox conditions [2] (Figure 1.). All of the aforementioned degradation products can undergo additional epimerization or form alternative degradation products [3]. Under certain conditions TC's are capable of undergoing intramolecular H-bonding, while keto-enol tautomerization may also occur but appears to be temperature dependent [4].



Compound	R_1	R_2	R_3	R_4
Tetracycline	H	CH_3	OH	H
Oxytetracycline	OH	CH_3	OH	H
Chlortetracycline	H	CH_3	OH	Cl
Demeclocycline	H	H	OH	Cl
Doxycycline	OH	CH_3	H	H
Minocycline	H	H	H	$N(CH_3)_2$

Figure 1. Structure of tetracycline antibiotics and the acid-catalyzed epimerization at C-4 position.

To ensure food safety and prevent the unnecessary exposure of antibiotic drugs to consumers, TC's are typically included in national chemical residue surveillance plans. The EU has established a maximum residue limit (MRL) for tetracycline, oxytetracycline and chlortetracycline at 100 µg/kg in the muscle and milk of all food producing species [5]. An MRL of 100 µg/kg has also been established for doxycycline in muscle, but it is not allowed for use in animals from which milk is produced for human consumption. The MRL's are based on the sum of the parent compound and its 4-epimer. In the US, tolerances are established for the sum of tetracycline residues (including chlortetracycline, oxytetracycline and tetracycline) in muscle and milk at 2000 and 300 µg/kg, respectively [6].

TC's are difficult to analyze due to their instability, their tendency to form chelation complexes with multivalent cations (i.e. metals), their ability to bind with proteins, and their ability to interact with charged silanol groups on silica-based sorbents [7]. As such, it is important to take all these issues into account when developing a method or when doing routine analysis of these compounds. Most reported methods for TC analysis use lengthy sample preparation procedures or do little to no sample cleanup. As a result, there is a need for a simple method for the analysis of TC antibiotics in foods of animal origin. Liquid-liquid extraction/partitioning is difficult to perform due to TC's charge and low affinity for organic solvents. Therefore, solid-phase extraction (SPE) combined with LC–MS/MS analysis is the most widely used method for the determination of TC residues. Aqueous-based extraction is the primary extraction mechanism. EDTA-McIlvaine's buffer (pH 4) is the most frequently used extraction solvent. At this pH TC's exist as zwitterions and are in their most stable state. In addition, at this pH it is possible to sufficiently deproteinize biological samples prior to SPE cleanup. However, deproteinization can also be carried out under mildly acidic conditions using trichloroacetic acid (TCA), hydrochloric acid (HCl) or phosphoric acid [8]. The inclusion of EDTA in the extraction solvent minimizes the interaction of TC's with chelating complexes present in the sample [8] (Figure 2.). This is particularly important when extracting milk which contains a large amount of calcium.

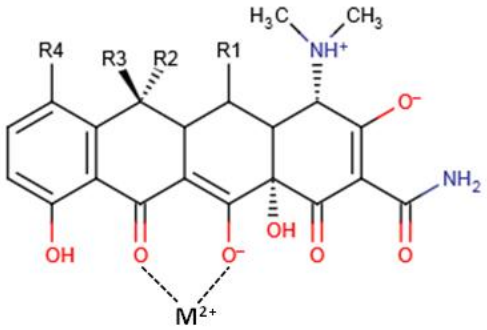


Figure 2. Chelation of divalent metal ions at the C-11 and C-12 position.

TC's chemical complexity and instability also causes problems during HPLC analysis, which often results in broad or tailing peaks and poor resolution. In addition, the difficulty in controlling or preventing epimer formation (at the C-4 position) during the LC process can result in poor peak resolution between the parent TC and its 4-epimer, which can lead to peaks of varying intensity and affect quantification. To overcome peak tailing it is common to prewash an LC column with EDTA prior to use or to use oxalic acid (a dicarboxylic acid with chelating properties) as mobile phase additive [8].

The aim of this study was to develop a simple but efficient procedure for the extraction, cleanup, and quantification of TC antibiotics in milk. Mixed-mode cartridges with a strong cation-exchange functionality were used for the isolation of the TC's from milk samples. HPLC separation was carried out using a Selectra® DA HPLC column prior to detection by mass spectrometry. This simple method allows for the rapid analysis of TC's in milk while achieving good accuracy, precision, and sensitivity without an evaporation step.

Procedure

1. Sample Extraction

a) Weigh 2 ± 0.1g of sample into a 15mL polypropylene centrifuge tube.
b) Add 10 mL of extraction buffer (50mM acetic acid + 10mM EDTA, pH 3.6) to each sample.
c) Shake or vortex samples for 15 minutes to deproteinize the sample and extract the tetracycline antibiotics.
    - For this work a SPEX® SamplePrep® GenoGrinder® was used (operated at 1500 rpm).
d) Centrifuge the samples for 10 min at ≥3000 rcf and 4°C.

2. Condition Cartridge

a) Add 3 mL of methanol to cartridge CSDAU206.
b) Add 3 mL of buffer (50mM acetic acid + 10mM EDTA, pH 3.6).

Note: Do not let the cartridge go dry otherwise repeat steps 2.a) and 2.b).

3. SPE Extraction

a) Load supernatant from step 1d).
b) Adjust vacuum for flow of 1–3 mL per minute.

4. Wash Cartridge

a) Add 3 mL of ultrapure H_2O and slowly draw through.
b) Add 3 mL of MeOH and slowly draw through.
c) Dry under vacuum for ≈1 minute to remove excess solvent.

5. Elute Cartridge

a) Elute the tetracyclines from the SPE cartridge using 3mL elution solvent (1M oxalic acid + 2% TEA in MeOH).
b) Vortex the samples for 2 min and transfer a 1 mL aliquot to an autosampler vial for analysis.

LC-MS/MS Conditions:

HPLC Conditions	
HPLC column	UCT Selectra® DA, 100 x 2.1 mm, 3 µm (p/n: SLDA100ID21-
Guard cartridge	UCT Selectra® DA, 10 x 2.0 mm, 3 µm (p/n: SLDAGDC21-3UM)
Guard Cartridge Holder	p/n: SLGRDHLDR
Column temp.	40°C
Injection volume	5 µL
Flow rate	300 µL/min
Mobile Phase:	A: 1mM oxalic acid in ultrapure H_2O
	B: 1mM oxalic acid in MeOH

Gradient		
Time (min)	**A (%)**	**B (%)**
0.0	95	5
1.0	95	5
5.0	60	40
10.0	60	40
12.0	0	100
16.0	0	100
16.2	95	5
21.0	95	5

MS Conditions	
Instrumentation	Thermo Scientific™ TSQ Vantage™ tandem mass spectrometer
Ionization mode	ESI^+
Spray voltage	5000 V
Vaporizer temperature	350°C
Capillary temperature	350°C
Sheath gas pressure	60 arbitrary units
Auxiliary gas pressure	10 arbitrary units
Ion sweep gas	2 arbitrary units
Declustering potential	2 V
Q1 and Q3 peak width	0.2 and 0.7 Da
Collision gas	Argon
Collision gas pressure	2.0 mTorr
Cycle time	2 sec

SRM Transitions							
Analyte	**t_R (min)**	**Precursor ion**	**Product ion 1**	**CE 1**	**Product ion 2**	**CE 2**	**S-lens (V)**
Thiabendazole-$^{13}C_6$	8.6	208.00	137.05	31	181.03	25	43
Oxytetracycline	8.4	461.13	426.07	17	200.96	32	95
Tetracycline	9.0	445.10	410.04	18	153.91	24	79

Minocycline	10.0	458.10	441.09	16	282.98	41	136
Demeclocycline	10.3	465.10	448.04	16	430.02	19	99
Chlortetracycline	12.5	479.10	462.05	11	444.02	18	102
Doxycycline	13.2	445.08	428.08	16	321.01	28	90
Anydrotetracycline	14.0	427.15	410.01	16	153.98	24	105

Results and Discussion

Polymeric SPE cartridges, particularly those containing polar modified polymeric sorbent, have been used to extract TC's from food samples. A major drawback of using these cartridges is the inability to rinse the sorbent with an organic solvent, which leads to "dirty" sample extracts containing matrix co-extractives. An alternative approach is to use ion-exchange SPE to retain the TC residues on the sorbent, which can then be rinsed with 100% organic solvent. This is very effective at removing matrix interferences and results in a clean sample extract. Due to the instability and complex physicochemical properties of TC's, the SPE procedure needs to be carefully optimized in order to achieve optimal results.

Polymeric cation-exchange sorbents (strong and weak) were evaluated using a variety of different loading conditions (buffer type, pH and ionic strength) but the TC's were found to be inadequately retained. Ultimately, a silica-based sorbent containing a strong cation exchange component was found to be the best choice. Several different SPE elution solvents were evaluated but were not found to be effective at eluting the TC's from the SCX sorbent:

- 2% NH_4OH in MeOH – this is a commonly used elution solvent for eluting basic compounds from SCX sorbent. The high pH neutralizes amino functional groups on the analyte(s) which allows them to be eluted from the sorbent. However, it was not effective at eluting the strongly retained TC's. In addition, NH_4OH is not recommended for use with TC's due to potential alkaline degradation.
- 2% TEA in MeOH – also provides a high pH to neutralize amino functional groups, but does not contain OH^- ions.
- Triethylammonium formate (1% TEA + 0.5% formic acid in MeOH) – At a low pH, the TEA is fully ionized and forms a protonated tertiary amine, which is capable of acting as a counter-ion to displace positively charged analytes from the SCX sorbent.

Accuracy & Precision Data for Tetracycline Antibiotics in Milk (100ppb, n=5)						
	Tetracycline	Oxytetracycline	Demeclocycline	Chlortetracycline	Doxycycline	Minocycline
Sample 1	74.7	53.4	86.7	87.6	88.8	129.2
Sample 2	70.8	52.0	87.7	85.4	91.4	128.9
Sample 3	75.5	51.9	88.7	85.7	86.4	126.6
Sample 4	87.4	58.1	80.8	87.1	99.7	136.3
Sample 5	84.4	66.9	74.6	83.8	91.5	153.4
Mean	**78.6**	**56.5**	**83.7**	**85.9**	**91.5**	**134.9**
RSD	**9.0**	**11.3**	**7.1**	**1.7**	**5.5**	**8.13**

An alternative elution approach to pH manipulation is the use of a high salt concentration (≥ 1 M) to disrupt the ionic interaction between the analyte(s) and ion-exchange sorbent. It was determined that MeOH containing 1M oxalic acid was required to elute the TC's from the ion-exchange sorbent. Oxalic acid was used because it is a good metal chelating agent, which is necessary for the elution of TC's from silica-base sorbent. Lower concentrations of oxalic acid were not as effective, which is similar to the results reported by Pena *et al.* [9]. Minocycline, containing an additional amino functional group, did not elute with 1M oxalic acid. Therefore, 2% TEA was incorporated into the extraction solvent to act as a

counter-ion and displace the minocycline from the sorbent. The eluted extracts can be analyzed directly by LC-MS/MS. If lower sensitivity is required (low ppb range), an evaporation step could be included although great care should be taken (low temperature and N_2 flow).

Oxalic acid is used as a mobile phase additive in TC analysis due to its good chelating properties, which significantly improves peak shape over traditional mobile phase additives (e.g. formic acid or acetate buffer). Oxalic acid also gives a suitable pH (≈2) for efficient ESI^+ ionization of the TC's. A low amount (1mM) of oxalic acid was used as mobile phase additive because oxalic acid is not as volatile as alternative buffers. However, oxalic acid is widely used in TC analysis and can be employed as long as the HPLC column is sufficiently rinsed after each run, no oxalic acid is allowed to remain stagnant in the HPLC column for long periods of time, and the MS source is periodically cleaned (i.e. routine maintenance). The inclusion of TEA in the final sample extract improved peak shape by acting as an ion-pairing agent and reducing peak tailing of the TC residues.

Other problems faced during LC-MS/MS analysis of TCs include isobaric interference and the formation of epimers and/or degradation products. Isobaric interference is observed between tetracycline and doxycycline as they have similar *m/z* values and common fragment ions that cannot be distinguished by a triple quadrupole mass spectrometer. Therefore, sufficient LC separation (≈4 min) was obtained between the two compounds to overcome this problem (not obvious in Figure 4 due to the use of time-segmented acquisition). Isobaric interference and degradation products can also be generated from other TCs (e.g. tetracycline and anhydrotetracycline, and CTC and demeclocycline). As can be seen in Figure 4, chlortetracycline and demeclocycline have 2 peaks each. For CTC there is a peak at 12.4 min corresponding to the analyte and a secondary peak at 10.36 min, which could correlate to domeclocycline (10.29 min), an epimer or an alternative degradation product. For demeclocycline, there is a peak at 10.3 min corresponding to the analyte and a secondary peak at 8.9 min (epimer or degradation product). Epimerization can potentially create difficulties in accurate quantification as the epimer peaks can be equal or larger than the parent TCs. Epimers have the same *m/z* values (parent and product ions), similar abundances and usually elute close to the parent TCs. It is often not possible to separate the epimers from the parent TCs and some analysts will use a fast gradient so that the epimers co-elute with the TCs. The Selectra DA® column is capable of separating out the epimers. However, epimerization was not a major problem in this work. Some minor peaks can be observed in the chromatograms but they do not affect the quantification. Lastly, if the final sample extracts (and calibration curve/QC standards) are prepared in an aqueous solution, particularly acidic solutions, epimerization is readily observed. However, if the final sample extracts are prepared in organic solvent the epimerization is drastically reduced, which limits the formation of a large epimer peaks and simplifies quantification.

Anhydrotetracycline was initially included in the method but was found to degrade rapidly on contact with the extraction buffer. The analyte is displayed in the chromatogram for informational purposes only and was not included in the final quantitative method. Thiabendazole-$^{13}C_6$ was included as an internal standard in the method but there was no improvement in results when it was incorporated into the calculations. Therefore, it was excluded from the calculations and only used as a QC standard. If an isotopically labeled internal standard for one (or more) of the TC's is available, its inclusion in the method would be an obvious advantage. However, at the time of this research no appropriate isotopically labeled internal standard could be commercially sourced.

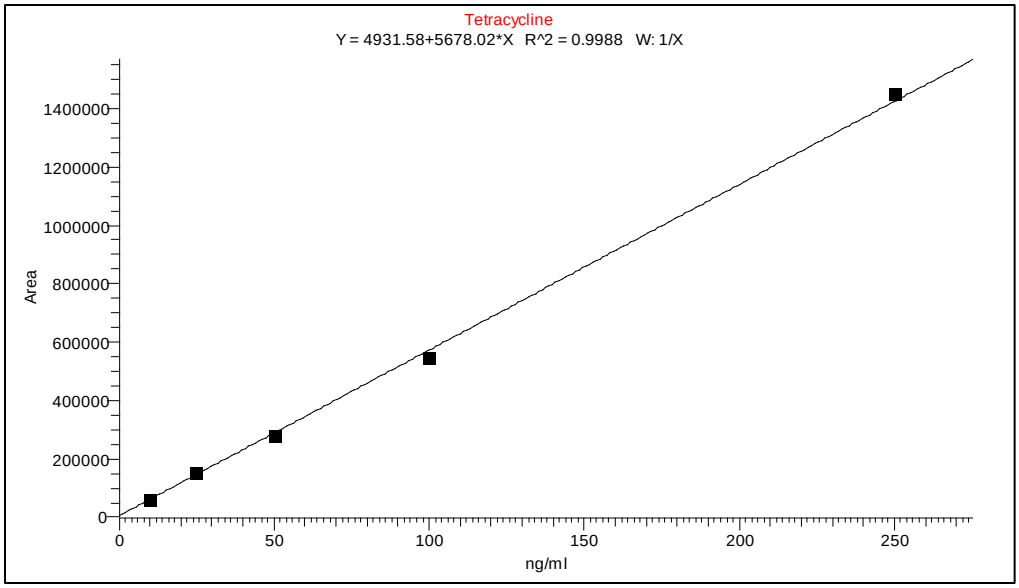


Figure 3. Calibration curve example (tetracycline).

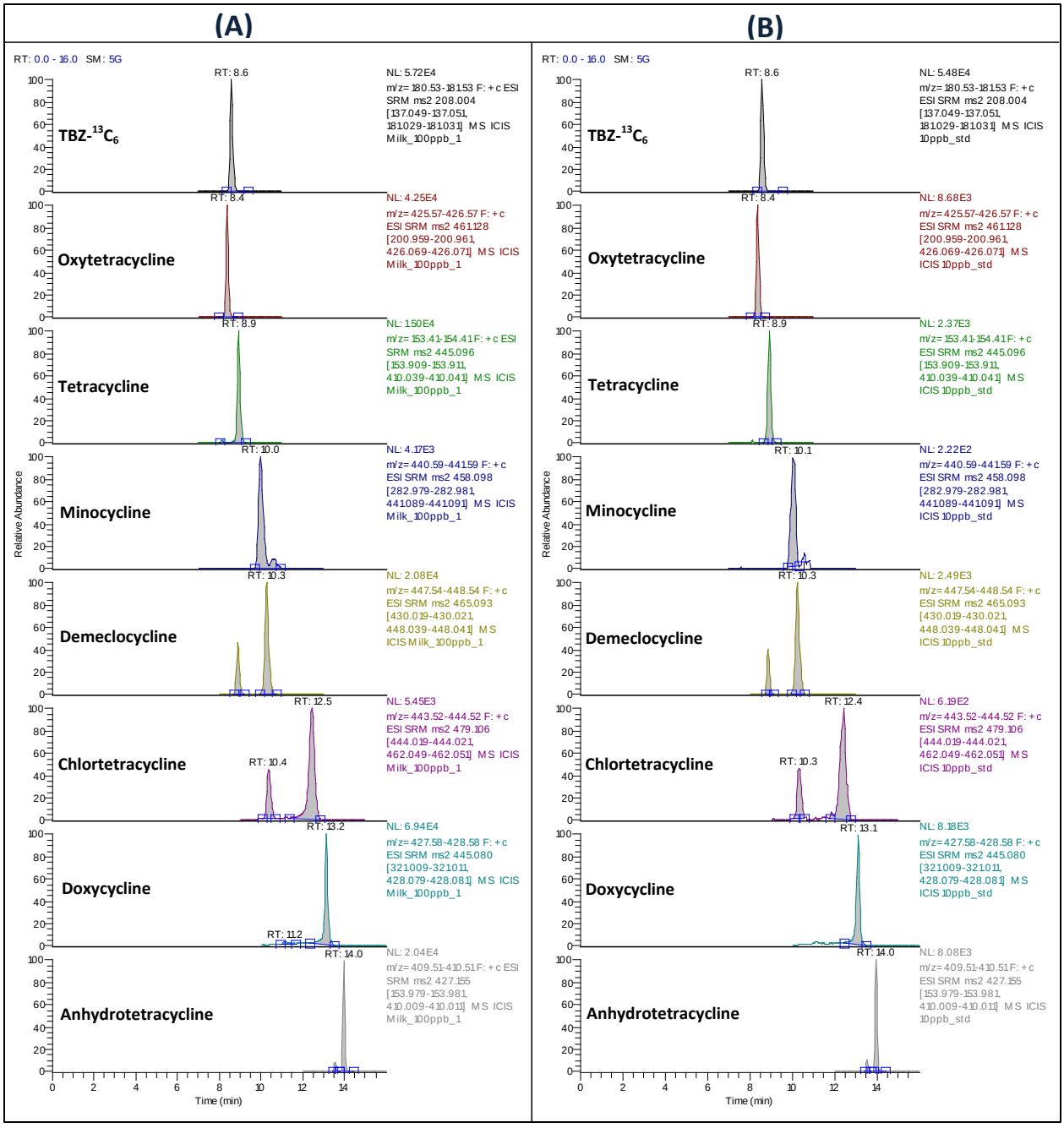


Figure 4. Chromatogram of **(A)** a milk sample fortified with tetracycline antibiotics at 100 ng/g (I.S. at 25 ng/g) and **(B)** a neat standard equivalent to 10 ng/g (I.S. at 25 ng/g).

Conclusions

A simple, fast, and cost effective SPE and LC-MS/MS method was developed for the quantitative detection of six tetracycline antibiotics (TC's) in milk. Samples were extracted with pH 3.6 acetate buffer containing EDTA, and SPE cleanup was carried out using mixed-mode cartridges containing a strong cation exchange component. Separation of the TC's was achieved within 16 min on a Selectra® DA HPLC column. The use of LC-MS/MS detection provided sufficient selectivity and sensitivity for the identification and quantification of the TC's. Good accuracy and precision were obtained for these difficult compounds. No evaporation step is included in the method which avoids any potential loss during this step. Based the lowest calibration point used, the method is capable of detecting TC residues at <10 ng/g. The accuracy and precision of the method could be further improved by incorporating an isotopically labeled internal standard for one of the TC's (if available). The method outlined here provides an attractive alternative to currently used methods for TC analysis.

References:

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