Production of secondary antibody for the development of screening method for the determination of tetracyclines residues in milk

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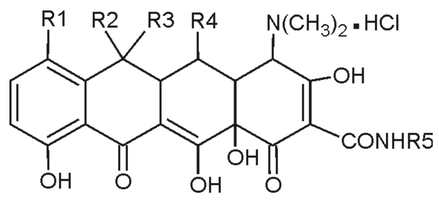
Introduction

Tetracyclines are a broad spectrum antibiotics. They are widely used for treatment and prevention of diseases in food-producing animals. The use of these antibiotics in animal healthcare has raised concerns as the presence of residues in food may lead to increase microbial resistance. And it cause harmful effect, such as allergic reactions, liver damage, yellowing of teeth and gastrointestinal disturbance. To protect consumers, many countries have set acceptable tolerance levels for these drugs.

The maximum residue level (MRL) established by European Union for tetracycline (TC), chlortetracycline (CTC) and oxyteracycline (OTC) in milk is 100 μg/kg (Commission Regulation (EU) No 37/2010). In Russia the MRL for these compounds is 10 μg/kg (Decision of the Customs Union Commission No. 299).

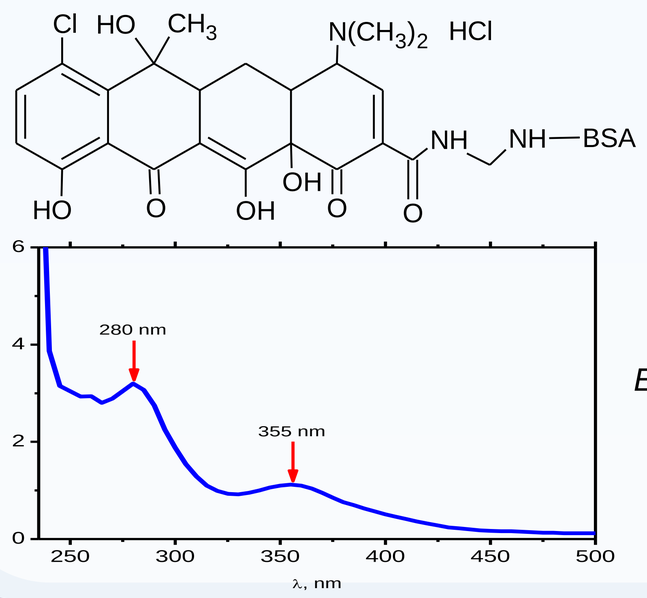
Therefore, it is necessary to develop a suitable analytical technique with specificity, sensitivity and simplicity. There are several methods for the determination of TCs in food staff, mainly chromatography including HPLC-MS, LC-MS. Enzyme-Linked Immunosorbent Assay (ELISA) is an immunological technique with simplicity, sensitivity, specificity that include enzyme to detect the presence of an antibody or an antigen in sample. This method is suitable for screening purpose.



	R1	R2	R3	R4	R5
chlorotetracycline	Cl	OH	CH3	H	H
oxytetracycline	H	OH	CH3	OH	H
tetracyline	H	OH	CH3	H	H

Materials and Methods

* TC, CTC, OTC, doxycycline (DC), epi-OTC, epi-TC, bovine serum albumin (BSA), 3,3',5,5'-tetramethylbenzidine (TMB), horseradish peroxidase (HRP), sodium periodate, sodium borohydride and dimethyl sulfoxide (DMSO) – Sigma (USA).
* The ELISA reader (Sunrise) were from Tecan (Austria).
* The 96-well polystyrene microtitre plates were from Greiner Bio One (Germany).
* TC, CTC and OTC were directly linked to BSA via the Mannich reaction (the *immunogens* and the *coating conjugates*, respectively).
* *Polyclonal specific rabbit sera* have been received by immunization of rabbits (male Chinchilla rabbits, intracutaneously, TC-BSA, OTC-BSA and CTC-BSA in mixed solution of physiological saline and complete Freund's adjuvant) with booster injections at 4 week intervals. Blood samples were obtained 7 days after each immunization and were assayed using the ELISA procedure.
* *Secondary diagnostic antibodies* was obtained by immunization of sheep with anti-CTC, anti-OTC and anti-TC antibodies. The booster injections were carried out at 4-6 weeks intervals. Blood samples were obtained 7 days after each immunization and were assayed using the ELISA procedure.
* Horseradish peroxidase was dissolved in deionized water. 0.1 M sodium periodate was added and the mixture stirred. HRP was separated on a column of PD-10 in carbonate buffer. Isolated immunoglobulins (anti rabbit IgG) in carbonate buffer was added. The mixture was stirred. Sodium borohydride was added and the mixture was stirred. Then the conjugate *anti–rabbit IgG-HRP* was purified by dialysis against PBS.
* TCs free milk samples were chosen by analysing the samples by HPLC-MS/MS. Before analysis milk samples were defatted by centrifugation.



Immunogen structure

Figure 1. Epitope density (12:1)

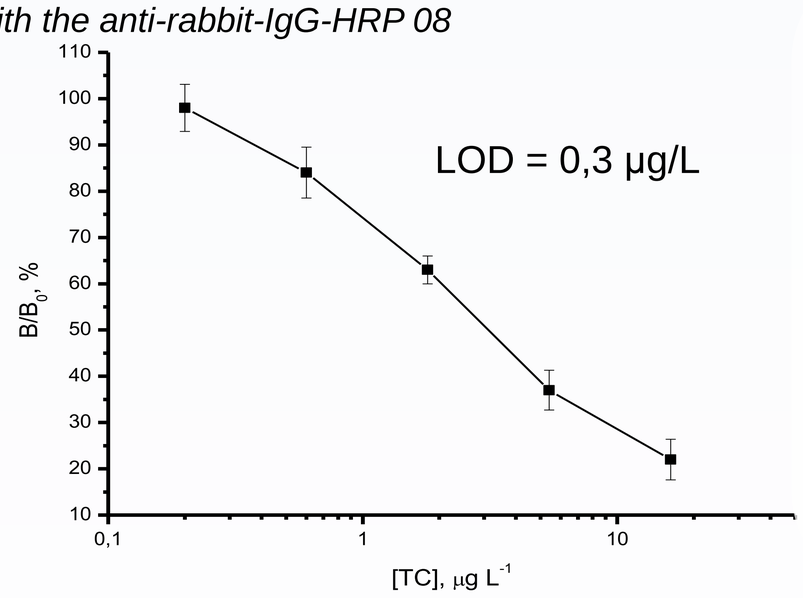
Table 1. The scheme of sheep immunization

Immunization No	Immunogen	Adjuvant	Sheep anti-rabbit IgG No
01	Σ Ig anti-CTC-BSA	complete Freund's	01
02	Σ Ig anti-CTC-BSA	incomplete Freund's	02
03	Σ Ig anti-CTC-BSA	incomplete Freund's	03
04	Σ Ig anti-CTC-BSA	incomplete Freund's	04
05	Σ Ig anti-CTC-BSA	incomplete Freund's	05
06	Σ Ig anti-CTC-BSA	incomplete Freund's	06
07	Sera anti-CTC-BSA, anti-TC-BSA, anti-OTC-BSA	incomplete Freund's	07
08	Σ Ig anti-CTC-BSA, anti-TC-BSA, anti-OTC-BSA	incomplete Freund's	08

Table 2. Repeatability, In-house Reproducibility and Recovery

Concentration of TC, μg L^{-1}	s_r, μg L^{-1}	RSD,%	s_{wR}, μg L^{-1}	RSD, %	Recovery , %
1	0,1	14	0,3	27	130
5	0,9	17	1,3	26	90
10	2,3	23	2,9	29	85

Figure 2. Typical calibration curve for TC constructed with the anti-rabbit-IgG-HRP 08



Linear range (80-20% binding) and cross-reactivity towards different TCs were shown in the Table 3.

Table 3. Cross-reactivity towards TCs

TCs	CR, %
Tetracycline	100
Chlortetracycline	125
Oxytetracycline	5
Doxytetracycline	15
Epi-Tetracycline	< 1
Epi-Oxytetracycline	29

Conclusion

1. A test-system based on enzyme-linked immunosorbent assay (ELISA) for the quantitative detection of tetracyclines (TCs) in milk was developed.
2. The sensitivity of the quantified detection of TCs by ELISA was 0.3 μg/L (the CCβ was 0.5 μg/L).
3. The recoveries in milk were ranged 90–130 %, respectively.

References

1. Commission Regulation (EU) No 37/2010, Off. J. Eur. Union L 15 (December (1))(2009).
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3. Decision of the Customs Union Commission No. 299 Uniform sanitary and epidemiological and hygienic requirements for safety and nutrition value of food products. P. 46-87.
4. Pastor-Navarro N., Morais S., et al. (2007) "Synthesis of haptens and development of a sensitive immunoassay for tetracycline residues Application to honey samples", Anal. Chim. Acta 594 : 211-218.