Validation report SYMMETRIC BTS

(Order nº: S1248/S1296)

(ProGnosis Biotech S.A., Larissa, Greece)

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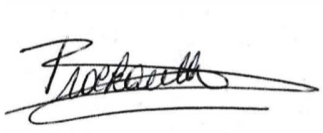

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1. Introduction

SYMMETRIC BTS (ProGnosis Biotech S.A., Larissa, Greece) is a qualitative one-step 5 min rapid lateral flow test kit for the detection of β-lactams, Tetracyclines and Sulfonamides in cows', ewes' and goats' milk.

This test is validated at ILVO-T&V (Technology & Food Science Unit of the Flanders research institute for agriculture, fisheries and food) according to ISO Technical Specification 23758 | IDF RM 251 (ISO/IDF, 2021), Commission Implementing Regulation 2021/808 and to the CRL guidelines for the validation of screening methods for residues of veterinary medicines (*Anonymous*, 2010). The following analytical parameters were checked: detection capability, test specificity, rate of false positives, repeatability of test and reader and test robustness. Further the suitability of SYMMETRIC BTS to screen different milk types (UHT milk and reconstituted milk powder) and milk from species other than the cow (goat and ewe) was evaluated. The test will also be included in a interlaboratory study organised by ILVO in fall 2023.
Last, a short reader comparison was performed between the S-Flow reader (use in the validation and the 3PR-Mini reader.

2. Test procedure

Test preparation
Plug in the One-touch Incubator and wait until the temperature has been stabilized at 40℃. All reagents and kit components should be at ambient temperature (21-25℃) before use (take out of fridge at least for half an hour). Open the plastic tube and take out as many test strips and microwells as needed. When not needed straight away, it is necessary to cover the reagents. Immediately close the plastic tube carefully and restore the remaining reagents in the fridge. Shake the milk vigorously or vortex to ensure milk sample homogeneity (no precipitation or clotting). The ideal temperature of the milk sample is between 4 and 18℃. In this validation study, raw milk temperature was standard 1-4℃.

Test procedure
Step 1: When the One-Touch Incubator is stable at 40℃, place the empty microwells into the incubator.
Step 2: Transfer 100 µL of raw milk to each microwell. Aspirate the sample up and down about 10 times to completely mix the lyophilized gold particles in the milk, while avoiding bubbles. In case of more than 3 samples, an 8-channel multipipette should be used.
Step 3: Immediately, place the appropriate number of sticks into the wells and push the START(RUN) button to start the 5-minute count down.
Step 4: After the 5-minute incubation, press START(STOP) to stop the sound signal and take the dipsticks out of the microwells.
Step 5: Remove the white cotton sample pad with your hands and place the strip in the plastic holder and put in the S-Flow scanner with the sticks facing up. Use S-Flow software to interpret results as soon as possible and no later than 1 minute after incubation visually or by use of the

S-Flow scanner and S-Flow software. Visual interpretation is also possible.

The control line should always be visible, if not the test is invalid. For the test lines (T1 – T3) following counts: Negative: If the test line is stronger than the control line, the milk sample contains no antibiotics or contains antibiotics at lower level than the detection limits. Positive: test line is weaker (less intense) than or equal to the control line, the milk sample contains antibiotics above or close to the detection limits.

2.1 Configuration of SYMMETRIC BTS test strip

The configuration of SYMMETRIC BTS is shown in Figure 1.

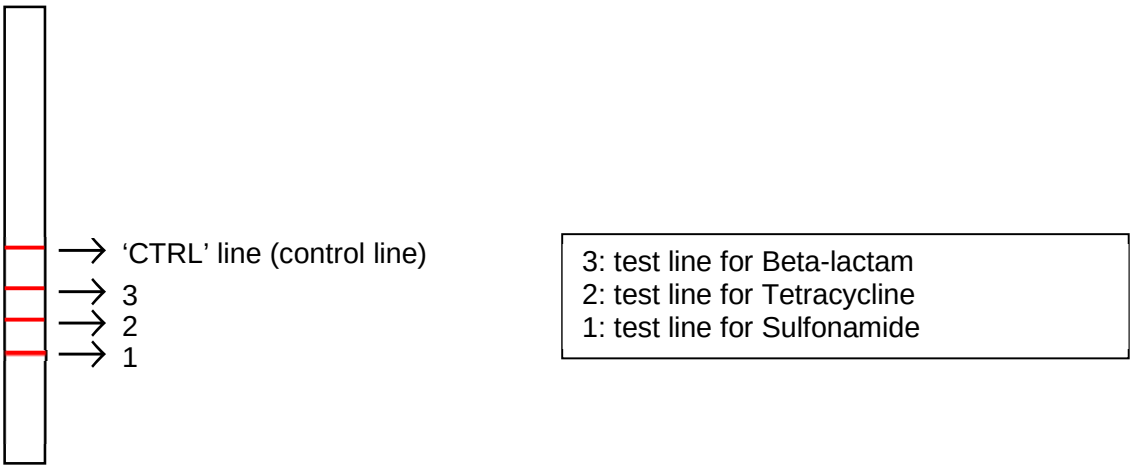

Fig. 1. Configuration of a SYMMETRIC BTS test strip.

2.2. Instrumental interpretation of the test

The reader is comparing the intensity of each test line with the intensity of the control (reference) line and calculates for each channel a ratio = intensity test line / intensity control line. This ratio for each test line is compared to a fixed cut-off value (ratio = 1.10).
The ratio cut-off levels are given in Table 1.

Table 1. Instrumental reading: interpretation of the test results.

Ratio	Interpretation	Ratio	Interpretation	Ratio	Interpretation
R>1.10	negative	0.9≤R≤1.1	weak positive	R<0.9	positive

Note: R: ratio.

Fig. 2. One-Touch Incubator and S-Flow Reader for instrumental reading.

2.3 Visual interpretation of the test

Visual reading of SYMMETRIC BTS test is also possible. The intensity of the test line is compared to the intensity of the reference (i.e. control) line. Negative: test line is darker than the control line; Positive: test line is lighter than or equal to the control line, the milk sample contains antibiotics above the detection limits. The interpretation is shown in Figure 3.
Visual reading was not checked in this validation study.

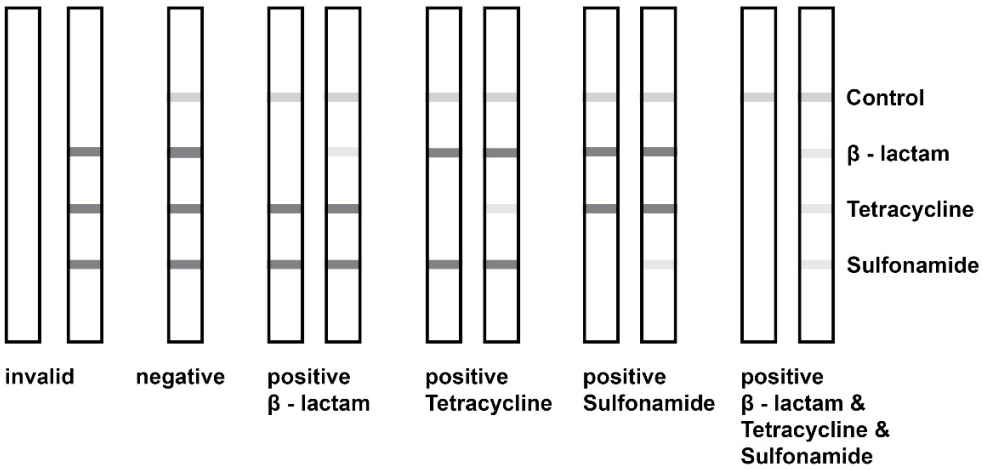

Fig. 3. Visual interpretation of the color formation on SYMMETRIC BTS (*Anon*., 2023).

3. Detection capability

Methods and Materials:

Spiking of antibiotic-free (blank) raw milk with β-lactams (penicillins and cephalosporins), tetracyclines and sulfonamides.

Blank milk was collected from 4 individual cows in mid-lactation which had not been treated with any veterinary drug for the last 2 months and which had a low to moderate number of somatic cells in the milk. The milk was collected in sterile containers and kept below 4℃ to limit the bacterial count. The maximum period for the cold storage of the fresh raw milk was 56 hours which is shorter than the local milk collection interval (3 days in Belgium). Milk of at least 4 animals is commingled and is considered as a sample of standard blank matrix.
At least four such samples are used for the determination of the detection capability when testing 20 replicates. If 40 or 60 replicates need to be tested to determine the detection capability, minimum eight or twelve different blank milk samples are used, respectively.

The detection capability of SYMMETRIC BTS was determined for all different compounds belonging to the β-lactam, tetracycline and sulfonamide family mentioned as marker residue in Table 1 of the annex of Commission Regulation (EU) No 37/2010. The spiking was performed as described in the ISO TS 23758 | IDF RM 251 (ISO/IDF, 2021). Each compound was individually spiked in blank raw milk at fixed concentrations. For each compound a minimum of 2 concentrations around the test sensitivity (test detection capability) were tested. The increment between the concentrations tested for each compound was dependent on the level of spiking and the closeness to the respective MRL (Table 2).

Each concentration was tested 20, 40 or 60 times in a time period of at least three days.

- o Tested concentration ≤0.5 MRL: 20 times
- o Tested concentration >0.5 – <0.9 MRL: 40 times
- o Tested concentration ≥0.9 – ≤1.0 MRL: 60 times
- o Tested concentration >MRL: 20 times

Table 2. Increment between the concentrations tested for each compound was dependent on the level of spiking.

Concentration (in µg/kg)	Increment (in µg/kg)
<1	0.2
1-10	1
11-20	2
21-50	5
51-100	10
101-250	25
251-500	50
501-1,000	100
1,001-5,000	500

The detection capability is defined as the lowest concentration tested where at least 19 out of 20 tests, 38 out of 40 tests or 57 out of 60 tests were positive, respectively.

Every day the following standards were also tested:

- blank raw milk free from antimicrobials (fresh and frozen) - twice
- blank raw milk spiked with benzylpenicillin at 1.5 µg/kg and sulfadoxine at 35 µg/kg - twice
- blank raw milk spiked with cefazolin at 45 µg/kg and oxytetracycline at 10 µg/kg - twice

These standards will also be used for the robustness analyses.

A positive and negative control with lot numbers BTSN0123 and BTSP0123 (exp. date 01/2024), BTSN0223 and BTSP0223 (exp. date 02/2024) and BTSN0323 and BTSP0323 (exp. date 03/2024) included in the kit were also tested daily. The positive control contains tetracycline at 100 µg/kg, benzylpenicillin at 4 µg/kg and sulfadiazine at 100 µg/kg.

Detection capability tests were performed with three different lot numbers of reagents: S1296005 (exp. date 01/2024), S1296006 (exp. date 02/2024) and S1296007 (exp. date 03/2024) following the manufacturer's instructions. The intensity of color formation of each test line was compared to the intensity of the control line and was interpreted by means of a S-Flow Reader and S-Flow Software. The cut-off value is 1.10 (>1.10: negative; ≤1.10: positive). All results (reader values) were collected in a database.

Certified reference material from following different reagent suppliers was used: Dr. Ehrenstorfer GmbH (Augsburg, DE), Toronto Research Chemicals (TRC) (Ontario, CA); HPC Standards GmbH (Borsdorf, DE); Sigma-Aldrich N.V. (Overijse, BE) and LGC Mikromol (Luckenwalde, DE). Detailed information of all standard material is given in Table 3.

Table 3. Standard material used in this validations study.

Active compound	Origin	Product number	Lot number	Solvent
4-epimer chlortetracycline	Dr. Ehrenstorfer	DRE-C13175500	1352919	MeOH
4-epimer oxytetracycline	Dr. Ehrenstorfer	DRE-C13179000	1185756	MeOH
4-epimer tetracycline	TRC	T291405	1-EDT-29-1	MeOH
	Dr. Ehrenstorfer	DRE-C13179500	1289803	MeOH
Amoxicillin	Dr. Ehrenstorfer	DRE-C10242500	G1193514	ACN/H_2O 50/50*
Ampicillin	Dr. Ehrenstorfer	DRE-C10243080	G1059464	ACN/H_2O 50/50*
Benzylpenicillin / Penicillin G	LGC Standards	DRE-C15935000	G1078242	H_2O
Cefacetrile	HPC Standards	679543	812729	ACN/H_2O 50/50*
Cefalexin	Dr. Ehrenstorfer	DRE-C11064000	G1152231	ACN/H_2O 50/50*
Cefalonium	LGC Mikromol	MM3169.00	1244778	ACN/H_2O 50/50
	Sigma-Aldrich	32904	BCCF4758	ACN/H_2O 50/50
Cefazolin	Dr. Ehrenstorfer	DRE-C11064100	G1238077	H_2O
Cefoperazone	Dr. Ehrenstorfer	DRE-C11064300	G1135405	ACN/H_2O 50/50
Cefquinome	Dr. Ehrenstorfer	DRE-C11064700	G1125857	ACN/H_2O 50/50*
Ceftiofur	Dr. Ehrenstorfer	DRE-C11065000	G1104213	500µl FA + H_2O ACN/Methanol
Cephapirin	Dr. Ehrenstorfer	DRE-C11064071	G1185416	H_2O
Chloramphenicol	Dr. Ehrenstorfer	DRE-C11120000	G974527	MeOH
Chlortetracycline	Sigma-Aldrich / Supelco	PHR1520	LRAD3654	H_2O

Clavulanic acid	Dr. Ehrenstorfer	DRE-C11668545	G1342649	H_2O
Cloxacillin	Dr. Ehrenstorfer	DRE-C11692100	G1053837	H_2O
Colistin	Sigma-Aldrich / Supelco	PHR1605	LRAC9149	H_2O
Dapsone	Dr. Ehrenstorfer	DRE-C11963000	G1143440	MeOH
Desacetylcephapirin	HPC	682120	815885	ACN/H_2O 50/50*
Desfuroylceftiofur	TRC	TRC-D289980	7-WAI-61-1	ACN/H_2O 50/50
Dicloxacillin	Dr. Ehrenstorfer	DRE-C12560500	G1226254	H_2O
Doxycycline	Dr. Ehrenstorfer	DRE-C13084280	1116543	MeOH
Enrofloxacin	Dr. Ehrenstorfer	DRE-C13170000	G1116914	1M NaOH
Erythromycin A	Sigma-Aldrich / Vetranal	46256	BCCB8689	MeOH
Lincomycin	Sigma-Aldrich / Supelco	PHR1657	LRAC9565	H_2O
Nafcillin	Dr. Ehrenstorfer	DRE-C15402400	1335481	H_2O
Neomycin	Sigma-Aldrich / Supelco	PHR1491	LRAC2086	H_2O
Oxacillin	Dr. Ehrenstorfer	DRE-C15755100	G1262742	H_2O
Oxytetracycline	Dr. Ehrenstorfer	DRE-C15820000	G1125763	H_2O
Phenoxymethylpenicillin / Penicillin V	Dr. Ehrenstorfer	DRE-C15935010	G1069505	H_2O
Sulfachloropyridazine	Dr. Ehrenstorfer	DRE-C16990100	G1112689	NaOH
Sulfadiazine	Sigma-Aldrich	S6387	MKCJ7742 MKCH4796	1M NaOH
Sulfadimethoxine	Dr. Ehrenstorfer	DRE-C16990550	G1161464	1M NaOH
Sulfadoxine	Dr. Ehrenstorfer	DRE-C16990600	G1210515	1M NaOH
Sulfamerazine	Dr. Ehrenstorfer	DRE-C16995100	G982705	1M NaOH
Sulfamethazine	Dr. Ehrenstorfer	DRE-C16996500	G1125859	1M NaOH
Sulfamethizole	Dr. Ehrenstorfer	DRE-C16998000	G1148000	1M NaOH
Sulfamethoxypyridazine	Dr. Ehrenstorfer	DRE-C16998150	G1200722	1M NaOH
Sulfamonomethoxine	Dr. Ehrenstorfer	DRE-C16998175	G1276331	1M NaOH
Sulfaquinoxaline	Dr. Ehrenstorfer	DRE-C16990000	G1161488	1M NaOH
Tetracycline	Dr. Ehrenstorfer	DRE-C17396150	G1127754	H_2O
Trimethoprim	Dr. Ehrenstorfer	DRE-C17875000	G1127755	MeOH

Note: *dissolved in a small amount of ACN/H_2O 50/50, and further diluted with H_2O.

Results:

A summary of SYMMETRIC BTS detection capabilities is given in Table 4.

Discussion:

SYMMETRIC BTS is capable to detect residues of all β-lactams, tetracyclines and sulfonamides with a MRL in milk (EU-Regulation 37/2010 and amendments).

All β-lactams, tetracyclines (parent drugs and their 4-epimers) and tested sulfonamides can be detected at least in 95% of the samples at their respective MRL except for ceftiofur (CCβ=300 µg/kg, MRL=100 µg/kg), desfuroylceftiofur (CCβ=400 µg/kg, MRL=100 µg/kg) and cefalexin (CCβ=500 µg/kg, MRL=100 µg/kg).

Doxycycline (no MRL in milk) was detected at least in 95% of the replicates at 14 µg/kg.

Table 4. Detection capability (in µg/kg) of SYMMETRIC BTS (ProGnosis Biotech S.A., Larissa, Greece) in raw bovine milk with instrumental reading (S-Flow Reader and S-Flow software) with cut-off ratio = 1.10. Detection capability defined as the lowest concentration tested giving minimum 19, 38 or 57 positive results out of 20, 40 or 60 samples, respectively.

Antibiotic Group/ antibiotic	EU MRL (µg/kg)	Detection capability (µg/kg)		
		BL channel	TET channel	SULFA channel
Penicillins				
benzylpenicillin*	4	2		
ampicillin	4	2		
amoxicillin	4	2		
oxacillin	30	3		
cloxacillin	30	3		
dicloxacillin	30	2		
nafcillin	30	6		
phenoxymethylpenicillin	(25[a])	2		
Cefalosporins				
ceftiofur	100[b]	**300****		
desfuroylceftiofur	100[b]	**400**		
cefquinome	20	6		
cefazolin	50	40		
cephapirin	60[c]	4		
desacetylcephapirin	60[c]	9		
cefacetrile	125	14		
cefoperazone	50	3		
cefalexin	100	**500**		
cefalonium	20	2		
Tetracyclines				
tetracycline	100[d]		12	
4-epimer of tetracycline	100[d]		45	
oxytetracycline	100[d]		9	
4-epimer of oxytetracycline	100[d]		35	
chlortetracycline	100[d]		10	
4-epimer of chlortetracycline	100[d]		35	
doxycycline	--[e]		14	
Sulfonamides				
Sulfamethazine	100[f]			1
Sulfamerazine	100[f]			1
Sulfadiazine	100[f]			3
Sulfamonomethoxine	100[f]			1
Sulfadimethoxine	100[f]			1

Sulfadoxine	100[f]	30
Sulfachlorpyridazine	100[f]	3
Sulfamethoxypyridazine	100[f]	12
Sulfaquinoxaline	100[f]	2
Sulfamethizole	100[f]	14

Notes: *: benzylpenicillin is also the marker residue for benzathine benzylpenicillin and of penethamate; **: with use of 500 µl formic acid as solvent and further dilution with H_2O. Bold and red font detection capabilities are above the drug MRL. MRL: Maximum Residue Limit, Regulation (EC) No 470/2009 of the European Parliament and of the Council and Commission Regulation (EU) No 37/2010 and amendments (actual situation). BL: β-lactam; TET: tetracycline, SULFA: sulfonamides. Detection capability defined as the lowest concentration tested giving a minimum of 19 positive results out of 20, 38 positive results out of 40 or 57 positive results out of 60, respectively.

[a]: No MRL in milk, MRL based on Commission Implementing Regulation (EU) 2018/470.

[b]: The MRL of 100 µg/kg is applied on the sum of all residues retaining the β-lactam structure expressed as desfuroylceftiofur;

[c]: The MRL of 60 µg/kg in milk is applied on the sum of cephapirin and desacetylcephapirin;

[d]: The MRL of 100 µg/kg in milk is applied on the sum of parent drug and its 4-epimer;

[e]: Not for use in animals from which milk is produced for human consumption.

[f]: The combined total residues of all substances within the sulfonamide group should not exceed 100 µg/kg

4. Test selectivity and rate of false positive results

4.1. Test selectivity

Methods and Materials:

The selectivity of the different test lines of SYMMETRIC BTS was tested by analysing milk spiked with β-lactam, tetracycline and sulfonamide compounds and by analysing milk spiked with compounds belonging to different antibiotic or chemotherapeutic families (1 per family) to check the selectivity of the β-lactam, tetracycline and sulfonamide test line. Raw milk was spiked at a high concentration (100×MRL or 100×RPA/MMPR in milk) in raw milk. All testing was completed in duplicate. In case of a positive result also lower concentrations were tested.

Following compounds were used: benzylpenicillin (penicillins), cefalonium (cephalosporins), oxytetracycline (tetracyclines), sulfadiazine (sulfonamides), neomycin B (aminoglycosides), enrofloxacin (quinolones), colistin (polymyxins), chloramphenicol (amphenicols), erythromycin (macrolides), lincomycin (lincosamides), clavulanic acid (β-lactamase inhibitors), trimethoprim (diamino pyrimidine derivatives) and dapsone (others chemotherapeutics).
More details about the standard material can be found in Table 3.

Results:

A summary of the test selectivity is given in Table 5.

Table 5. Ratios obtained for β-lactams, tetracyclines and sulfonamides and compounds of other families spiked in raw milk and tested with SYMMETRIC BTS

Family	Compound	MR(P)L/ RPA/ MMPR (µg/kg)	Conc. spiked in milk (µg/kg)	BL		TET		SULFA	
				Ratio	Result	Ratio	Result	Ratio	Result
Penicillins	benzylpenicillin	4	400	+	0.00	-	1.35	-	1.52
Cefalosporins	cefalonium	20	2,000	+	0.00	-	1.38	-	1.58
Tetracyclines	oxytetracycline	100[a]	10,000	-	2.67	+	0.01	-	2.75
Sulfonamides	sulfadiazine	100[b]	10,000	-	2.84	-	2.24	+	0.01
Aminoglycosides	neomycin B	1,500	150,000	-	2.16	-	2.01	-	2.47
Quinolones	enrofloxacin	100[c]	10,000	-	2.95	-	2.41	-	2.87
Polymyxins	colistin	50	5,000	-	2.77	-	2.45	-	3.01
Amphenicols	chloramphenicol	0.3[d]/0.15[e]	30	-	2.75	-	2.36	-	2.72
Macrolides	erythromycin A	40	4,000	-	2.83	-	2.32	-	2.86
Lincosamides	lincomycin	150	15,000	-	2.94	-	2.42	-	2.90
β-lactamase inhibitors	clavulanic acid	200	20,000	+	0.01	-	1.35	-	1.71
Diamino pyrimidine derivatives	trimethoprim	50	5,000	-	2.90	-	2.48	-	1.87
Others	dapsone	5[f]	500	-	2.85	-	2.46	-	2.34

Notes: MRL: Maximum Residue Limit, Regulation (EC) No 470/2009 of the European Parliament and of the Council and Commission Regulation (EU) No 37/2010 and amendments (current situation). Conc.: concentration. BL: β-lactam channel; TET: tetracycline channel; SULFA: sulfonamide channel.
[a]: The MRL of 100 µg/kg in milk is applied on the sum of parent drug and its 4-epimer;
[b]: The combined total residues of all substances within the sulfonamide group should not exceed 100 µg/kg;
[c]: The MRL of 100 µg/kg in milk is applied on the sum of enrofloxacin and ciprofloxacin;
[d]: Prohibited substance, MRPL (Minimum Required Performance Limit, Commission Decision (EC) No 2003/181/EC) till 27 November 2022;
[e]: Prohibited substance, RPA or reference point for action for chloramphenicol from 28 November 2022 on, Commission Regulation (EU) 2019/1871;
[f]: Prohibited substance, Minimum Method Performance Requirement (MMPR) (*Anon.*, 2020)

Discussion:

Clavulanic acid, a β-lactamase inhibitor, gave an interference at the beta-lactam channel at high concentrations. This interference is expected since this molecule contains a β-lactam structure resembling that of the penicillin, except that the fused thiazolidine ring of the penicillins is replaced by an oxazolidine ring (*Anon.*, 2005). The 95% detection capability of clavulanic acid was determined at 400 µg/kg.
On the tetracycline channel and sulfonamide channel no interference was obtained.

SYMMETRIC BTS is a highly specific test for detection of beta-lactams, tetracyclines and sulfonamides in milk and does not detect compounds from the aminoglycosides, quinolones, polymyxins, amphenicols, macrolides, lincosamides and diamino pyrimidine derivatives, nor dapsone.

4.2. Test for false-positive results

Methods and materials:
300 blank farm and 300 tanker load milk samples were tested with SYMMETRIC BTS. In case of positive results, the samples were tested with other microbiological and receptor screening tests to determine whether it is a false-positive result. When it remained inconclusive, the sample was analysed with LC-MS/MS.

Results and discussion:
All 300 farm and 300 tanker load milk samples tested negative for β-lactams, tetracyclines and sulfonamides on SYMMETRIC BTS. So in total no false positive results (0%) are obtained upon 600 blank samples on all channels. The results are summarized in Table 6.
For one farm milk sample very high ratio values were obtained (BL: 8.01, TET: 11.71 and SULFA: 13.47). It was observed that the control line was not well formed due to a bad milk flow. Repeating the analysis of the sample gave similar results. The fat and protein content and somatic cell count of the sample were normal.

Table 6. SYMMETRIC BTS results for blank farm and tanker milk samples.

	Farm milk (n=300)			Tanker milk (n=300)		
	Ratio			Ratio		
mean	2.79	2.51	2.75	2.68	2.40	2.89
min	1.92	1.95	1.95	1.59	1.58	1.89
max	8.01	11.71	13.47	3.93	3.17	4.08
s_r	0.48	0.78	0.91	0.23	0.17	0.32
CV%	17.13	31.05	33.27	8.40	7.26	10.96

Notes: mean: mean ratio; min: lowest ratio; max: highest ratio; s_r ; Standard Deviation; CV(%): Relative Standard Deviation; BL: β-lactam channel; TET: tetracycline channel, SULFA: sulfonamide channel.

5. Reader and test repeatability

5.1 Repeatability of the reader

Methods and Materials:
Samples of 10 blank,10 low positive samples and 10 high positive samples for each channel were measured twice. For the spiked samples, any compound found positive could be used for the testing of the reader repeatability.

Results:
The results of the repeatability of the reader on SYMMETRIC BTS results are summarized in Table 7. For the spiked milk only the relevant data for the different channels are presented.

Table 7. Repeatability of the reader

Reader repeatability	β-Lactam channel			Tetracycline channel			Sulfonamide channel		
	Mean ratio	SD	CV%	Mean ratio	SD	CV%	Mean ratio	SD	CV%
Blank milk	2.92	0.05	1.84	2.62	0.07	2.71	3.06	0.08	2.53
Low pos	0.89	0.03	2.94	0.96	0.03	2.65	0.94	0.03	2.91
High pos	0.46	0.01	2.79	0.22	0.01	4.93	0.51	0.04	7.85

Notes: mean: mean ratio on the respective channel; s_r: Standard deviation of repeatability; CV(%): Relative standard deviation.

Discussion:

The repeatability of the reader is very good; very low standard deviations of repeatability are obtained. The highest variance value is 7.85% for high positive samples with ratio values far from the cut-off.

5.2 Repeatability of the test

Methods and Materials:

Ten blank, 10 low positive samples and 10 high positive samples for each channel were analysed in duplicate. For the spiked samples, any compound found positive could be used.

Results:

The results of the repeatability of SYMMETRIC BTS are summarized in Table 8. For the spiked milk only the relevant data for the different channels are presented.

Table 8: Repeatability of the test

Test repeatability	β-Lactam channel			Tetracycline channel			Sulfonamide channel		
	Mean ratio	SD	CV%	Mean ratio	SD	CV%	Mean ratio	SD	CV%
Blank milk	2.66	0.13	4.80	2.24	0.14	6.06	2.57	0.14	5.37
Low pos	0.89	0.03	3.37	0.93	0.06	6.93	0.84	0.05	6.38
High pos	0.43	0.04	8.31	0.42	0.04	8.64	0.43	0.06	13.20

Notes: mean: mean ratio on the respective channel; s_r: Standard deviation of repeatability; CV(%): Relative standard deviation.

Discussion:

The repeatability of the test is good, low standard deviation values are obtained. The highest variance value of 13.20% is still acceptable since this variance is noted for high positive (low ratio) values far away from the cut-off value.

6. Test robustness

6.1. Influence of changes in the test protocol on the test results

In order to determine the robustness of the assay, small changes to the protocol were introduced concerning the timing of the incubation, delay in reading, milk volume and removal of the filter pad.

6.1.1. Influence of the length of the incubation step on the test results

In order to determine the robustness of the assay, the timing of the incubation step in the protocol was changed. The normal incubation takes 5 minutes.

Methods and Materials:

Blank and spiked milk samples containing benzylpenicillin at 1.5 µg/kg and sulfadoxine at 35 µg/kg or containing cefazolin at 45 µg/kg and oxytetracycline at 10 µg/kg were analysed (4 samples) with a test protocol with incubation timings different from the test protocol (reference = 5 minutes).

Results:

The results of the influence of the length of the incubation step on SYMMETRIC BTS results are summarized in Table 9.

Table 9. Impact of the length of the incubation steps on SYMMETRIC BTS results (ratio).

	Length of incubation step								
	4 minutes and 30 seconds			5 minutes (REF)			5 minutes and 30 seconds		
	BL	TET	SULFA	BL	TET	SULFA	BL	TET	SULFA
Blank milk									
mean	2.86	2.53	2.69	2.75	2.36	2.67	2.87	2.55	2.66
min	2.65	2.37	2.53	2.66	2.22	2.64	2.66	2.30	2.42
max	3.10	2.67	2.98	2.97	2.55	2.70	2.96	2.72	2.89
Milk spiked with benzylpenicillin at 1.5 µg/kg and sulfadoxine at 35 µg/kg									
mean	0.82	1.60	0.58	0.86	1.62	0.58	0.82	1.59	0.56
min	0.71	1.53	0.50	0.83	1.50	0.55	0.72	1.47	0.50
max	0.94	1.66	0.61	0.90	1.71	0.60	0.88	1.64	0.63
Milk spiked with cefazolin at 45 µg/kg and oxytetracycline at 10 µg/kg									
mean	0.74	0.73	1.63	0.78	0.73	1.70	0.77	0.78	1.65
min	0.65	0.66	1.51	0.70	0.69	1.61	0.70	0.73	1.58
max	0.78	0.78	1.78	0.84	0.78	1.76	0.82	0.84	1.74

Notes: mean: mean ratio; min: minimum ratio; max: maximum ratio; BL: β-lactam channel; TET: tetracycline channel; SULFA: sulfonamide channel.

Discussion:

Variations in the length of the incubation step did not significantly impact results; all negative results remained negative and all positive results stayed positive.

6.1.2. Delay of reading

Methods and Materials:
Blank and spiked milk samples containing benzylpenicillin at 1.5 µg/kg and sulfadoxine at 35 µg/kg or containing cefazolin at 45 µg/kg and oxytetracycline at 10 µg/kg were analysed (4 samples) with a test protocol with a delay of the reading after the incubation. A delay of 5 and 10 minutes was tested and the results compared with no delay in reading (= reference). The kit manufacturers advises to read the result as soon as possible and no later than 1 minute after incubation.

Results:
The results of the influence of the delay of reading are summarized in Table 10.

Table 10. Ratios obtained when testing blank and spiked milk samples and reading SYMMETRIC BTS strips directly after incubation or with a delay of 5 or 10 minutes respectively.

	Delay of reading								
	No delay (REF)			5 min			10 min		
	BL	TET	SULFA	BL	TET	SULFA	BL	TET	SULFA
Blank milk									
mean	2.80	2.42	2.52	2.77	2.46	2.55	2.76	2.53	2.62
min	2.70	2.32	2.50	2.55	2.30	2.28	2.72	2.46	2.39
max	2.93	2.56	2.54	2.98	2.81	3.05	2.81	2.58	2.72
Milk spiked with benzylpenicillin at 1.5 µg/kg and sulfadoxine at 35 µg/kg									
mean	0.87	1.52	0.57	0.91	1.59	0.65	0.99	1.75	0.56
min	0.83	1.46	0.54	0.83	1.45	0.64	0.91	1.61	0.51
max	0.90	1.59	0.59	0.97	1.67	0.66	1.04	1.82	0.61
Milk spiked with cefazolin at 45 µg/kg and oxytetracycline at 10 µg/kg									
mean	0.60	0.62	1.44	0.59	0.63	1.50	0.59	0.65	1.51
min	0.53	0.57	1.38	0.46	0.53	**1.03**	0.53	0.58	1.13
max	0.65	0.64	1.54	0.67	0.75	1.86	0.65	0.69	1.74

Notes: REF: reference; mean: mean ratio; min: minimum ratio; max: maximum ratio; BL: β-lactam channel; TET: tetracycline channel; SULFA: sulfonamide channel.

Discussion:
Delay in reading of the test strips did not significantly impact the interpretation of the results; all negative results remained negative and all positive results stayed positive on the respective channels. One test strip gave a false positive result (ratio 1.03) on the sulfonamide channel for a milk sample containing cefazolin and oxytetracycline at detectable levels, when a delay in reading of 5 minutes was applied. After a 10 minute delay, a borderline negative (ratio 1.13) was obtained. When delaying the reading, also slightly higher (less positive) ratios on the β-lactam channel were obtained. Therefore it is recommended to follow the manufacturers' instructions and read the strips within 1 minute after incubation.

6.1.3. Volume of the milk

Methods and Materials:
Blank and spiked milk samples containing benzylpenicillin at 1.5 µg/kg and sulfadoxine at 35 µg/kg or containing cefazolin at 45 µg/kg and oxytetracycline at 10 µg/kg were analysed (4 samples) with a test protocol with different volumes of milk. A volume of 90, 100 (protocol = reference), and 110 µl of milk was tested.

Results:
The results of the influence of the different volumes of milk are summarized in Table 11.

Table 11. Ratios obtained when testing different volumes (90, 100 and 110 µl, respectively) of milk with SYMMETRIC BTS.

	Volume of milk								
	90 µl			100 µl (REF)			110 µl		
	BL	TET	SULFA	BL	TET	SULFA	BL	TET	SULFA
Blank milk									
mean	2.63	2.23	2.57	2.76	2.43	2.82	2.75	2.51	2.66
min	2.59	2.13	2.46	2.54	2.31	2.49	2.61	2.40	2.55
max	2.69	2.37	2.65	2.99	2.55	3.41	2.87	2.63	2.81
Milk spiked with benzylpenicillin at 1.5 µg/kg and sulfadoxine at 35 µg/kg									
mean	0.99	1.66	0.70	0.86	1.58	0.61	0.71	1.60	0.54
min	0.84	1.49	0.64	0.82	1.55	0.56	0.69	1.57	0.50
max	1.07	1.74	0.73	0.89	1.64	0.66	0.74	1.68	0.57
Milk spiked with cefazolin at 45 µg/kg and oxytetracycline at 10 µg/kg									
mean	0.77	0.66	1.75	0.72	0.69	1.73	0.76	0.73	1.79
min	0.73	0.60	1.62	0.68	0.66	1.50	0.73	0.69	1.69
max	0.81	0.73	1.81	0.75	0.73	1.94	0.79	0.75	1.91

Notes: REF: reference; mean: mean ratio; min: minimum ratio; max: maximum ratio; BL: β-lactam channel; TET: tetracycline channel; SULFA: sulfonamide channel.

Discussion:
A volume of milk differing 10 µl (10%) from the prescribed volume of 100 µl did not impact the interpretation of test results; the negative results remained negative and positive results stayed positive.

6.1.4. Removal of the filter pad

Methods and Materials:
Blank and spiked milk samples containing benzylpenicillin at 1.5 µg/kg and sulfadoxine at 35 µg/kg or containing cefazolin at 45 µg/kg and oxytetracycline at 10 µg/kg were analysed (4 samples) with a test protocol with and without removal of the filter pad. Manufacturers' guidelines advice the immediate removal of the filter pad.

Results:
The results of the influence of the not removing of the filter pad are summarized in Table 12.

Table 12. Ratios obtained with SYMMETRIC BTS when removing (=reference) or not removing the filter pad before reading.

	Removal of filter pad					
	Yes (REF)			NO		
	BL	TET	SULFA	BL	TET	SULFA
Blank milk						
mean	2.80	2.42	2.52	2.92	2.55	2.94
min	2.70	2.32	2.50	2.78	2.36	2.85
max	2.93	2.56	2.54	3.03	2.70	3.01
Milk spiked with benzylpenicillin at 1.5 µg/kg and sulfadoxine at 35 µg/kg						
mean	0.87	1.52	0.57	0.89	1.58	0.47
min	0.83	1.46	0.54	0.83	1.54	0.04
max	0.90	1.59	0.59	0.92	1.65	0.62
Milk spiked with cefazolin at 45 µg/kg and oxytetracycline at 10 µg/kg						
mean	0.60	0.62	1.44	0.68	0.63	1.65
min	0.53	0.57	1.38	0.58	0.58	1.55
max	0.65	0.64	1.54	0.76	0.68	1.78

Notes: REF: reference; mean: mean ratio; min: minimum ratio; max: maximum ratio; BL: β-lactam channel; TET: tetracycline channel; SULFA: sulfonamide channel.

Discussion:
Not removing the filter pad did not impact the interpretation of test results significantly; the negative results remained negative and positive results stayed positive. However, it is worth noting that for one sample spiked with 35 µg/kg of sulfadoxine, a very positive result was obtained (ratio 0.04) when not removing the sample pad, reanalysing the sample gave similar results.

6.2. External influences

6.2.1. Impact of the milk temperature

Methods and Materials:
Tests were performed (4 samples) with milk of 1 - 4℃ (= reference) and of 20℃ in order to check if the milk temperature is influencing SYMMETRIC BTS results. Besides blank milk also spiked milk samples containing benzylpenicillin at 1.5 µg/kg and sulfadoxine at 35 µg/kg or containing cefazolin at 45 µg/kg and oxytetracycline at 10 µg/kg were used.

Results:
The results of the impact of the milk temperature are summarized in Table 13. For the spiked milk only the relevant data for the different channels are presented.

Table 13. Impact of the milk temperature on the SYMMETRIC BTS result.

	Milk temperature					
	1 - 4℃ (REF)			20℃		
	BL	TET	SULFA	BL	TET	SULFA
Blank milk						
mean	2.75	2.36	2.67	2.95	2.54	2.87
min	2.66	2.22	2.64	2.82	2.41	2.66
max	2.97	2.55	2.70	3.17	2.67	3.01
Milk spiked with benzylpenicillin at 1.5 µg/kg and sulfadoxine at 35 µg/kg						
mean	0.86	1.62	0.58	0.83	1.62	0.59
min	0.83	1.50	0.55	0.80	1.57	0.57
max	0.90	1.71	0.60	0.88	1.67	0.61
Milk spiked with cefazolin at 45 µg/kg and oxytetracycline at 10 µg/kg						
mean	0.78	0.73	1.70	0.79	0.75	1.77
min	0.70	0.69	1.61	0.70	0.69	1.64
max	0.84	0.78	1.76	0.86	0.80	1.91

Notes: REF: reference; mean: mean ratio; min: minimum ratio; max: maximum ratio; BL: β-lactam channel; TET: tetracycline channel; SULFA: sulfonamide channel.

Discussion:
A milk temperature of 20℃ did not significantly im pact the SYMMETRIC BTS results. Blank milk was always tested as negative while the spiked milk samples gave clear positive results on their respective channel.

6.3. Milk quality and milk composition influences

Methods and Materials:

Somatic cell count
Normal milk samples and milk samples with a high somatic cell count (>10^6 per ml) were analysed and the ratios obtained were compared in order to study the impact of the somatic cell count on the SYMMETRIC BTS result. The milk samples with a high number of somatic cells were selected at the milk control station based on Fossomatic 7 (FOSS, Hillerød. DK) measurements.

Total bacterial count
Normal milk samples and milk samples with a high total bacterial count (TBC >1.10×10^6 CFU per ml) were analysed and the ratios obtained were compared in order to study the impact of the total bacterial count on the SYMMETRIC BTS result. The milk samples with a high total bacterial count were obtained by keeping normal milk samples during 4-6 hours at room temperature. The final bacterial count was determined by performing a spiral plate count (Eddy Jet. IUL sa. Barcelona, ES) on Plate count agar plates after 3 days incubation at 30℃.

Fat content
Normal milk samples and milk samples with a low (≤1.93 g per 100 ml) or a high (≥6.12 g per 100 ml) fat content were analysed and the ratios obtained were compared in order to study the

impact of the fat content on the SYMMETRIC BTS result. The milk samples with a low and high fat content were natural milk samples with a low and a high fat content selected at the milk control station based on infrared spectroscopic results with a MilcoScan 7 (FOSS, Hillerød, DK).

Protein content

Normal milk samples and milk samples with a low (≤2.94 g per 100 ml) or a high (≥4.00 g per 100 ml) protein content were analysed and the ratios obtained were compared in order to study the impact of the protein content on the SYMMETRIC BTS result. The milk samples tested were natural milk samples with a low and a high protein content. These samples were selected at the milk control station based on infrared spectroscopic results with a MilcoScan 7 (FOSS, Hillerød, DK).

pH:

Milk samples with a normal pH (6.7 - 6.9) and milk samples with a low pH (6.0) or a high pH (7.5) were analysed and the ratios obtained were compared in order to study the impact of the milk pH on the SYMMETRIC BTS result. The low and high pH milk samples were prepared by adding 1 M HCl or 1 M NaOH. Respectively, to milk with normal pH. Afterwards the pH of the milk was brought exactly to 6.0 and 7.5 by fine-tuning with 0.1 M HCl and/or 0.1 M NaOH.

Results:

With respect to the impact of the milk quality (high somatic cell count, high total bacterial count) and composition (fat and protein content and pH), the mean, the highest and lowest reader value for each milk type are given in Figures 4 to 6 and Table 14.

The legend for the different situations in figures 4 to 6.

1 = Reference: normal raw milk;
2 = SCC >10^6/ml
3 = TBC ≥1.10×10^6 CFU/ml
4 = Low fat content (≤1.93 g/100 ml);
5 = High fat content (≥6.12 g/100 ml);
6 = Low protein (≤2.94 g/100 ml);
7 = High protein (≥4.00 g/100 ml);
8 = Low pH (pH = 6.0)
9 = High pH (pH = 7.5)

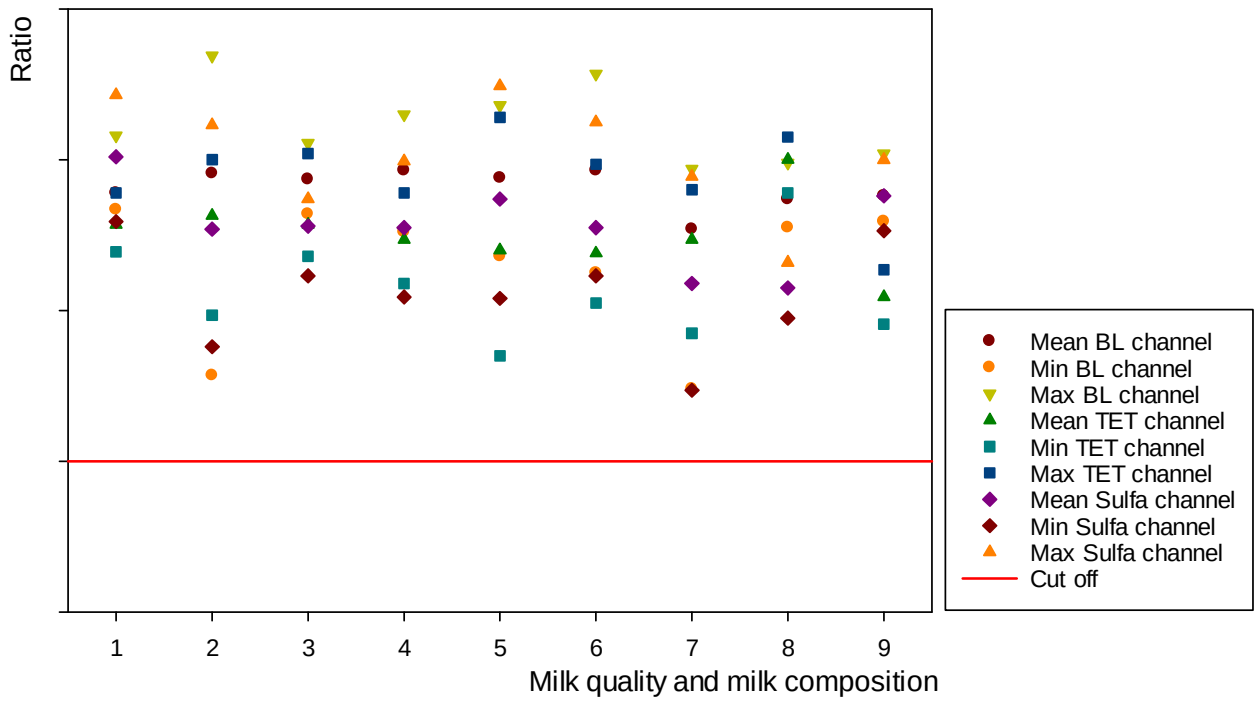

Fig. 4. Results for blank milk, 10 samples. BL: β-lactam channel; TET: tetracycline channel; SULFA: sulfonamide channel.

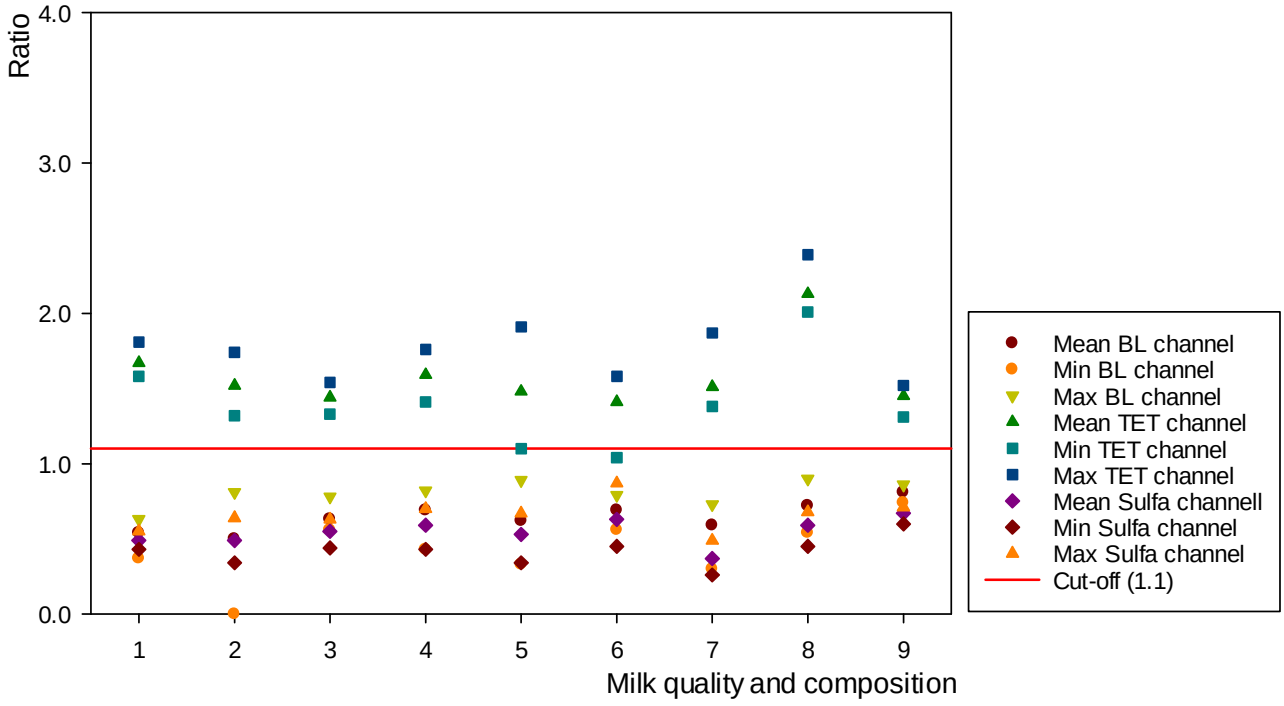

Fig. 5. Results for milk spiked with benzylpenicillin at 1.5 µg/kg and sulfadoxine at 35 µg/kg, 10 samples. BL: β-lactam channel; TET: tetracycline channel; SULFA: sulfonamide channel.

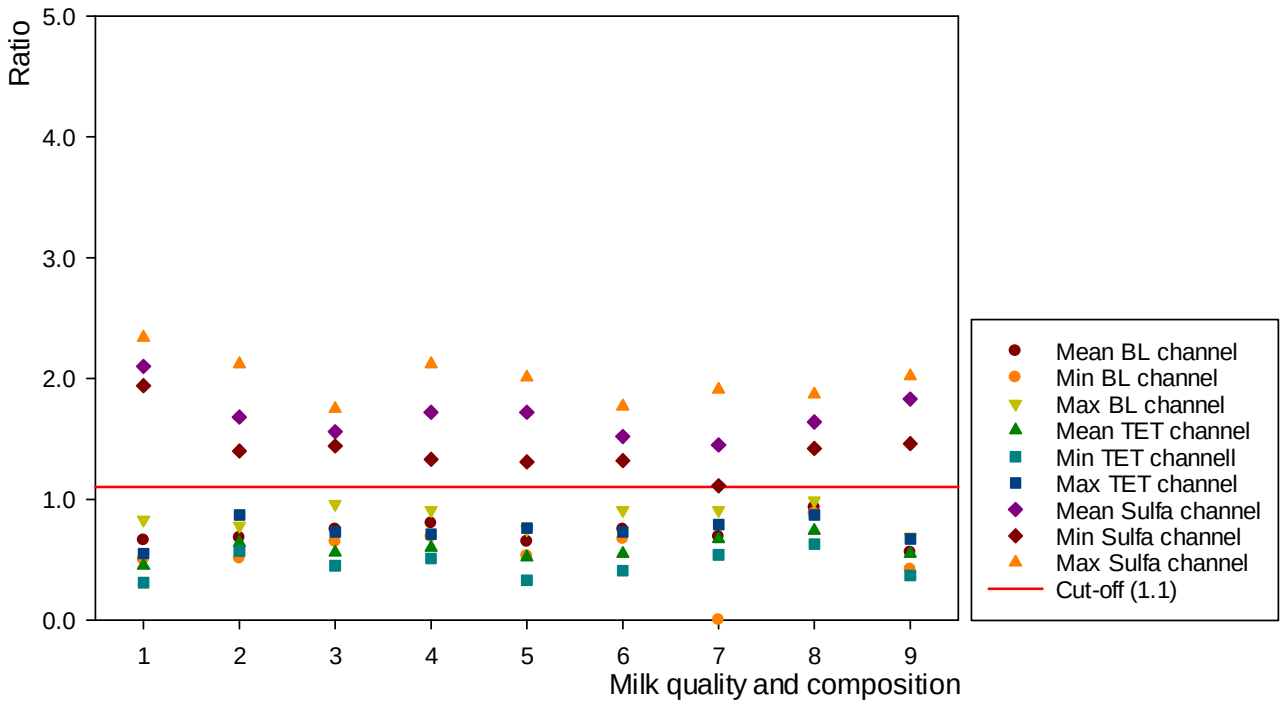

Fig. 6. Results for milk spiked with cefazolin at 45 µg/kg and oxytetracycline at 10 µg/kg, 10 samples. BL: β-lactam channel; TET: tetracycline channel; SULFA: sulfonamide channel.

Discussion:

The milk quality and composition had no significant influence on the performance of the SYMMETRIC BTS. No false positives were obtained with the blank milk. It is worth noting that for blank milk, lower ratio values were obtained for milk with a high protein content on all three test channels, for milk with a high somatic cell count on the β-lactam and sulfonamide channel and for milk with a high fat content on the tetracycline channel. And one false positive results out of 10 samples was obtained for milk with a low protein content spiked with benzylpenicillin and sulfadoxine on the tetracycline channel (ratio: 1.04).

For all spiked samples, positive results were obtained on the respective test lines.

Table 14. SYMMETRIC BTS results for blank and spiked normal raw cows' milk and for blank and spiked milk of special quality or composition (10 samples).

	Ratio								
	β-lactam channel			Tetracycline channel			Sulfonamide channel		
	mean	min	max	mean	min	max			
Blank raw cows' milk									
normal milk = reference	2.78	2.67	3.16	2.57	2.39	2.78	3.02	2.59	3.43
SCC >10^6/ml	2.91	1.57	3.69	2.63	1.97	3.00	2.54	1.76	3.23
TBC ≥1.10×10^6 cfu/ml	2.87	2.64	3.11	2.57	2.36	3.04	2.56	2.23	2.74
low fat ≤1.93 g/100 ml	2.93	2.52	3.30	2.47	2.18	2.78	2.55	2.09	2.99
high fat ≥6.12 g/100 ml	2.88	2.36	3.36	2.40	1.70	3.28	2.74	2.08	3.49
low protein ≤2.94 g/100 ml	2.93	2.25	3.57	2.38	2.05	2.97	2.55	2.23	3.25
high protein ≥4.00 g/100 ml	2.54	1.48	2.94	2.47	1.85	2.80	2.18	1.47	2.89
low pH (pH = 6.0)	2.74	2.55	2.98	3.00	2.78	3.15	2.15	1.95	2.32
high pH (pH = 7.5)	2.76	2.59	3.04	2.09	1.91	2.27	2.76	2.53	3.00
Milk spiked with benzylpenicillin at 1.5 µg/kg and sulfadoxine at 35 µg/kg									
normal milk = reference	0.54	0.37	0.63	1.67	1.58	1.81	0.49	0.43	0.55
SCC >10^6/ml	0.50	0.00	0.81	1.52	1.32	1.74	0.49	0.34	0.64
TBC ≥1.1x10^6 cfu/ml	0.63	0.56	0.78	1.44	1.33	1.54	0.55	0.44	0.63
low fat ≤1.93 g/100 g	0.69	0.43	0.82	1.59	1.41	1.76	0.59	0.43	0.70
high fat ≥6.12 g/100 g	0.62	0.33	0.89	1.48	1.10	1.91	0.53	0.34	0.67
low protein ≤2.94 g/100 ml	0.69	0.56	0.79	1.41	**1.04**	1.58	0.63	0.45	0.87
high protein ≥4.00 g/100 ml	0.59	0.30	0.73	1.51	1.38	1.87	0.37	0.26	0.49
low pH (pH = 6.0)	0.72	0.54	0.90	2.13	2.01	2.39	0.59	0.45	0.68
high pH (pH = 7.5)	0.81	0.74	0.86	1.45	1.31	1.52	0.67	0.60	0.71
Milk spiked with cefazolin at 45 µg/kg and oxytetracycline at 10 µg/kg									
normal milk = reference	0.66	0.50	0.83	0.45	0.31	0.55	2.10	1.94	2.34
SCC >10^6/ml	0.68	0.51	0.78	0.64	0.57	0.87	1.68	1.40	2.12
TBC ≥1.1x10^6 cfu/ml	0.75	0.65	0.96	0.56	0.45	0.73	1.56	1.44	1.75
low fat ≤1.93 g/100 ml	0.80	0.69	0.91	0.60	0.51	0.71	1.72	1.33	2.12
high fat ≥6.12 g/100 ml	0.65	0.53	0.75	0.52	0.33	0.76	1.72	1.31	2.01
low protein ≤2.94 g/100 ml	0.75	0.67	0.91	0.55	0.41	0.73	1.52	1.32	1.77
high protein ≥4.00 g/100 ml	0.69	0.00	0.91	0.67	0.54	0.79	1.45	1.11	1.91
low pH (pH = 6.0)	0.93	0.88	0.99	0.74	0.63	0.87	1.64	1.42	1.87
high pH (pH = 7.5)	0.56	0.42	0.69	0.55	0.37	0.67	1.83	1.46	2.02

Notes: min: minimum; max: maximum.

6.4. Type of milk and animal origin influences

Methods and Materials:
Raw milk, UHT milk and reconstituted milk powder were analysed in order to determine if the SYMMETRIC BTS is a suitable test for these types of milk. Also raw goats' and raw ewes' milk samples were analysed to determine if SYMMETRIC BTS is a suitable test for these milk types.

Results:
With respect to the impact of the milk type (UHT and reconstituted milk powder), goats' and ewes' milk, the mean, the highest and lowest reader value for each milk type are given in Figures 7 to 9 and Table 15.

The legend for the different situations in figures 7 to 9.

1 = Normal raw milk = reference
2 = UHT milk
3 = Reconstituted milk powder
4 = Goats' milk
5 = Ewes' milk

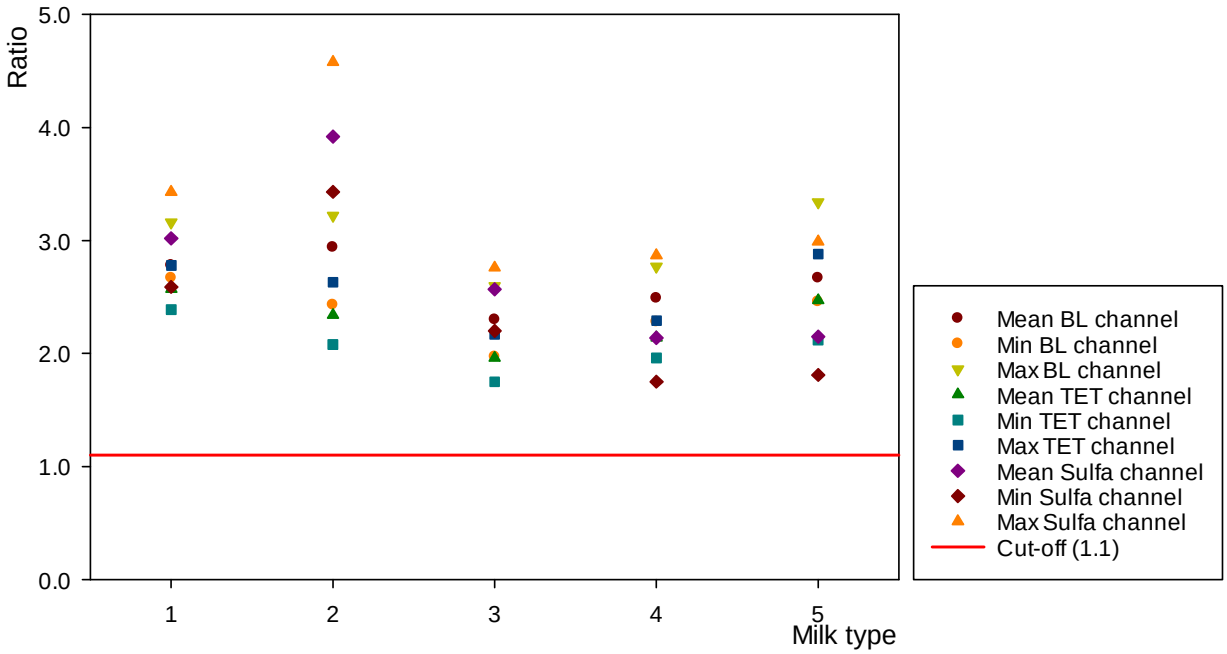

Fig. 7. Results for blank milk, 10 samples. BL: β-lactam channel; TET: tetracycline channel; SULFA: sulfonamide channel.

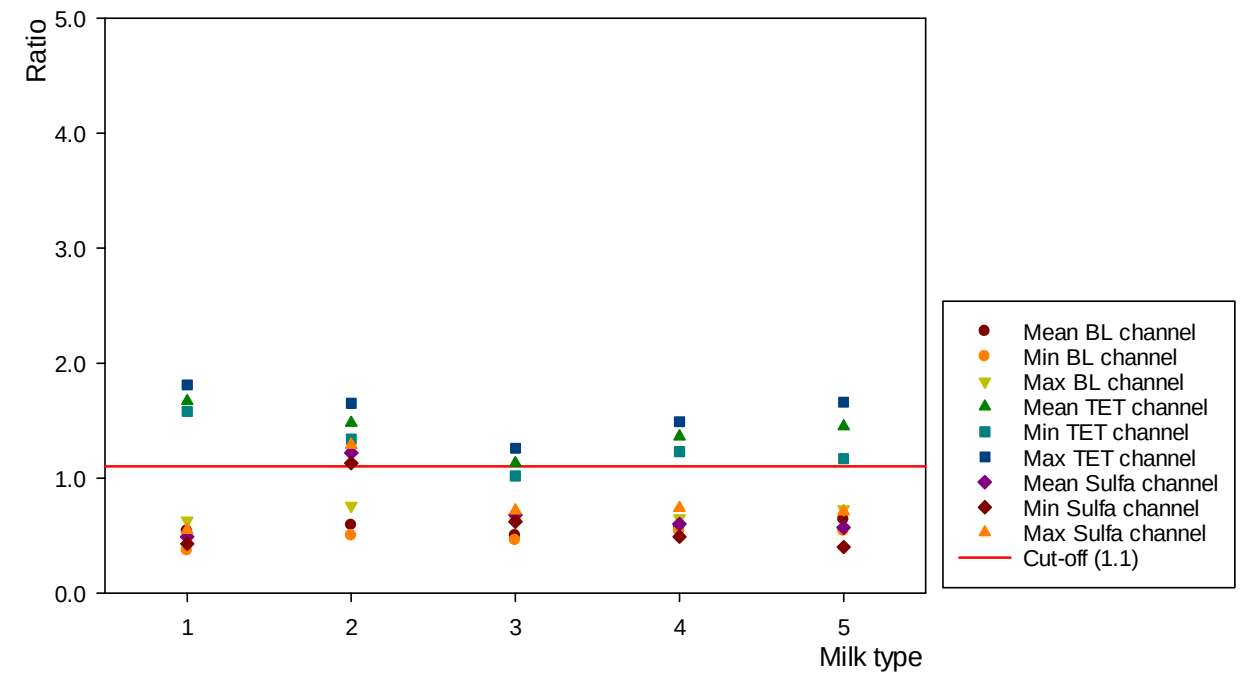

Fig. 8. Results for milk spiked with benzylpenicillin at 1.5 µg/kg and sulfadoxine at 35 µg/kg, 10 samples. BL: β-lactam channel; TET: tetracycline channel; SULFA: sulfonamide channel.

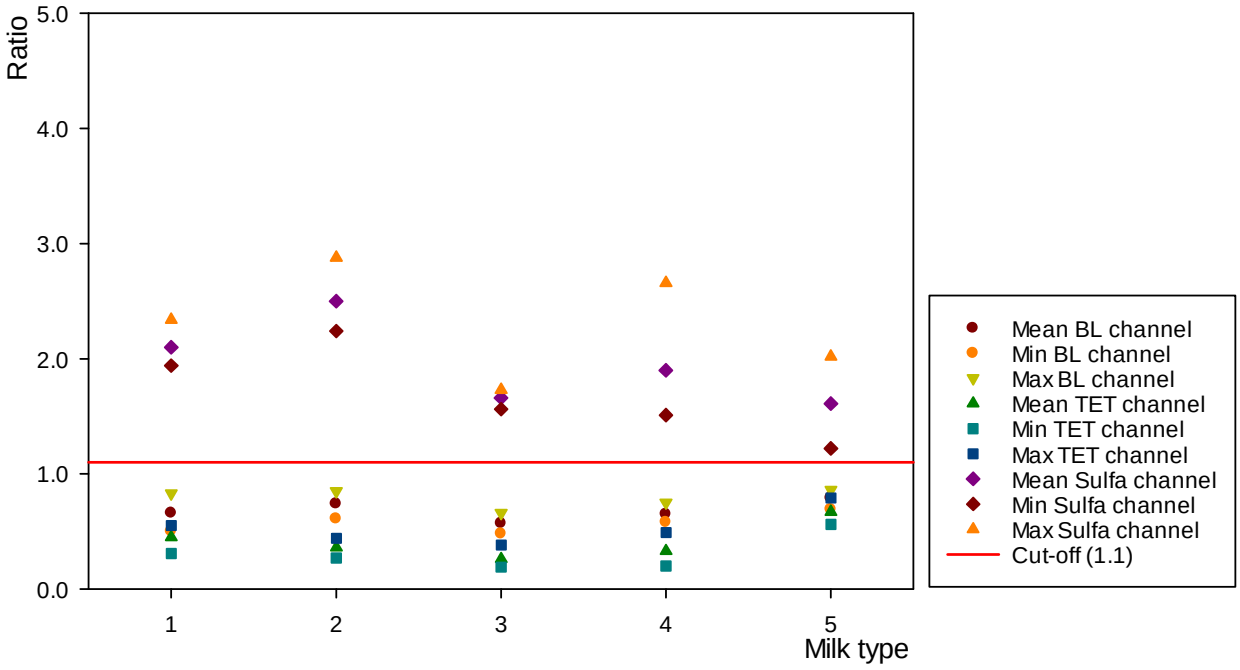

Fig. 9. Results for milk spiked with cefazolin at 45 µg/kg and oxytetracycline at 10 µg/kg, 10 samples. BL: β-lactam channel; TET: tetracycline channel; SULFA: sulfonamide channel.

Table 15. SYMMETRIC BTS results for blank and spiked normal raw cows' milk and for blank and spiked milk of special milk type or other animal origin (10 samples, except for 20 samples of blank goats' and ewes' milk).

	Ratio								
	BL			TET			SULFA		
	mean	min	max	mean	min	max	mean	min	max
Blank raw cows' milk									
Normal milk = reference	2.78	2.67	3.16	2.57	2.39	2.78	3.02	2.59	3.43
UHT	2.94	2.43	3.22	2.34	2.08	2.63	3.92	3.43	4.58
Reconstituted milk powder	2.30	1.97	2.60	1.96	1.75	2.17	2.57	2.20	2.76
Goats' milk (n=20)	2.49	2.28	2.77	2.14	1.96	2.29	2.14	1.75	2.87
Ewes' milk (n=20)	2.67	2.46	3.34	2.47	2.12	2.88	2.15	1.81	2.99
Milk spiked with benzylpenicillin at 1.5 µg/kg and sulfadoxine at 35 µg/kg									
Normal milk = reference	0.54	0.37	0.63	1.67	1.58	1.81	0.49	0.43	0.55
UHT	0.59	0.50	0.76	1.48	1.34	1.65	**1.22**	**1.13**	**1.29**
Reconstituted milk powder	0.50	0.46	0.62	1.13	**1.02**	1.26	0.68	0.62	0.72
Goats' milk	0.58	0.54	0.65	1.36	1.23	1.49	0.60	0.49	0.74
Ewes' milk	0.64	0.54	0.73	1.45	1.17	1.66	0.57	0.40	0.71
Milk spiked with cefazolin at 45 µg/kg and oxytetracycline at 10 µg/kg									
Normal milk = reference	0.66	0.50	0.83	0.45	0.31	0.55	2.10	1.94	2.34
UHT	0.74	0.61	0.85	0.36	0.27	0.44	2.50	2.24	2.88
Reconstituted milk powder	0.57	0.48	0.66	0.26	0.19	0.38	1.66	1.56	1.73
Goats' milk	0.65	0.58	0.75	0.33	0.20	0.49	1.90	1.51	2.66
Ewes' milk	0.79	0.69	0.86	0.67	0.56	0.79	1.61	1.22	2.02

Notes: min: minimum; max: maximum. BL: β-lactam channel; TET: tetracycline channel; SULFA: sulfonamide channel.

Discussion:

There could be interest to use SYMMETRIC BTS, although developed for the testing of raw cows' milk, to test UHT milk or reconstituted milk powder. People could also have interest to test milk from an animal species different from the cow (goat and ewe).

For all tested milk types and species different from raw cows' milk, all blank milk tested negative. But it is worth noting that on the tetracycline channel lower ratio values were obtained for reconstituted milk powder and on the sulfonamide channel lower ratios were obtained for goats' and ewes' milk for blank samples. False positive results were obtained on the tetracycline channel for reconstituted milk powder spiked with benzylpenicillin and sulfadoxine. All 10 tested samples showed very low ratio values on the tetracycline channel (mean ratio value: 1.13; maximum ratio value: 1.29), and 3 out of 10 samples gave positive values (ratios: 1.02, 1.05 and 1.09).

For the spiked samples, all positive results were obtained on the respective channels except for UHT milk where all 10 spiked samples gave borderline negative results for the sulfonamides test line for milk spiked with 35 µg/kg of sulfadoxine (ratios between 1.13 and 1.29). This hampering of detection of sulfonamides in UHT milk indicates a slightly higher ccβ in UHT milk, but at MRL (=100 µg/kg) no problems are expected.

But it can be concluded that SYMMETRIC BTS is also suitable to use for UHT milk and reconstituted milk powder and also to test goats' and ewes' milk for the detection of β-lactam, tetracycline and sulfonamide residues.

6.5. Stability of reagents - Daily control samples

Methods and material:
The following control samples were analyzed daily with SYMMETRIC BTS to check the stability of the reagents and consistency of results:

- blank raw milk free from antimicrobials (fresh and frozen) - twice
- blank raw milk spiked with benzylpenicillin at 1.5 µg/kg and sulfadoxine at 35 µg/kg - twice
- blank raw milk spiked with cefazolin at 45 µg/kg and oxytetracycline at 10 µg/kg - twice

Each day, also a negative and positive control as provided in the kit were analysed. The positive standard contains: benzylpenicillin 4 µg/kg, tetracycline 100 µg/kg and 100 sulfadiazine 100 µg/kg.

Results:
The results of the daily control samples and negative and positive control samples are presented in Figure 10 to 12. A summary is provided in Table 16.

Table 16. SYMMETRIC BTS results (ratio values) for the daily standards.

standard	β-lactam channel				Tetracycline channel				Sulfonamide channel			
	mean	min	max	s_r	mean	min	max	s_r	mean	min	max	s_r
Blank milk												
Fresh	2.84	2.36	3.39	0.23	2.43	2.07	3.00	0.21	2.95	2.05	3.80	0.34
Frozen	2.68	2.23	3.15	0.18	2.22	1.93	2.61	0.14	2.82	1.63	3.37	0.31
Milk spiked with benzylpenicillin at 1.5 µg/kg and sulfadoxine at 35 µg/kg												
	0.54	0.35	0.88	0.09	1.46	1.33	1.60	0.07	0.57	0.44	0.82	0.07
Milk spiked with cefazolin at 45 µg/kg and oxytetracycline at 10 µg/kg												
	0.69	0.46	0.82	0.06	0.59	0.33	0.72	0.07	1.88	1.24	2.50	0.22
Controls included in the kit												
Negative	2.53	2.07	3.51	0.27	1.63	1.33	2.20	0.18	2.89	1.96	4.07	0.37
Positive	0.01	0.00	0.09	0.02	0.01	0.00	0.14	0.03	0.08	0.00	0.25	0.06

Notes: s_r: standard deviation; min: lowest ratio; max: highest ratio.

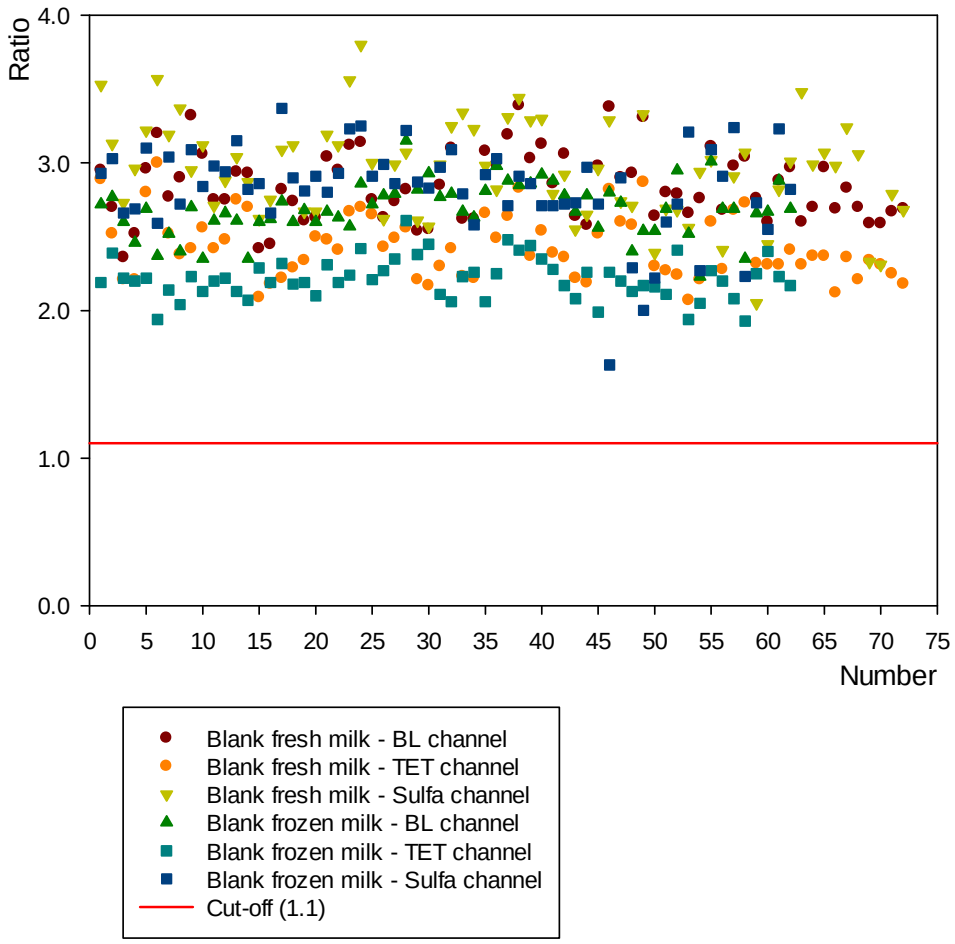

Fig. 10. SYMMETRIC BTS results (ratio) for the blank control samples (fresh and frozen) of the daily standard. BL: β-lactam channel; TET: tetracycline channel; SULFA: sulfonamide channel.

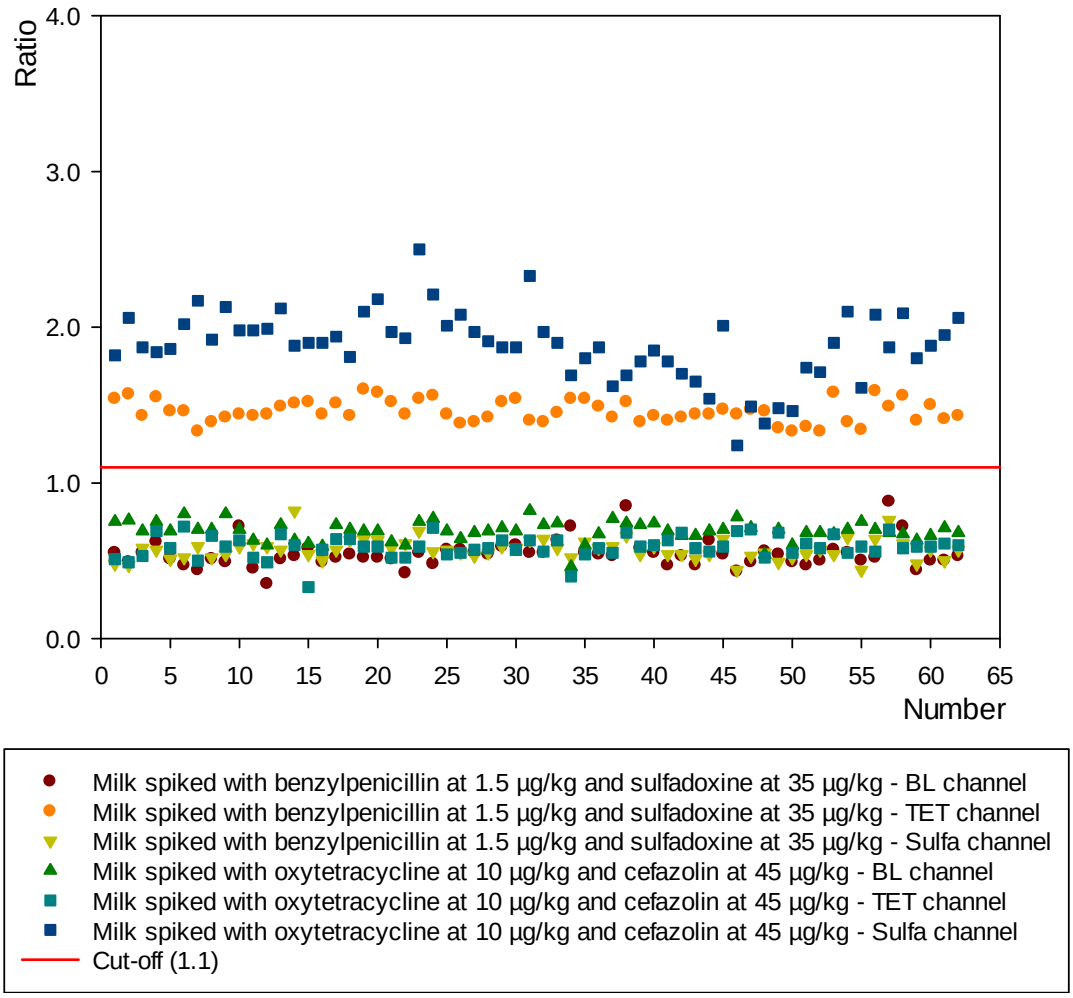

Fig. 11. SYMMETRIC BTS results (ratio) for the spiked samples with benzylpenicillin at 1.5 µg/kg and sulfadoxine at 35 µg/kg or containing cefazolin at 45 µg/kg and oxytetracycline at 10 µg/kg of the daily standards. BL: β-lactam channel; TET: tetracycline channel; SULFA: sulfonamide channel.

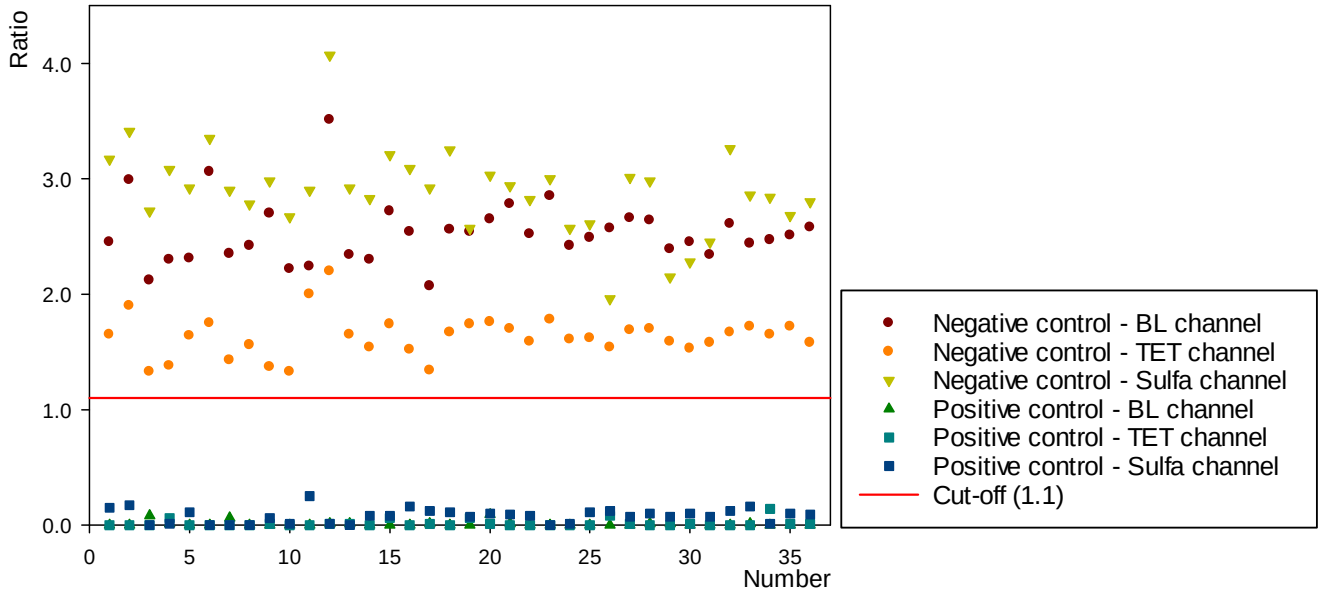

Fig. 12. SYMMETRIC BTS results (ratio) for the negative and positive controls inserted in the kit. BL: β-lactam channel; TET: tetracycline channel; SULFA: sulfonamide channel.

Discussion:
In general stable ratio values were obtained for daily control samples with SYMMETRIC BTS reagents over the test period on both tests lines. Always correct values were obtained for the different daily standards. All blank milk standards gave a negative result on all channels. All spiked milk samples gave positive results.
The negative and positive controls inserted in the kit always gave correct results. The concentrations in the positive controls of the kit are at MRL, so far above the respective ccβ-values.

7. Reliability of the instrumentation

It is very important that no remains of the filter pad are present on the strip to avoid 'INVALID' results. If the test strips are not well positioned (vertically) in the reader, the reading can be discontinued due to an software error.

8. Reader comparison S-Flow Reader and 3PR-Mini Reader

The validation of SYMMETRIC BTS was performed with the S-Flow Reader and S-Flow software (ProGnosis Biotech S.A., Larissa, Greece). In this part of the report, a short comparison study is performed between the S-Flow Reader and the 3PR Mini reader (ProGnosis Biotech S.A., Larissa, Greece). For both readers, S-Flow software (ProGnosis Biotech S.A., Larissa, Greece) is used.

Test protocol and results:

Both blank raw farm milk samples and milk samples spiked with different concentrations of beta-lactams, tetracyclines and sulfonamides were used to obtain a range of negative and (low and high) positive results. These spiked samples, together with blank milk samples were tested with SYMMETRIC BTS.
The SYMMETRIC BTS strips were dried for 15 minutes and read with both readers with the smallest time interval as possible, with half of the strips read first by the S-Flow Reader, the other half read first by the 3PR Mini reader. Both readers calculated the ratio for each test line with the reference line. 130 Results for the beta-lactam test line, and 106 results for the tetracycline test line and the sulfonamide test line were collected with values for both readers. The results are shown in figures 13 to 15. The best-fitting linear curve is calculated based on all data.

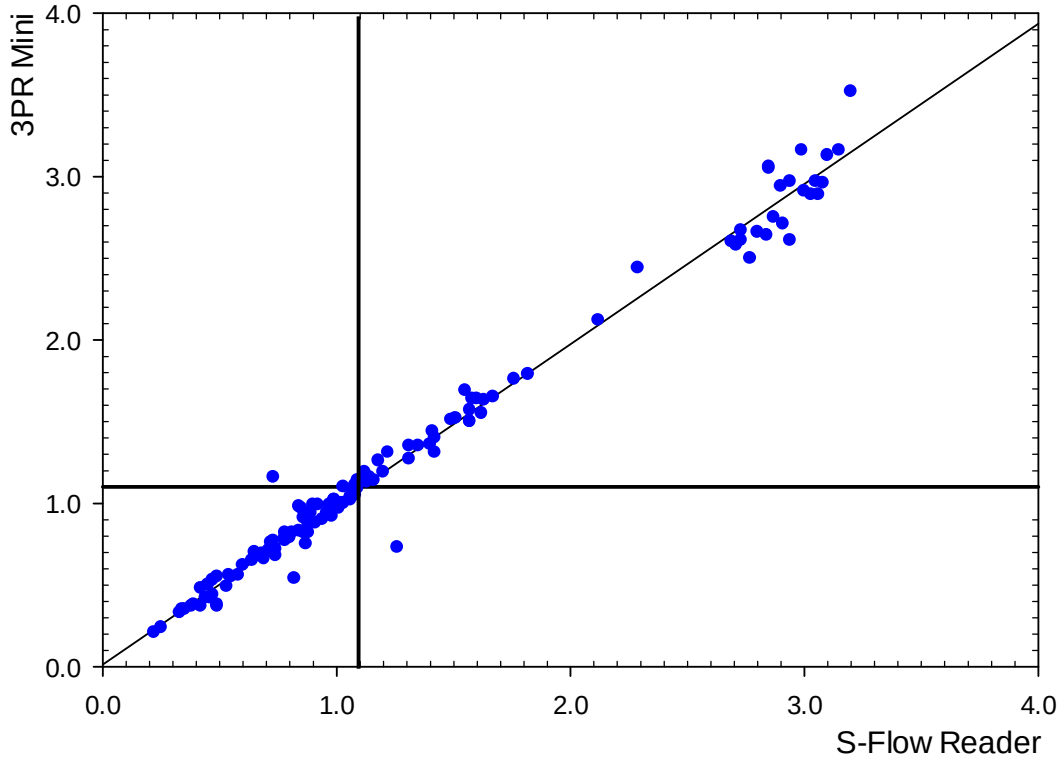

Figure 13: Results (ratio) for the β-lactam test line. Comparison in reading with S-Flow Reader and the 3PR Mini reader.

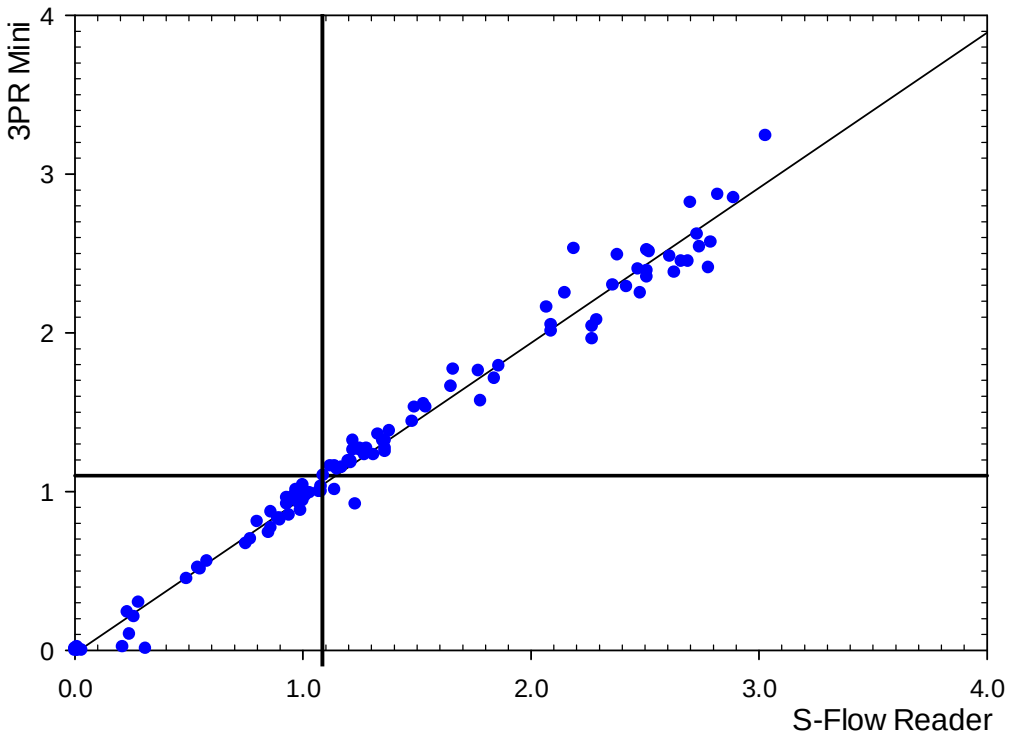

Figure 14: Results (ratio) for the tetracycline test line. Comparison in reading with S-Flow Reader and the 3PR Mini reader.

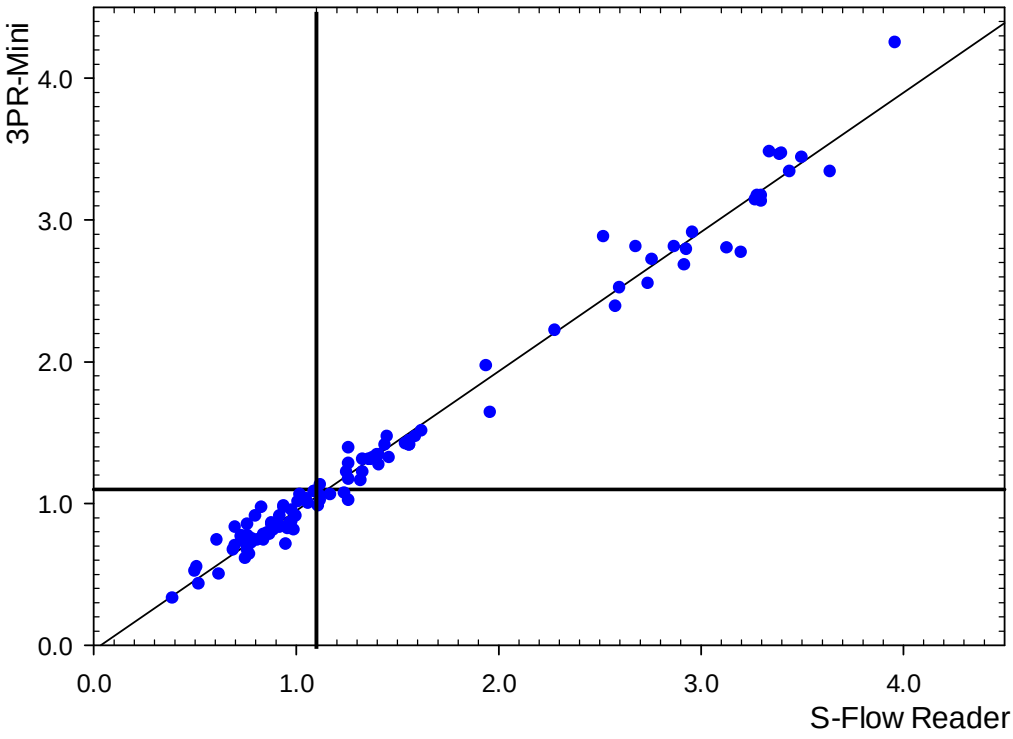

Figure 15: Results (ratio) for the sulfonamide test line. Comparison in reading with S-Flow Reader and the 3PR Mini reader.

Discussion:
In the validation study of SYMMETRIC BTS, the S-Flow Reader with cut-off 1.10 was used to determine the detection capability. In the figures 13 to 15, it is seen that best fitting curve between the data for all three channels goes through the intercept nearly at 0 and also goes nearly through the crosspoint of both lines for a ratio of 1.10, indicating that there is no or only a limited difference between the interpretation of results with both readers. This is confirmed with the raw data, where most data are interpreted equally. Only a few samples with ratio values around the cut-off of 1.1 have a different interpretation.

Based on these results can be claimed that when using the 3PR Mini reader with S-Flow software and a cut-off of 1.10, very similar detection capabilities will be obtained as using the S-Flow Reader for SYMMETRIC BTS.

8. Final conclusion

The validation of SYMMETRIC BTS (ProGnosis Biotech S.A., Larissa, Greece) was executed following ISO Technical specification TS 23758 / IDF RM 251.

The validation results of the SYMMETRIC BTS (ProGnosis Biotech S.A., Larissa, Greece) show that the test is a fast, simple and reliable highly specific test for screening of raw cows' milk for residues of β-lactam antibiotics (penicillins and cefalosporins), tetracyclines and sulfonamides in raw commingled cows' milk (Commission Regulation 37/2010 and amendments). All β-lactams, tetracyclines (parent drugs and their 4-epimers) and sulfonamides can be detected at least in 95% of the samples at their respective MRL except for ceftiofur (CCβ =300 µg/kg, MRL=100 µg/kg), desfuroylceftiofur (CCβ=400 µg/kg, MRL=100 µg/kg) and cefalexin (CCβ=500 µg/kg, MRL=100 µg/kg).
Doxycycline (no MRL in milk) was detected at least in 95% of the replicates at 14 µg/kg.

SYMMETRIC BTS is a highly specific test for detection of beta-lactams (penicillins and cefalosporins), tetracyclines and sulfonamides. Interference by clavulanic acid, a β-lactamase inhibitor, was observed on the β-lactam channel. The 95% detection capability of clavulanic acid was determined at 400 µg/kg.

No false positive results (0%) were obtained testing 300 blank farm and 300 blank tanker load milk samples on both channels. The repeatability of both reader and test are good.

The test showed to be robust when changes in the test protocol (incubation times, incubation temperature, delay of reading, milk temperature, volume of milk, removal of sample pad, were introduced. The milk quality and composition had no significant influence on the performance of the SYMMETRIC BTS.

The test could also be used to screen UHT or reconstituted milk powder or goats' and ewes' milk on the presence of residues of beta-lactams, tetracyclines and sulfonamides. For UHT

milk slightly higher ccβ-values for sulfonamides (Table 4) are observed, but at MRL it is expected that all sulfonamides listed in Table 4 will be detected in at least 95% of the replicates.

A short comparison study between the S-Flow Reader (used throughout the validation) and the 3PR Mini reader, both with S-Flow software and a cut-off of 1.10, shows that very similar detection capabilities will be obtained with both readers.

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REFERENCES

Anonymous. 2005. Martindale: The complete drug reference. Ed. Sweetman S. C. Royal Pharmaceutical Society of Great Britain, Pharmaceutical Press, London, United Kingdom, 34th Edition.

Anonymous. 2010. Guidelines for the validation of screening methods for residues of veterinary medicines (initial validation and transfer). Community Reference Laboratories Residues (CRLs). 20/01/2010: 1-18.

Anonymous. 2020. EURL Guidance on minimum Method Performance Requirements (MMPRs) for specific pharmacologically active substances in specific animal matrices September 2020: 1-8.

Anonymous. 2023. Manual ProGnosis Biotech S.A. SYMMETRIC BTS (S1248/S1296), version N2.

Commission Decision (EC) No 2003/181/EC of 13 March 2003 as regards the setting of minimum required performance limits (MRPLs) for certain residues in food of animal origin. Off. J. Eur. Union 2003 L71: 17-18.

Commission Implementing Regulation (EU) 2018/470 of 21 March 2018 on detailed rules on the maximum residue limit to be considered for control purposes for foodstuffs derived from animals which have been treated in the EU under Article 11 of Directive 2001/82/EC. Off. J. Eur. Union 2018 L79: 16-18.

Commission Implementing Regulation (EU) No 2021/808 of 22 March 2021 on the performance of analytical methods for residues of pharmacologically active substances used in food-producing animals and on the interpretation of results as well as on the methods to be

used for sampling and repealing Decisions 2002/657/EC and 98/179/EC. Off. J. Eur. Union 2021 L180: 84-109.

Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. Off. J. Eur. Union 2010 L15: 1-72.

Commission Regulation (EU) No 2019/1871 of 7 November 2019 on reference points for action for non-allowed pharmacologically active substances present in food of animal origin and repealing Decision 2005/34/EC. Off. J. Eur. Union 2019 L289: 41-46.

ISO/IDF. 2021. ISO Technical specification TS 23758 | IDF RM 251 - Guidelines for the validation of qualitative screening methods for the detection of residues of veterinary drugs in milk and milk products. First edition: 1-42.

Regulation (EC) No 470/2009 of the European Parliament and of the Council of 6 May 2009 laying down Community procedures for the establishment of residue limits of pharmacologically active substances in foodstuffs of animal origin, repealing Council Regulation (EEC) No 2377/90 and amending Directive 2001/82/EC of the European Parliament and of the Council and Regulation (EC) No 726/2004 of the European Parliament and of the Council laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. Off. J. Eur. Union 2009 L152: 11-22.

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