https://doi.org/10.17221/262/2018-CJFS

Detection of antibacterial residues in milk by HPLC-DAD and microbial inhibitor tests

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Citation: Christoforidou S., Karageorgou E., Ioannidou M. , Psomas E., Maggira M., Samouris G. (2020): Detection of antibacterial residues in milk by HPLC-DAD and microbial inhibitor tests. Czech J. Food Sci., 38: 63–71.

Abstract: The objective of this study was to assess the detection sensitivity of four commercial microbial inhibition assays (MIAs) in comparison with the results by High Pressure Liquid Chromatography with Diode Array Detection (HPLC-DAD) for residues of three tetracyclines, trimethoprim and sulfadiazine in fortified raw milk samples. MIAs showed fairly good results at detecting sulfadiazine residues, whereas concerning tetracyclines and trimethoprim residues, most of the kits showed insufficient results. HPLC analysis revealed high recoveries of examined compounds with detection limits below their respective maximum residue levels (MRLs). The HPLC method was validated determining linearity, accuracy, precision, detection capability and decision limit. Subsequently, both methods were used to monitor several cow and sheep milk samples for the presence of antibacterial agents. Results obtained showed that MIAs could be successfully used for the analysis of different milk types. However, milk spoilage which was observed in some cases could decrease the sensitivity of the kits, a fact that does not seem to influence the effectiveness of HPLC-DAD method.

Keywords: cow milk; chromatography; microbial inhibition assays; tetracyclines; trimethoprim; sulphadiazine

Improper administration of antimicrobial agents and not respecting withdrawal time can result in the presence of antibiotic residues in milk causing problems in the dairy industry (Molina et al. 2003) and raising public health concerns (Bilandžić et al. 2011). In the European Union, veterinary drug residue monitoring is enforced according to the requirements laid down in Council Regulation 470/2009/CE and Council Regulation 37/2010/UE (Rama et al. 2017).

Several methods have been described for the determination of antibacterial substances in milk. Screening tests, such as microbial inhibition assays (MIAs), rapid test kits, immunoassays and biosensors (Wang 2009) are usually preferred due to their simplicity, large number of samples per kit, low cost and possibility of automation (Reig & Toldrá 2011), despite their lack of specificity and the need to be optimized to avoid false negative results (CAC/GL 16, 1993). On the other hand, confirmatory methods, including mainly separation techniques like liquid chromatography (Wang 2009), have superior specificity, quantify the analyte and provide structure elucidation when being coupled with mass spectrometers (CAC/GL 16, 1993; Nicolich et al. 2006).

Liquid chromatography is the most frequently applied technique, usually followed by either photodiode array (PDA) UV detection (Karageorgou & Samanidou 2010; Yahaya et al. 2015) or mass spectrometry (Karageorgou et al. 2013).

Prior to a chromatographic analysis, a sample pretreatment technique should be applied for the effective isolation of antibiotics from the complex milk matrix. Solid phase extraction using molecularly imprinted polymers as the sorbent material (Quesada-Molina

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Supported by the Hellenic Agricultural Organization "DEMETER".

et al. 2012), matrix solid-phase dispersion (MSPD), and dispersive solid phase extraction (DSPE) applying the quick, easy, cheap, effective, rugged, and safe (QuEChERS) methodology are commonly used (Karageorgou & Samanidou 2011).

The most widely used methods for the detection of antibiotic residues in milk are the MIAs based on the International Dairy Federation (IDF) reference method (IDF 1991, bulletin No. 258), but also many other methods have emerged as tools for the detection of residues such as plate diffusion methods using *E. coli* and *Yersinia* spp. strains (Navrátilová et al. 2014; Navrátilová et al. 2017). However, there is no MIA that can detect all substances at the MRLs (maximum residue levels) laid down by the European Union Regulations. Most of them are targeted at β-lactams for the reason that they are the most commonly used veterinary drugs in the therapy of cows in many countries (Alkan 2007). However, these tests are less sensitive to other antimicrobial agents such as macrolides, sulfonamides, tetracyclines or chloramphenicol (Botsoglou & Fletouris 2001) as the *Bacillus stearothermophilus* sensitivity to antibiotics such as tetracyclines and sulfonamides is described as insufficient compared to that obtained for β-lactams (Althaus et al. 2003). More specifically, its sensitivity to tetracycline ranges between 400 and 500 μg kg^{-1}, whereas the MRLs for tetracyclines in milk are set to 0.1 mg kg^{-1} (IDF 1991, bulletin No. 258; Jevinova et al. 2003). In 2018, authors' laboratory achieved the detection of β-Lactams and chloramphenicol residues in raw milk. In this study an HPLC-DAD method was developed and compared with Microbial Inhibition Assays (Karageorgou et al., 2018). However there were not studied other groups of antibiotics.

Concerns and perceptions about antibiotic residues in milk have contributed to several studies that were aimed at milk safety and purity by the application of screening methods on the antibiotic residue detection (Ibraimi et al. 2013). Only in 2015, 60 notifications reporting the detection of veterinary medicinal products, including antibiotics, in food or feed in the EU were published via the European Rapid Alert System for Food and Feed preliminary annual report (RASFF). In Greece, according to results reported by the Ministry of Rural Development and Food from 2009 to 2013, tetracyclines and sulfonamides are some of the most frequent residues having been detected in animal products.

The present study follows up on the authors' previous research regarding the detection of β-Lactams and Chloramphenicol residues in raw milk (Karageorgou et al., 2018). The present study was conducted to assess the detection sensitivity of four MIAs available in the Greek market in comparison with the results obtained by HPLC-DAD method for three tetracyclines, trimethoprim and sulfadiazine in fortified raw milk samples. The selection of the aforementioned antibacterial drugs was based on their high frequency detection in animal products and their impacts on public health when being consumed.

MATERIAL AND METHODS

Instrumentation

HPLC-DAD instrumentation. The HPLC system was the Perkin Elmer Series 200, with a PDA detector and 200 μL loop (Perkin-Elmer, USA). Degassing of the mobile phase was achieved by a Perkin Elmer Series 200 vacuum degasser. A Kinetex C_{18} –2.6 μm, 150 × 4.6 mm analytical column, purchased from Phenomenex (Torrance, USA), was used for the separation of examined analytes.

A glass vacuum filtration apparatus (Alltech Associates) was employed for the filtration of solvents, using cellulose nitrate 0.45 μm membrane filters (Sartorius Stedim Biotech GmbH; Gottingen, Germany). A Vortex Genie 2 (Scientific Industries Inc., USA) and an ultrasonic bath AM-9 Aquasonic Cleaners (Sherwood, AR, USA) were used for the sample pretreatment. All evaporations were performed with an evaporator from Barkey GmbH & Co. KG (Germany).

Two SPE products were investigated: Plexa (60 mg 3 mL^{-1}), Agilent Technologies Inc. (USA) and Oasis-HLB (200 mg 6 mL^{-1}) by Waters (USA). Moreover, two mL dispersive SPE tubes QuEChERS containing 150 mg magnesium sulphate, 50 mg PSA and 50 mg C_{18}EC also purchased from Agilent Technologies were used in MSPD mode, only for the isolation of sulfadiazine and trimethoprim.

MIAs and instruments used. Four commercial MIAs available in the Greek market were evaluated, namely (a) Delvotest SP-NT (DSM Food Specialties, Netherlands), (b) Eclipse 3G (Zeu-Immunotec, Spain), (c) BRT MRL Screening test (AIM, Germany) and (d) Charm Blue Yellow II (Charm Sciences Inc., USA). For confidential reasons, the kits will be from now on referred to as "A", "B", "C" and "D" non-respectively.

The standard solution preparation and the milk sample fortification at the appropriate concentrations were achieved by calibrated pipettes Eppendorf Research plus 0.5–10 μL, 10–100 μL and 20–200 μL (Merck, Germany). After inoculation, microplates were incubated at 64 ± 2 °C for 2.5–3.5 h according to the manufacturers' guidance, until the colour change of the negative control. The interpretation of the results was done either visually or photometrically by the use of an ELISA reader TECAN Infinite F50 (Elisa, Austria) and specific scanners Epson Perfection V600 Photo and Epson Perfection V30 (Epson Europe B.V., Netherlands), kindly provided by the manufacturers of the kits.

Reagents and materials

Tetracycline hydrochloride (TC), oxytetracycline hydrochloride (OTC), chlortetracycline hydrochloride (CTC), sulfadiazine (SDZ) and trimethoprim (TMP) were purchased from Sigma-Aldrich (Germany). Methanol, acetonitrile, and water were of HPLC grade whereas acetone, oxalic and formic acid were of analytical grade (Sigma-Aldrich, Germany).

The raw bovine milk sample was obtained from small dairy farms in northern Greece and was first analysed by the HPLC method so as to be confirmed free of antibiotic residues and sanitizers that could interfere with sensitivity of the kits. Subsequently, the initial milk sample was prepared in aliquots of 50 mL and was stored at –20 °C until the day of analysis.

Preparation of standard solutions

For the chromatographic analysis of tetracyclines, sulfadiazine and trimethoprim, all stock standard solutions were prepared in methanol at a concentration of 100 ng μL^{-1} and were stable at 4 °C for four months. Working aqueous standards were freshly prepared every three days by further dilution at various concentrations. Aliquots of 150 μL and 100 μL were injected onto the column for tetracyclines and sulfadiazine/trimethoprim, respectively, and quantitative analysis was based on peak area measurements.

For the analysis by MIAs, all stock standard solutions were freshly prepared on the day of analysis at a concentration of 100 ng μL^{-1}. Tetracyclines and sulfadiazine were dissolved in methanol (Sigma-Aldrich, Germany), whereas trimethoprim was dissolved in sterile demineralized water. Subsequent dilutions were prepared in sterile demineralized water and milk to yield appropriate working standard solutions at various concentrations ranging from 50% to 150% MRL. During the analysis, tetracycline solutions were isolated from light to avoid their degradation (Chen et al. 2008).

Chromatography

Regarding tetracyclines, target analytes were separated by gradient elution using: A: oxalic acid 0.01 M and B: ACN. The initial volume ratio was 80 : 20 (*v/v*). Over the next 10 min, the ratio was changed to 70 : 30 (*v/v*) and was kept isocratic for 10 minutes. During the last 10 min of analysis, the mobile phase returns to its initial composition. The flow rate at 0.6 mL min^{-1} provides the inlet pressure of approximately 3 500 psi. The analytical column was operated at ambient temperature and the detector was set at 270 nm.

As far as sulfadiazine and trimethoprim are concerned, target analytes were separated by gradient elution using: (a) HCOOH 0.1%, (b) MeOH and (c) ACN. The initial volume ratio was 80 : 15 : 5 (*v/v*). Over the next 8 min, this ratio became 70 : 20 : 10. During the last 5 min of analysis, the mobile phase returns to its initial composition. The flow rate at 0.7 mL min^{-1} provides the inlet pressure of approximately 4 200 psi. The analytical column was operated at ambient temperature and the detector was set at 265 nm.

The evaluation software for all the analytes was Total Chrom V6.2.0.0.1 (Perkin-Elmer, USA).

Sample preparation prior to HPLC-DAD analysis

In the case of sulfadiazine and trimethoprim, the optimum sorbent was applied to milk on dispersive extraction by QuEChERS in MSPD format, where the extraction was enhanced ultrasonically.

Regarding tetracyclines, the MSPD procedure was applied using the Plexa sorbent. The SPE sorbent material was preconditioned by flushing with 2 mL of methanol and 2 mL of water. Then it was emptied into a beaker, where 500 μg of milk and 500 μL of the standard solution of examined tetracyclines were added. In the modified MSPD method used herein, homogenization was enhanced by sonication for 10 minutes. Afterwards the sample was transferred into an empty cartridge, it was compressed and vacuum-dried. Subsequently, an interference was removed by washing the sorbent bed once with 5 mL water (1% acetone), and the analytes were eluted with 1 mL oxalic acid 0.01 M and 1 mL methanol. The samples were filtrated with PVDF Durapore syringe filters (13 mm × 0.45 μm) (Millex Millipore Corporation, USA) prior to evaporation until dryness under nitrogen stream, and the residues were dissolved in 500 μL of water. Aliquots of 150 μL were injected into the HPLC system. In the case of milk samples, the same procedure was followed by adding 500 μL of distilled water instead of 500 μL standard solution.

Concerning sulfadiazine and trimethoprim, the optimum sorbent was applied to milk on dispersive extraction by QuEChERS in MSPD format, where the extraction was enhanced ultrasonically. The MSPD

https://doi.org/10.17221/262/2018-CJFS

procedure was applied using the Oasis sorbent. The SPE sorbent material was preconditioned by flushing with 2 mL of methanol and 2 mL of water, and it was emptied into a beaker, where 500 µg of milk and 500 µL of standard solution were added in the case of spiked samples, with half the quantity of a QuEChERS tube, i.e. 125 mg were added. In the modified MSPD method, homogenization was enhanced by sonication for 10 minutes. Afterwards the sample was transferred into an empty cartridge, it was compressed and vacuum-dried. Subsequently, the analytes were eluted with 2 mL methanol and 2 mL ACN. The samples were filtrated prior to evaporation until dryness, and the residues were dissolved in 500 µL of water. Aliquots of 100 µL were injected into the HPLC system. In the case of milk samples, the same procedure was followed by adding 500 µL of distilled water instead of 500 µL standard solution.

Analysis by means of MIAs

Four MIAs were used to assess the detection of antibiotic residues in raw bovine milk. Each commercial kit was carried out following the manufacturers' instructions. The assay was performed in microplates containing an agar medium spread with *Geobacillus stearothermophilus var. calidolactis* spores. The milk samples were applied onto the agar surface and in case they contained inhibitor substances, reduction or total inhibition of *Geobacillus stearothermophilus var. calidolactis* growth occurred. Analyses were done in triplicate for every antibiotic concentration tested. In each assay one positive and one negative control were used in quadruplicate for the result interpretation. All controls were freshly prepared on the day of analysis according to the manufacturers' instructions and the results were read both visually and photometrically. The detection limits of the test kits are presented in Table 1.

Table 1: Detection limits of commercial MIAs for tetracyclines, sulfadiazine and trimethoprim

Antibiotic name	Spiked antibiotic concentrations (μg kg^{-1})						Type of analysis	MRL (μg kg^{-1})
Tetracycline	50	75	100	150	200	250		100
"A"	–	+	+	+	+	+	photometrical and visual	
"B"	–	–	+	+	+	+	concentration of 100 µg kg^{-1} (only visual)	
"C"	–	–	–	–	–	–	photometrical and visual	
"D"	–	–	–	+	+	+	photometrical and visual	
Oxytetracycline	50	75	100	150	200	250		100
"A"	–	–	+	+	+	+	concentration of 100 µg kg^{-1} (only visual)	
"B"	–	–	+	+	+	+	concentration of 100 µg kg^{-1} (only visual)	
"C"	–	–	–	–	–	+	photometrical and visual	
"D"	–	–	–	–	+	+	photometrical and visual	
Chlortetracycline	50	75	100	150	200	250		100
"A"	–	–	–	+	+	+	concentration of 150 µg kg^{-1} (only visual)	
"B"	–	–	–	+	+	+	concentration of 150 µg kg^{-1} (only visual)	
"C"	–	–	–	–	–	–	photometrical and visual	
"D"	–	–	–	+	+	+	photometrical and visual	
Sulfadiazine	50	75	100	150	200	250		100
"A"	–	+	+	+	+	+	photometrical and visual	
"B"	+	+	+	+	+	+	photometrical and visual	
"C"	–	–	+	+	+	+	photometrical and visual	
"D"	–	+	+	+	+	+	photometrical and visual	
Trimethoprim	25	40	50	75	100	125		50
"A"	–	–	–	–	–	–	photometrical and visual	
"B"	+	+	+	+	+	+	photometrical and visual	
"C"	–	–	–	–	–	–	photometrical and visual	
"D"	–	–	–	–	–	–	photometrical and visual	

MRL – maximum residual level; "A" – delvotest SP-NT; "B"– eclipse 3G; "C" – BRT MRL screening test; "D" – charm blue yellow

https://doi.org/10.17221/262/2018-CJFS

RESULTS AND DISCUSSION

Chromatography. The multistep gradient elution programs yielded optimum separation of tetracyclines (OTC, TC, and CTC), sulfadiazine (SZD) and trimethoprim (TMP) within 13 minutes. Quantitation of target analytes was performed as follows: OTC, TC and CTC at 270 nm, SDZ and TMP at 265 nm.

Sample preparation prior to HPLC-DAD analysis. Concerning tetracyclines, the optimum SPE sorbent material was Plexa, which provided high absolute recoveries (87–104%) in experiments with standard solutions. Results are shown in Table 2. Preconditioning of the Plexa sorbent was found to improve its performance. The contents of a QuEChERS tube were divided into two aliquots and blended with the Plexa sorbent. Sonication resulted in higher recovery rates of the target analytes. Absolute recoveries after ultrasound assisted MSPD ranged from 87% to 104% for all compounds. All examined analytes were well resolved from the complex milk matrix. Typical chromatograms of blank and spiked milk samples after MSPD are illustrated in Figure 1 (A and B).

Regarding sulfadiazine and trimethoprim, the optimum SPE sorbent material was Oasis, which obtained high absolute recoveries (70% and 65% for sulfadiazine and trimethoprim, respectively). Results are shown in Table 3. Preconditioning of the Oasis sorbent combined with 125 mg QuEChERS was found to improve its performance. Sonication resulted in higher recovery rates. All examined analytes were well resolved from the complex milk matrix. Typical chromatograms of blank and spiked milk samples after MSPD are illustrated in Figure 1 (C and D).

HPLC-DAD method validation. The developed HPLC-DAD method was validated in terms of sensitivity, linearity, decision limit (*CCa*), detection capability (*CCb*), accuracy and precision according to European Decision 200/657/EU. Samples of raw milk, which were analyzed and found not to contain detectable residues of the analytes, were used as blank samples.

Calibration curves were constructed using fortified milk samples after MSPD procedure. Correlation coefficients varied between 0.986 and 0.991 for tetracyclines, 0.997 for sulfadiazine and 0.992 for trimethoprim. All observed LODs were lower than the respective MRL values (Table 4).

The precision of the method based on within-day repeatability was assessed by replicate measurements (n = 3) from these spiked milk samples at concentration

Table 2. Optimization of MSPD procedure for tetracyclines

Analyte	Trial	Sorbent	Elution	Washing Step	Observations
Tetracyclines	1	Plexa + 125 mg QuEChERS	1 mL oxalic acid (0.01 M) and 1 mL MeOH successively	5 mL H_2O (1% acetone)	amount of QuEChERS sorbent interferes with the sufficient elution of target analytes
	2	Plexa	1 mL oxalic acid (0.01 M) and 1 mL MeOH successively	5 mL H_2O (1% acetone)	all analytes are well resolved from milk matrix. absolute recoveries ranged 87–104%

QuEChERS – kinds of solid phase extraction cartridges, formed from „quick, easy, cheap, effective, rugged, and safe“

Table 3. Optimization of MSPD procedure for sulfadiazine (SDZ) and trimethoprim (TMP)

Analyte	Trial	Sorbent	Elution	R (%)	
				SDZ	TMP
Sulfadiazine/ Trimethoprim	1	Plexa Oasis	1 mL MeOH + 1 mL ACN successively	30 43	25 29
	2	Plexa + 125 mg QuEChERS Oasis + 125 mg QuEChERS	1 mL MeOH + 1 mL ACN successively	40 50	32 30
	3	Oasis + 125 mg QuEChERS	2 mL MeOH + 1 mL ACN successively	56	52
	4	Oasis + 125 mg QuEChERS	2 mL mix MeOH : HCOOH (0.1%) (1 : 1 v/v)	40	32
	5	Oasis + 125 mg QuEChERS	2 mL MeOH	60	40
	6	Oasis + 125 mg QuEChERS	2 mL MeOH + 2 mL ACN successively	70	65

MSPD – matrix solid-phase dispersion; for abbreviation of QuEChERS see Table 2; R – recovery

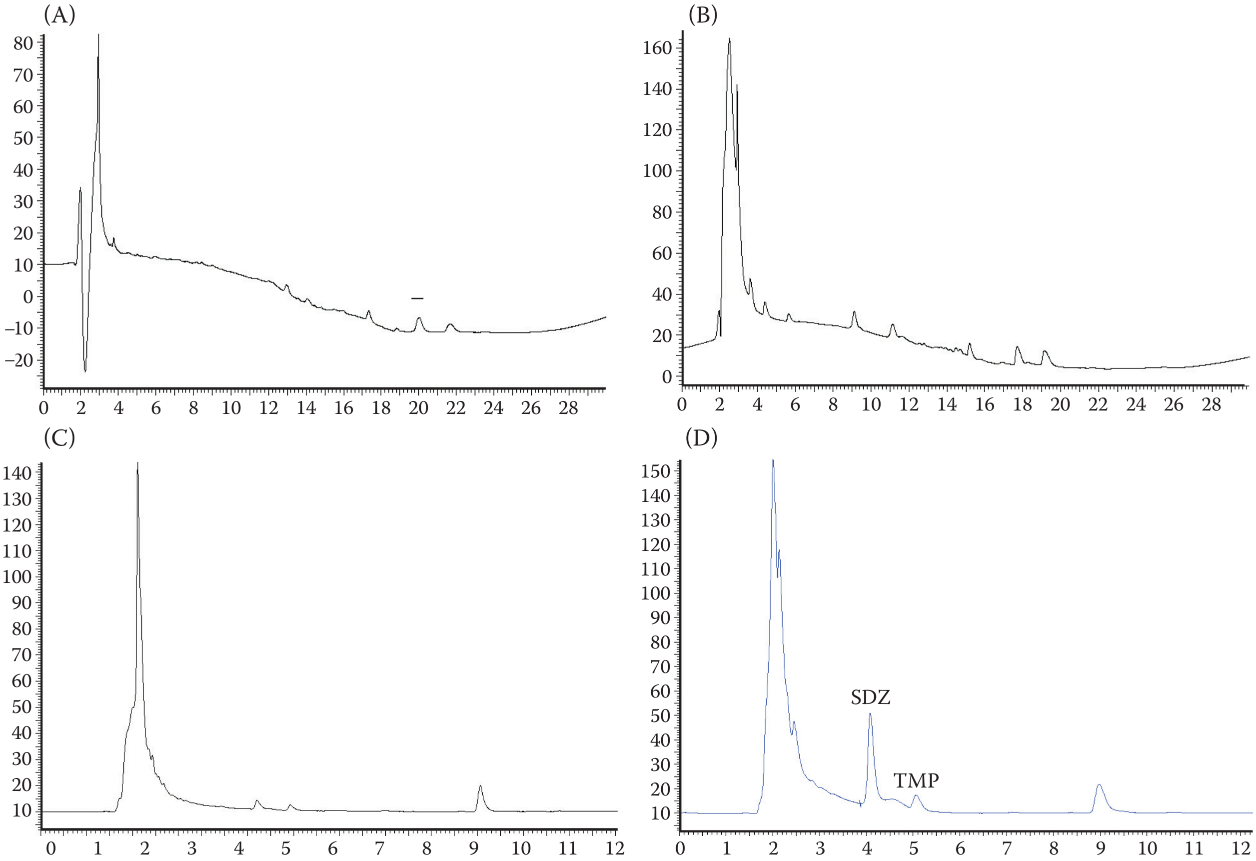

Figure 1. (A) Chromatogram of blank milk sample at 270 nm. (B) Chromatogram of spiked milk sample with standard solution of target analytes (500 μg kg^{-1}) at 270 nm. Peaks: 1. OTC: 3.6 min, 2. TC: 4.3 min, 3. CTC: 9.1 min. (C) Chromatogram of blank milk sample at 265 nm. (D) Chromatogram of spiked milk sample with standard solution of target analytes (500 μg kg^{-1}) at 265 nm. Peaks: SDZ: 4.3 min, TMP: 5.6 minutes

levels of 0.5 × MRL, MRL and 1.5 × MRL. Relative recovery rates from the spiked samples were determined at the same concentrations. The between-day precision of the method was established using milk samples at the same concentration range as above. RSD values were lower than 7.3% for all the examined tetracyclines and sulfadiazine and lower than 7.7% for trimethoprim. Mean apparent recovery rates were in the range of 91.5–102.2% for all tetracyclines, 92.0–110.8% for sulfadiazine and 99.6–110.1% for trimethoprim (Table 4).

The *CCa* values revealed after spiking 20 blank milk samples at MRL and *CCb* values by analyzing 20 blank spiked samples at the corresponding *CCa* level for each analyte are presented in Table 4, together with all values derived from the validation procedure for examined parameters.

Comparative evaluation of MIAs. Regarding sulfadiazine, all kits presented fairly good results being interpreted either photometrically or visually, as they were all able to detect the analyte at concentrations lower than the MRL values. However, none of the MIAs was able to detect any of the spiked concentrations of trimethoprim in milk, except for kit "B" which was able to detect trimethoprim at all the examined concentrations. Concerning tetracyclines, the sensitivity of the kits depends on the type of tetracycline under examination and the type of result interpretation (photometric or visual). More precisely, kit "C" was not able to detect any antibiotic substances of the tetracycline group at concentrations lower or higher than the MRL values, whereas none of the MIAs was able to detect chlortetracycline at the MRL value. Kits "A" and "B" were able to detect oxytetracycline at the concentration of 100 μg kg^{-1} only visually, whereas the sensitivity of kit "D" surpassed the MRL value. Concerning tetracycline, kit "A" showed the best sensitivity as it was able to detect tetracycline at the concentration of 75 μg kg^{-1} either visually or photometrically, whereas kits "B" and "D" could detect the analyte at the concentration of 100 and 150 μg kg^{-1}, respectively.

Table 4. Validation parameters for the determination of tetracyclines (OTC, TC, CTC), sulfadiazine (SDZ) and trimethoprim (TMP) in milk

Compounds	Validation parameters (values obtained)										
	Linearity R^2	Slope	Intercept	MRL (µg kg^{-1})	LOD (S/N = 3.3) (µg kg^{-1})	Intra-Assay Recovery (*n* = 9)	RSD (%)	Inter-Assay Recovery (*n* = 9)	RSD (%)	*CCa* (µg kg^{-1})	*CCb* (µg kg^{-1})
OTC	0.991	17.98	1 609	100	10	96.0–101.9	7.3	97.9–100.5	3.3	107.3	110.9
TC	0.986	22.07	1 915	100	10	92.3–102.3	4.8	97.8–101.6	4.3	104.5	106.1
CTC	0.989	26.87	2 040	100	10	91.5–100.4	6.3	92.7–100.5	2.4	104.9	109.4
SDZ	0.997	363.76	10 537	100	7	92.0–110.7	5.2	95.2–110.8	7.3	106.1	111.4
TMP	0.992	80.533	1 306.8	50	7	99.7–104.4	7.7	99.6–110.1	5.3	54.0	60.7

MRL – maximum residual level; LOD – limit of detection; S/N – signal to noise ratio; *CCa* – decision limit; *CCb* – detection capability; OTC – oxytetracycline hydrochloride; TC – tetracycline hydrochloride; CTC – chlortetracycline hydrochloride; SDZ – sulfadiazine; TMP – trimethoprim

Quality control of raw milk samples by means of HPLC-DAD method and MIAs. Raw milk samples collected from cattle and sheep dairy farms located in Greece were tested for the presence of tetracyclines, trimethoprim and sulfadiazine residues. The samples were initially checked for the presence of the aforementioned antibacterial agents by local Milk Quality Control Laboratories of the Hellenic Agricultural Organization "DEMETER".

Firstly, the samples were analyzed by MIAs in duplicate and the results were interpreted photometrically and visually. Before the analyses, all samples were examined for improper consistency that could intervene with the sensitivity of the kits. The results of the analysis are presented in Table 5.

Regarding the analysis of negative milk samples, MIAs showed similar results to those obtained by the laboratories of HAO "DEMETER". During the monitoring of positive milk samples, all commercial kits detected antibacterial residues in seven out of eleven samples. The false negative results could possibly be attributed to the milk sample spoilage, which could lead to inactivation of antibiotics and subsequently to false negative results due to a loss of the sensitivity of the kits (Žvirdauskiene & Šalomskiene 2007).

Subsequently, milk samples that were found to be positive and those which were equivocally negative according to the results by MIAs were analyzed by the HPLC-DAD method. Results obtained confirmed the presence of antibacterial substances which exceeded the permissible levels in most cases. More specifically, the analysis of raw milk samples performed by the HPLC-DAD method showed that three out of the eleven positive samples were contaminated by residues other than tetracyclines, sulfadiazine and trimethoprim. In most of the samples which were positive for the tetracycline residues, the determined concentrations exceeded the MRL values (100 µg kg^{-1}). Sulfadiazine was detected only in three out of the eleven samples at concentrations below the MRL, whereas trimethoprim was detected only in one sample. Experimental results are presented in Table 5.

CONCLUSION

During their comparative evaluation, all MIAs presented fairly good sensitivity at detecting sulfadiazine residues, but they were not successful in detecting trimethoprim residues in milk, except for kit "B". Concerning tetracyclines, no kit was able to detect chlortetracycline at the MRL values, whereas only kits "A"

Table 5. Results of milk sample analysis by MIAs and HPLC-DAD method

Geographical region	Milk type	Sample Code	MIAs				HPLC-DAD antimicrobials detected
			“A”	“B”	“C”	“D”	
1	cow’s milk	5L	+	+	+	+	antimicrobial agents other than OTC, TC, CTC, SDZ and TMP
		8L	+	+	+	+	SDZ, TMP < MRL
		11L	+	+	+	+	OTC, TC > MRL
2	sheep’s milk	8G	–	–	–	–	antimicrobial agents other than OTC, TC, CTC, SDZ and TMP
		10G	–	–	–	–	OTC < MRL
3	cow’s milk	1P	+	+	+	+	antimicrobial agents other than OTC, TC, CTC, SDZ and TMP
		2P	+	+	+	+	TC > MRL SDZ < MRL
		3P	–	–	–	–	OTC, TC > MRL SDZ < MRL
		5P	+	+	+	+	OTC, TC > MRL
		6P	–	–	–	–	OTC > MRL
		7P	+	+	+	+	TC < MRL

MAIs – microbial inhibition assays; HPLC-DAD – High Pressure Liquid Chromatography with Diode Array Detection; “A” – delvotest SP-NT; “B”– eclipse 3G; “C” – BRT MRL screening test; “D” – charm blue yellow II; for other abbreviations see Table 4

and “B” were able to detect tetracycline and oxytetracycline at the concentration of 100 μg kg^{-1}. This fact is in agreement with the findings of previous studies as *B. stearothermophilus* was reported to be sensitive enough to the group of β-lactam antibiotics, whereas its sensitivity to other antibiotics was described as insufficient (Botsoglou & Fletouris 2001). The results obtained during the comparative evaluation of the kits regarding tetracyclines showed differences in the detection limits of the commercial MIAs when being interpreted visually or photometrically as the photometric detection levels were slightly higher than those obtained visually.

Taking into account the quality control of milk samples, MIAs could be successfully applied to the analysis of different milk types. However, regarding the analysis of the positive milk samples by MIAs, the fact that some results of this study were in contrast with the results reported by the milk quality control laboratories of the Hellenic Agricultural Organization “DEMETER” and the HPLC method could be attributed to milk sample spoilage as described before.

The ultrasound-assisted MSPD method was successfully applied to the multi-residue analysis of tetracyclines, sulfadiazine and trimethoprim residues in milk by HPLC-DAD. The sensitivity of this method allowed quantifying all antimicrobials in milk samples, whereas milk spoilage does not influence the effectiveness of the analysis.

On modern dairy cattle farms, most antimicrobials are administered for the therapy and protection from infectious diseases. However, their presence in foods of animal origin is a serious issue due to their toxicological risk for public health and the risk of non-compliance with regulatory requirements. Avoiding antibiotic residues in milk and dairy products is a fundamental aspect of quality food production. Thus, the development of more sophisticated multi-residue antibiotic screening tests combined with the implementation of risk-reduction programs at the farm level is necessary in order to ensure food quality and public health safety.

Acknowledgment. The authors would like to thank the companies “DSM Food Specialties” and “Charm Sciences, Inc.”, which kindly provided their scanners to be used herein.

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Received: September 13, 2019
Accepted: July 8, 2019