Chapter 16

Biosensors for the Detection of Antibiotic Residues in Milk

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Additional information is available at the end of the chapter

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Abstract

Milk and dairy products are important nutrients for all age groups. However, the use of antibiotics for the treatment of food-producing animals generates the risk to human health, as these compounds and their metabolites can be transferred into milk. Rapid testing of the presence of antibiotics in raw milk to grant its quality has become a major task for farmers and dairy industry. The conventional analytical methods are either too slow or do not enable quantitative detection of antibiotic residues, so alternative methods that are rapid, cost effective, and easy to perform should be considered. The present chapter gives an overview of the recent developments and issues of the construction of different biosensors for the detection of antibiotic residues in milk.

Keywords: Biosensor, antibiotic residues, milk, detection limit

1. Introduction

Milk and dairy products, generally considered to be healthy and nutritionally balanced natural food, comprise essential nutrients for all age groups and are an important part of our everyday diet. However, the use of antimicrobial drugs for the treatment of food-producing animals generates the risk to human health due to the transmission of the residues and metabolites of these compounds into the food chain. Hypersensitive consumers may be subject to allergic reactions or even more severe health problems. At present, up to 10% of people have already been diagnosed to be hypersensitive or allergic to antibiotics [1, 2]. In addition to direct health problems, the presence of antibiotic residues in milk has an adverse effect on milk fermentation

processes in dairy industry, as many starter cultures used for the production of fermented food products (cheese, yoghurt, etc.) may be inhibited by antimicrobial substances and the product quality will be impaired [1, 2]. Scientists and health experts also fear that wide application of antimicrobial agents is contributing to the rise and spread of antibiotic-resistant bacterial infections [3].

The overall sales of veterinary antimicrobial agents, used in food-producing animals in EU (24 countries, excluding Croatia, Greece, Malta, and Romania), were 7974.2 tons of pure ingredients in 2012. The sales of the most commonly used antibiotic classes are shown in Table 1 [4]. Based on the sales data, the classes of antibiotics shown in Table 1 are of main concern regarding the need for practical analyses of antibiotic residues in food, including milk.

Active Ingredient	Sales (Tons)	% of Total Sales
Tetracyclines	2942.8	36.9
Penicillins	1776.9	22.3
Sulphonamides	824.5	10.3
Macrolides	638.0	8.0
Polymyxins	545.0	6.8
Aminoglycosides	290.2	3.6
Lincosamides	235.0	3.0
Pleuromutilins	228.9	2.9
Fluoroquinolones	136.0	1.7
Trimethoprim	128.7	1.6
Others	228.2	2.9
Total	**7974.2**	**100**

Table 1. Sales of veterinary antibiotics used in food-producing animals in EU (24 countries, excluding Croatia, Greece, Malta, and Romania) in 2012 [4]

To protect consumers, strict legislative regulations have been imposed for the treatment of animals with antibiotics and maximum residue limits (MRLs) in foods of animal origin, which are not to be exceeded, established for residues of antibiotics or their metabolites. The residue levels in milk and other body fluids are dependent on the physicochemical properties of a particular drug, which determines the pharmacokinetics and time course of drug concentrations in the body [5]. Table 2 lists the MRL values in milk in EU for the most common antibiotics used for the treatment of dairy cows [6].

Since the introduction of EU regulations on MRLs of pharmacologically active substances in foodstuffs of animal origin (Regulation 2377/90 now replaced with the EU Regulations 470/2009 and 37/2010), it has been clear that the concept of regulating MRL values in foods can

be implemented successfully if methods for quantification of these substances are available for on-site use during monitoring and testing.

Antibiotic Classes	Pharmacologically Active Substance	Maximum Residue Limit (μg/l) in Milk
Tetracyclines	Tetracycline, oxytetracycline, chlortetracycline	100*
Penicillins	Benzylpenicillin, amoxicillin, ampicillin	4
	Cloxacillin, oxacillin, dicloxacillin, nafcillin	30
Sulphonamides	Sulfadiazine, sulfamethazine, sulfadoxine, sulfamethoxazole, sulfamerazine	100**
Macrolides	Erythromycin A	40
	Spiramycin	200
	Tilmicosin, tylosin	50
Polymyxins	Colistin	50
Aminoglycosides	Dihydrostreptomycin, streptomycin	200
	Kanamycin A	150
	Gentamycin	100
	Neomycin B (incl. framycetin)	1500
Lincosamides	Lincomycin	150
	Pirlimycin	100
Pleuromutilins	Tiamulin	n.a.
Fluoroquinolones	Enrofloxacin	100
	Danofloxacin	30
	Marbofloxacin	75
	Flumequine	50
Diaminopyrimidines	Trimethoprim	50

*Sum of the parent compound and its 4-epimer

**Sum of all sulphonamides should not exceed 100 μg/l

Table 2. The MRL values for the most commonly used antibiotics in milk [6].

Routine testing of milk for the detection of residues of different antibiotics to grant the quality and safety of milk has become a major task for farmers and dairy industry. At present, two major technologies are commonly used for milk analyses: qualitative milk screening tests [7-9] and various chromatography-based techniques [7, 10]. Qualitative microbial inhibition tests have been proven to be very suitable for milk screening purposes [7-9]. These tests comprise

spores of specific bacteria, sensitive to particular antibiotics on agar gel including nutrients for bacterial growth and a pH indicator. After milk is added to the test, it is incubated at the appropriate temperature to germinate and grow the spores. In the absence of antibiotic residues, the growth of bacteria can be detected visually either by the change of opacity of the agar medium or by the color change of the pH indicator, resulting from acid production and change of pH. In the presence of antibiotic residues (or any other inhibitors), the growth of bacteria is suppressed and there are no observable changes of the color. The main advantages of these tests are their low cost, simple performance, and broad selection toward different antibiotics. Although these tests are considered to be rapid, they take 3–24 hours to perform in an incubator. The bacterial strains used in tests should be constantly monitored to ensure that they have not become resistant to the antibacterials. The interpretation of test results is quite subjective and may lead to false negative or positive results. The presence of natural inhibitors in abnormal milk (e.g., milk of mastitic cows or colostrum) can be the cause of false positive results [7-9].

In addition to microbial inhibition tests; there are different rapid tests, based either on immunoassay or enzymatic operation, available for the screening of a number of antibiotic residues in milk. These tests provide results usually within 30 min [11]. As already said, milk tests are specific to the particular class of antibiotics. As a rule, the detection limits of milk tests are in the range of the established MRL values [7, 8, 11-13].

Chromatography is the most reliable technique for quantitative detection of antibiotic residues [7, 10]. Nowadays more than 80% of the analytical techniques for the determination of veterinary drugs use high-performance liquid chromatography in combination with mass spectrometry (HPLC/MS) [10]. However, chromatography-based methods require expensive equipment and trained personnel with high experience. In addition, HPLC techniques demand laborious pre-treatment of samples for the extraction of the compound analyzed, from the sample matrix.

As the milk matrix is one of the most complex ones, the application of biosensors, enabling a selective detection of particular compounds in natural or only minimally pre-treated samples is a good option for the on-site assessment of milk quality [7, 14-16]. Biosensors are compact devices transferring the selective biochemical recognition into a measurable physical signal, which can be translated into an indication of the safety or quality of milk. Physically biosensors comprise of bio-recognition and signal transduction elements. Biosensors offer an opportunity for the development of quick and portable devices for real-time analysis in complex matrixes, operating fully automatically or manually, so the user does not require special skills. At present, most of the biosensing methods focus on the detection of single antibiotic groups, but there are also studies dealing with the simultaneous determination of different groups of antibiotics.

The present review gives an overview of the developments and construction issues of antibiotic biosensors, applicable for the analysis of bovine milk quality, during the last 15 years. Due to the large number of different technologies used, these biosensors are classified into five separate groups according to the bio-recognition employed. The basic parameters of performance of different developments are collected into concise tables.

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2. Receptor and enzyme-based biosensors

In these biosensors, specific receptors or enzymes are utilized to generate a bio-recognition reaction, whose signal is then detected with a suitable transducer. Receptor/enzyme-based biosensors usually employ optical or electrochemical signal detection principles [7, 15]. For optical detection, surface plasmon resonance (SPR) has been used most commonly. The application of SPR technology secures low detection limits, even below the established MRL values. The main drawbacks of SPR biosensors are their high cost; nonspecific binding of compounds of sample matrix to the sensor surface; and assay time (including chip preparation, incubation of receptors, detection, and system regeneration), which could take even a couple of days [17-23].

A receptor-based SPR biosensor, where a conjugate of cephalosporin C and a H1 monoclonal antibody (mAb) was attached to the sensor surface before injecting the milk sample mixed with DD-carboxypeptidase (EC 3.4.16.4), was developed for β-lactam antibiotics (β-Ls) by Gustavsson et al. [18]. In the presence of β-L residues in the sample, this receptor (DD-carboxypeptidase) did not bind to the complex on the sensor surface, making the sensor response inversely proportional to the β-L concentration in the sample. The limit of detection for the studied β-Ls was below or near the corresponding MRL. The same workgroup also developed an alternative SPR-based biosensor assay for β-Ls, using the catalytic properties of carboxypeptidase from *Streptomyces* R39 [19, 20, 23]. The enzyme catalyzes the hydrolysis of a tri-peptide (acetyl-L-Lys-D-Ala-D-Ala) into a di-peptide (acetyl-L-Lys-D-Ala). In the presence of β-Ls, the enzymatic activity is inhibited and less di-peptide will be formed. The assay described measured the amount of remaining enzymatic substrate (tri-peptide). The detection limits of this biosensor system for most of the studied β-Ls were below or equal to the established MRL values, except for cloxacillin (CLOX) and ceftiofur (CEFT).

A frequently used receptor for the detection of β-L residues is penicillin-binding protein (PBP), which covalently binds to penicillin G (PEN) and other β-Ls. High molecular mass PBPs exhibit DD-carboxypeptidase activity and catalyze the final steps of peptidoglycan cross-linking [17]. PBPs are used as binding reagents in receptor and enzyme-based assays, specific to β-Ls, and the biosensor system measures the inhibition of the enzymatic activity of DD-carboxypeptidase. A SPR-based biosensor to measure the inhibition of the binding of digoxigenin-labelled ampicillin (DIG-AMP) to a soluble PBP 2x* from *Streptococcus pneumonia* was proposed by Cacciatore et al. [17]. The nonspecific binding was minimized and kept on a constant level by applying heat-treatment and centrifugation steps and the addition of carboxymethylated dextran. Sample pre-preparation enabled to detect some of the studied β-Ls at MRL levels. The assay, however, does not distinguish between different β-Ls and is intended to be used as a screening method prior to identification and quantification of the individual analytes by other methods, such as by HPLC/MS [17]. To detect β-Ls, Lamar and Petz [21] immobilized PBP 2x* from *S. pneumonia* to a micro-plate. DIG-AMP complex was added to the samples. The amount of DIG-AMP bound via its AMP part to the PBP was decreasing along with the increase of β-L concentrations in the sample. In the detection step, anti-DIG *Fab* fragments marked with horseradish peroxidase (HRP) were added. The more DIG-AMP complex was bound to the

receptor protein, the more antibody fragments were bound via the DIG part of the complex. A maximum color development with a chromogen agent was achieved, when no β-L residues were present and the amount of bound *Fab* fragments with peroxidase was highest. With this system, it was possible to detect the studied β-L residues at levels corresponding to 50% of their respective MRL values in milk. To eliminate the matrix interferences and increase sensitivity, it was necessary to remove fat from the milk samples. Setford et al. [22] integrated PBP with screen-printed electrodes to measure PEN. Although the system was simple to perform and easy to use, it was only possible to distinguish between PEN concentrations below or above 1.3 MRL levels.

A group of enzyme-based biosensors for the detection of β-Ls are called penicillinase (EC 3.5.2.6, PCNase) biosensors. PCNase is produced by bacteria providing resistance toward β-L antibiotics [24]. All β-L antibiotics have a common element in their molecular structure: a four-atom ring known as a β-Lactam ring. PCNase catalyzes the opening of the β-Lactam ring turning PEN into penicilloic acid and so deactivating the molecule's antibacterial properties [24]. The measurement of the hydrolysis of β-L ring by detecting the pH change has been used in biosensors for β-L antibiotics [25-28]. Chen et al. [25] proposed a PEN sensor, where hematein (pH indicator) was co-immobilized with multi-walled carbon nanotubes (MWCNTs) and PCNase onto glassy carbon electrode. MWCNTs were used to enhance electron transfer. In case PEN was present in the sample, the pH value decreased due to PEN hydrolysis into penicilloic acid catalyzed by PCNase. Once $[H]^+$ was accepted, hematein as a pH-sensitive redox probe was reduced to hematoxylin, what induced the increase of electrochemical signal. Severe interferences from the matrix of raw milk, caused by the adsorption of milk proteins and fat onto the electrode surface, probably forming a barrier between the enzyme and analyte molecules, were observed. To eliminate these effects, proteins and fat were separated from milk samples using salting-out and centrifugation processes. However, the interferences from the milk matrix were still observable and the detection limit remained quite high—9 mg/l. Hence, this sensor is not applicable for the detection of PEN in milk but only in water samples. Wu et al. [26] reported a similar PEN biosensor using single-graphene nanosheets (SGCs) instead of MWCNTs. Hematein was attached directly to graphene by adsorption, then ionic liquid was added due to its good biocompatibility, favoring further immobilization of PCNase. They found that at higher concentrations of PEN, the PCNase activity was lowered due to accumulation of the acidic products, decreasing the system sensitivity. The PEN detection limit was declared to be at 0.04 pg/l. The main drawback of pH-dependent biosensors is the fact that they exhibit only a limited range of applicability. For example, in complex fermentation media these biosensors are useless, as there can be dramatic pH changes caused by the fermentation itself.

Concalves et al. [29] proposed a PEN sensor using a cysteine-based self-assembled monolayer to immobilize PCNase onto a gold electrode. Ferrocene was added to establish the effectiveness of the biosensor development, although cysteine and PCNase inhibit the electron transfer to ferrocene. The PCNase used was a metallo-β-lactamase. The reaction could be monitored cronoamperometrically without any redox pH probe (e.g., hematein). The obtained detection limit was 1.5 μg/l.

Ismail and Adeloju [30] developed a potentiometric biosensor for PEN by exploiting the non-conducting polytyramine-PCNase film, which was placed on Pt electrode. In potentiometric setup, the main nonspecific factors influencing the detection sensitivity are buffer concentration and pH. As PCNase was used in this sensor system, pH was fixed at 7.0 to ensure the optimal enzymatic activity. The detection limit of the sensor in buffer solutions was 0.3 μM (100 μg/l). In milk samples, the PEN recovery was not reproducible, being 110±80% at 1 mg/l, 92±32% at 5 mg/l, and 78±16% at 10 mg/l PEN. The same workgroup [27] proposed also a bilayer potentiometric biosensor for PEN. The benefit of using a bilayer configuration, where the enzyme is immobilized in both layers, is a considerable enhancement of the sensitivity through the efficiency of electron transfer between the enzyme and the electrode. In the bilayer configuration, polypyrrole film was used for PCNase immobilization. The PEN detection limit was not improved in the buffer solutions, being 0.3 μM. In milk, the detection limit was found to be 5 mg/l; unfortunately, at higher PEN concentrations the recoveries in milk were very low (30–60%).

A biosensor array, based on the analyses of the patterns of change of oxidation kinetics of lactose and its metabolites, has been proposed for a rapid multiplex detection of the most common veterinary antibiotics in raw milk [31]. In this biosensor array, different oxidoreductases were used to catalyze the oxidation of lactose and its hydrolysis products galactose and glucose in separated sample flow channels. The combination of different reaction parameters of different biosensors forms a pattern of milk sample and in the presence of antibiotics this combination forms the fingerprints of particular antibiotics.

The condensed overview of receptor/enzyme-based biosensors for the determination of antibiotic residues in milk is given in Table 3.

Biosensor Assay	Bio-Selective Element	Antibiotic Residues	LOD	Linear Range	Principle of Detection	Ref.
Surface plasmon resonance based inhibition sensor	DD-carboxypeptidase	PEN, AMP, AMOX, OXA, CLOX, CEFL, CEFA, CEFT	1.5 μg/l 1.2 μg/l	n.a. n.a.	The assay measures the amount of remaining enzymatic substrate (tri-peptide or di-peptide) using antibodies against the tri-peptide or di-peptide.	[19,20]
Surface plasmon resonance	DD-carboxypeptidase and monoclonal H1 antibody	PEN, OXA, CLOX, CEFL, CEFA	1-2 μg/l (PEN), 11-12 μg/l (OXA), 7-8 μg/l (CLOX),	n.a.	Microbial receptor protein with DD-carboxypeptidase activity. Using H1 antibody as substrate for the enzyme reaction.	[18]

Biosensor Assay	Bio-Selective Element	Antibiotic Residues	LOD	Linear Range	Principle of Detection	Ref.
			6-7 μg/l (CEFL), 3-4 μg/l (CEFA)			
Surface plasmon resonance bio-specific interaction assay	PBP	PEN, AMP, AMOX, CLOX, CEFL, CEFO	2 μg/l (PEN), 2 μg/l (AMP), 2 μg/l (AMOX), 15 μg/l (CLOX), 50 μg/l (CEFL), 25 μg/l (CEFO)	n.a.	Inhibition of the binding of digoxigenin-labelled AMP to soluble PBP.	[17]
Chemometric non-competitive binding assay	PBP	AMP, PEN, CEFQ, CLOX, CEFZ, CEFO	+/– tests: ≥ 1 μg/l (AMP), ≥ 1 μg/l (PEN), ≥ 1 μg/l (CEFQ), ≥ 3 μg/l (CLOX), ≥ 7 μg/l (CEFZ), ≥ 5 μg/l (CEFO)		Inhibition of digoxigenin-labelled AMP binding to soluble PBP.	[21]
Electrochemical	PBP	PEN	Half quantitative—enables to distinguish between no PEN and 1.3 MRL of PEN in samples		Assay utilizes immobilized PBP in a competitive binding assay format. No sample pre-treatment required.	[22]
Electrochemical	PBP	PEN, CEFA, CLOX, OXA	4 μg/l (PEN), 19.5 μg/l (CLOX), 6.6 μg/l (CEFA), 13.6 μg/l (OXA)	12–91 μg/l (PEN), 3–232 μg/l (CLOX), 14–123 μg/l (CEFA), 20–109 μg/l (OXA)	Quantification through competitive binding between the target and HRP-labelled specific tracer for the binding sites of the immobilized PBP.	[32]
Electrochemical	PBP	SMR, SDZ, SCP, OTC,	Half quantitative—enables to discriminate		Quantification through competitive	[33]

Biosensor Assay	Bio-Selective Element	Antibiotic Residues	LOD	Linear Range	Principle of Detection	Ref.
		TC, CEFT, CTC, SPY, CEFA	between the presence and absent of antibiotic residues at the MRL concentration level but not to identify the type of antibiotic detected		binding between the target and HRP-labelled specific tracer. Hydroquinone was used as an electron transfer mediator and peroxide as an enzyme substrate.	
Electrochemical pH sensor	PCNase	PEN	24 μM	24 μM to 0.89 mM	pH change, caused by the hydrolyses of PEN by PCNase, is measured with a pH-sensitive hematein probe.	[25]
Electrochemical pH sensor	PCNase	PEN	1 nM	1.3×10^{-13} – 7.5×10^{-3} M	pH change, caused by the hydrolyses of PEN by PCNase, is measured with a pH-sensitive hematein probe.	[26]
Fluorescence pH sensor	PCNase	PEN	n.a.	n.a.	Measures pH changes caused by hydrolyses of PEN to penicilloic acid by PCNase using photosensitive polymer matrices on optical imaging fibers.	[34]
Cronoamperometric sensor	Metallo-PCNase	PEN	1.5 ppb	3.3–16.7 μg/l	Catalytic hydrolysis of PEN was monitored using enzyme-catalyzed hydrolysis reaction.	[29]
Potentiometric sensor	PCNase	PEN	0.3 μM (≥ 5 ppm in milk)	7.5–146 μM	Change of electrode sensitivity was measured with a pyrrole-PCNase single layer and bilayer sensor.	[27]

Biosensor Assay	Bio-Selective Element	Antibiotic Residues	LOD	Linear Range	Principle of Detection	Ref.
Potentiometric sensor	PCNase	PEN	0.3 μM (≥ 20 ppm in milk)	3–283 μM	Polytyramine-PCNase film for potentiometric detection of PEN.	[30]
Biosensor array	Oxidoreductases	PEN	50 ppb		Different reaction parameters of parallel biosensors form a pattern of milk sample and in the presence of antibiotics this pattern forms the fingerprints of particular antibiotics.	[31]

PEN—penicillin G; AMP—ampicillin; AMOX—amoxicillin; OXA—oxacillin; CLOX—cloxacillin; CEFL—cephalexin; CEFA—cefapirin; CEFO—cefoperazone; CEFQ—cefquinome; CEFZ—cefazolin; SMR—sulfamerazine; SDZ—sulfadiazine; SCP—sulfachlorpyridazine; OTC—oxytetracycline; TC—tetracycline; CEFT—ceftiofur; CTC—chlortetracycline; SPY—sulfapyridine; PCNase—penicillinase; PBP—penicillin binding protein; LOD—limit of detection; n.a.—data not available.

Table 3. Receptor/enzyme-based biosensors for the detection of antibiotic residues in milk.

3. Microbial biosensors

There are a few biosensors for detecting antibiotic residues in milk based on the application of enzymatic activity of microorganisms [35-37]. Systems for the monitoring of β-Ls are based on similar principles as microbiological inhibition tests [8, 10], with the difference that the bio-recognition reaction signal is detected quantitatively or semi-quantitatively. The microbial biosensors are based on the measurement of the inhibition of bacterial growth due to the presence of antibiotics [7-10].

Ferrini et al. [35] presented a hybrid biosensor combining classical microbiological screening of antibacterials with electrochemical detection and reading. In this system, *Bacillus stearothermophilus* var. *calidolactis* was used as a test microorganism and its growth was followed electrochemically measuring the quantity of CO_2 produced. The presence of microbial inhibitors (e.g., antibiotics) in the milk sample prohibits the growth of test strain and thus decreases the CO_2 production rate. This variation in CO_2 production was recorded during the initial 120 min in comparison to a control milk sample. The detection limits were at MRL levels.

A *Bacillus cereus 66* assay based on its β-lactamatic activity and using iodine as reaction indicator has been proposed by Das et al. [36]. The system was examined for different β-Ls and other antibiotics to study its selectivity. In case antibiotics were not present, cultures did

not show any color change in the test ampoules meaning that the basic enzyme production by microorganisms was not sufficient to reduce the starch iodine mixture. In the presence of antibiotics, a color change was observed within 15–25 min. The growth of *B. cereus 66* was inhibited by β-Ls at ≥ 100 mg/l, which is much higher than the allowed MRL values for any of the studied β-Ls. Other antibiotics studied showed inhibition at very high concentrations ranging from 2.5 to 1000 mg/l, indicating the low sensitivity of the system.

For the detection of quinolones (Qs) and tetracyclines (TCs), an electrochemical microbial biosensor was proposed by Pellegrini et al. [37]. The detection was based on the measurement of CO_2 production rate in relation to the inhibition of microbial growth by antibiotics. The microorganism used in this study was *Escherichia coli* (ATCC 11303). *E. coli* was chosen for its good sensitivity to Qs and TCs. The inhibition degrees were evaluated after 120 min. Qs and TCs residues were detectable at 25 μg/l. The biosensor was not sensitive toward other studied antibiotics (macrolides, β-Ls, aminoglycosides, and sulfonamides).

The condensed overview of microbial-based biosensors for the determination of antibiotic residues in milk is given in Table 4.

Biosensor Assay	Bio-Selective Element	Antibiotic Residues	Assay Time	LOD	Principle of Detection	Ref.
Electrochemical assay	*B. stearothermophilus* var. *calidolactis*	PEN, AMP, OXA, CLOX, diCLOX	~ 120 min	At MRL levels	CO_2 detection (microbial growth inhibition)	[35]
Iodometric assay	*Bacillus cereus 66*	PEN, AMP, CLOX, AMOX, CEFL, CFZ	~ 10 min (+incubation 4 hours)	PEN, AMP, AMOX, CLOX ≥ 100 mg/l; CEFL, CFZ 2.5–1000 mg/l	Color change detection	[36]
Electrochemical assay	*Escherichia coli*	TC, OTC, CTC, NALA, ENRO, MAR, NOR, CIPRO, FLU, DAN	~ 120 min	≤ 25 μg/l	CO_2 detection (microbial growth inhibition)	[37]

PEN—penicillin G; AMP—ampicillin; AMOX—amoxicillin; OXA—oxacillin; CLOX—cloxacillin; diCLOX—dicloxacillin; CEFL—cephalexin; CFZ—cefazolin; TC—tetracycline; OTC—oxytetracycline; CTC—chlortetracycline; NALA—nalidixic acid; ENRO—enrofloxacin; MAR—marbofloxacin; NOR—norfloxacin; CIPRO—ciprofloxacin; FLU—flumequine; DAN—danofloxacin; LOD—limit of detection.

Table 4. Microbial biosensors for the detection of antibiotic residues in milk.

4. Immunosensors

The largest group of biosensors, used for the detection of antibiotic residues in milk, is based on the exploitation of immunochemical biorecognition reactions. The most frequently applied immunosensors are the electrochemical and optical ones, the latter most often being an SPR biosensor. Although immunosensors are very selective, the speed of analysis depends on the incubation time required to form antigen/antibody complex. In addition, the full regeneration of the sensor can also be quite time consuming.

For the detection of β-L residues in milk, a SPR biosensor based on a commercial anti-ampicillin (AMP) antibody, which had much higher affinity toward open β-lactam rings than the closed ones, was constructed by Gaudin et al. [38]. In order to open the β-lactam rings and increase the assay sensitivity, two different methods for sample pre-treatment—enzymatic (with the help of PCNase) and chemical (basic hydrolysis)—were tested. The application of pre-treatment enabled to achieve AMP detection limits at 33 μg/l and 12.5 μg/l after enzymatic and chemical pre-treatment, respectively. Another SPR immunosensor for the analysis of AMP was described by Zhang et al. [39]. This biosensor was a competitive binding assay between AMP covalently immobilized on the sensor surface and AMP-containing sample, mixed with monoclonal anti-AMP antibody. The sensor detected the amount of free antibody bound to the sensor surface after the injection of milk sample. The limit of detection of AMP with this sensor was 2.5 μg/l.

A portable SPR immunosensor for the determination of fluoroquinolone (FQ) antibiotics (enrofloxacin (ENRO), ciprofloxacin (CIP), and norfloxacin (NOR)) in milk was proposed by Fernandez et al. [40]. The assay worked in indirect inhibition format based on binding of the polyclonal anti-FQ-haptenized protein (FQ-BSA) antibody to the SPR sensing surface, activated with FQ-BSA, while the presence of FQs in sample inhibited the binding. The limit of detection was 2.0 μg/l. An earlier version of this portable SPR biosensor was constructed for the simultaneous detection of three different antibiotic classes (FQs, sulfonamides, and phenicols) [41]. This sensor was based on a similar competitive assay format. The limits of detection were 1.7 μg/l for ENRO, 2.1 μg/l for sulfapyridine (SPY), and 1.1 μg/l for chloramphenicol (CAP). Rebe Raz et al. [42] developed a microarray biosensor, based on an imaging SPR platform for the simultaneous detection of aminoglycosides in milk. The detection of antibiotics was carried out by combining seven different specific immunoassays on one sensor chip and was based on the competitive inhibition of antibody binding. The immunosensor showed ppb (μg/l)-level sensitivity toward the target compounds if 10 times diluted milk samples were used. A SPR immunosensor for CAP residues in milk, designed also as a binding inhibition assay, was proposed by Ferguson et al. [43]. The detection limit of CAP in milk with this assay was quite low—0.05 μg/l. For the screening of streptomycin (STR) residues in milk, Haasnoot et al. [44] tested both direct and competitive binding SPR immunoassays, based on monoclonal anti-dihydro STR antibodies. The limit of detection for STR was 20 μg/l, both for the direct and competitive binding assays. One more competitive STR immunosensor, exploiting commercial

Qflex™ antibodies, was reported by Ferguson et al. [45]. This assay enabled the analysis of STR in whole bovine milk (fat content 3.5%) at concentration level 30 μg/l.

A parallel affinity immunosensor array (PASA) for the analysis of 10 different antibiotics in milk (see Table 5), using multi-analyte immunoassays with an indirect competitive ELISA format, was presented by Knecht et al. [46]. Hapten conjugated with different antibiotics was attached to modified microscope glass slides to prepare disposable microarrays. Specific monoclonal antibodies against each antibiotic allowed the simultaneous detection of each individual analyte. Antibody binding was detected by a secondary antibody, labelled with horseradish peroxidase (HRP) generating enhanced chemiluminescence. The detection limits ranged from 0.12 μg/l up to 32 μg/l. Kloth et al. [47] proposed an improved PASA system, enabling the multiplexed analysis of even 13 antibiotic residues in milk (see Table 5). In these re-generable microarray chips, hapten-antibiotic conjugates were coupled onto epoxy-activated polyethylene glycol (PEG) chip surfaces. The simultaneous detection of the 13 antibiotics in raw milk samples close to the corresponding MRL values was possible within 6 min.

A disposable amperometric magneto-immunosensor, using a polyclonal sheep anti-tetracycline (TC) antibody immobilized on the surface of protein G-functionalized magnetic beads (ProtG-MBs) and screen-printed carbon electrodes (SPCEs) for the detection of TCs in milk, was described by Conzuelo et al. [48]. TC detection was performed through competitive binding between TC in the sample and a HRP-labelled specific tracer (TC-HRP) for binding sites of the capture antibodies. The detection limits were 8.9 μg/l for TC, 1.2 μg/l for oxytetracycline (OTC), 66.8 μg/l for chlortetracycline, and 0.7 μg/l for doxycycline. Conzuelo et al. [49] proposed a similar immunosensor for the specific detection and quantification of sulfonamide (SAs) residues in milk. They used polyclonal rabbit antibodies immobilized onto the electrode surface, modified with 4-aminobenzoic acid. The limit of detection was 0.15 μg/l. Another direct competitive immunoassay for the determination of SPY was based on antibody immobilized onto the surface of protein G-modified glassy carbon plates [50]. The limit of detection of this immunosensor for SPY was on a similar level—0.13 μg/l.

A wavelength interrogated optical sensor (WIOS) technology has been employed for the development of biosensors for SAs, FQs, β-Ls, and TCs. A competitive immunosensor for the simultaneous detection of three antibiotics—SPY, CIP, and OTC—in raw milk has been described by Suarez et al. [51]. These three assays were performed in indirect formats with three specific haptens. Raw milk samples were mixed with different antiserum receptors that specifically reacted with particular antibiotics and further spiked with three antibiotics at corresponding MRLs (100 μg/ml). In contact with the sensing surface, excess antibodies not bound to antibiotics were attached to the hapten-coated sensing region. The attached antibodies were revealed with a secondary antibody. Adrian et al. developed WIOS immunosensors for the detection of SAs [52] and for the simultaneous screening of SAs and other most frequently occurring antibiotic classes: FQs, β-Ls, and TCs [53]. These sensors relied on a competitive immunoassay format, where haptenized proteins for SAs, FQs, β-Ls, and TCs were immobilized on the chip surface, where they formed independent sensing zones. Milk samples

were mixed with the specific antibodies and bio-receptors were added. The detection limits of these sensors were 0.5 μg/l for SPY, 1.3 μg/l for CIP, 3.1 μg/l for AMP, and 34.2 μg/l for OTC.

An impedimetric immunosensor for CIP that comprised polyclonal anti-CIP antibody immobilized on an electrogenerated N-hydroxysuccinimide-functionalized polypyrrole film was developed by Ionescu et al. [54]. The antibody-antigen affinity reaction resulted in an extremely sensitive and specific impedance response, even with CIP concentrations as low as 10 pg/ml. A flow injection impedimetric immunosensor for the direct detection of PEN in milk samples, based on immobilized monoclonal anti-PEN on self-assembled thioctic acid monolayer on gold electrode, was developed by Thavarungkul et al. [55]. Binding of PEN to anti-PEN on the electrode surface causes the impedance to increase. The limit of detection of this immunosensor was 1 pg/l, much lower than the corresponding MRL in milk, but the sensor preparation took as long as 2 days.

A competitive amperometric immunoassay for PEN in milk was developed by Merola et al. [56]. This immunosensor was based on the competitive binding of free PEN and BSA-PEN conjugate immobilized on the sensor membrane, to anti-PEN-biotin-avidin-peroxidase complex. The limit of detection of this immunosensor was as low as 5 ng/l. Another amperometric immunosensor for PEN in milk has been described by Wu et al. [57]. This biosensor was based on the interaction of PEN with the covalently bound new methylene blue (NMB) and HRP-labelled PEN polyclonal antibody (HRP-PEN-Ab) on a glassy carbon electrode. Cyclic voltammetry and impedance spectroscopy enabled to gain the detection limit 0.6 μg/l. An electrochemical magneto immunosensor for CIP has been proposed by Pinacho et al. [58]. Magnetic beads for the attachment of CIP, which are modified with antibody (Ab171) and HRP-BSA, are collected after incubation with the sample onto magnetic electrode and detected electrochemically following the H_2O_2 oxidation, catalyzed by HRP. The detection limit for CIP 9 ng/l was almost as low as gained in Ref. [56].

A nanogold resonance-scattering (RS) spectral assay for the determination of PEN was developed by Jiang et al. [59]. The binding of PEN to anti-PEN, immobilized on the surface of gold nanoparticles, leads to the cleavage of nanoparticles. Uncovered nanoparticles aggregated in the presence of PEN and generated the resonance scattering (RS) effect, measured at 560 nm. The detection limit of the assay was 0.78 μg/l. Another immunosensor, based on the application of the aggregation of nanoparticles, was developed by Chen et al. [60] for the detection of kanamycin (KAN) in milk. This sensor, using superparamagnetic iron oxide (SPIO) nanoparticles, acted as a magnetic relaxation switch. The target analyte KAN competed with KAN immobilized on the surface of the SPIO nanoparticles and hence affected the formation of SPIO aggregates. The dispersed and aggregated states of the SPIO modulate the spin–spin relaxation time (T_2) of the neighboring water molecules, which change due to the effect of the target analyte. The limit of detection with this biosensor for KAN was 0.1 μg/l.

Piezoelectric immunosensors for PEN, AMP, and the total content of penicillin antibiotics have been developed by Karaseva et al. [61]. The receptor coating of the sensors was prepared by the immobilization of PEN- or AMP-hapten-protein conjugates on the polypyrrole film via glutaraldehyde. The limits of detection obtained were 0.8 μg/l for PEN, 3.9 μg/l for AMP, and 1.7 μg/l for total penicillins.

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Finally, a surface acoustic wave (SAW) biosensor has been introduced by Gruhl et al. [62] for the rapid detection of PEN in milk. This was a binding inhibition assay, using the interaction of PEN in the sample and PEN epitopes immobilized on the sensor surface with monoclonal anti-PEN. The binding of antibodies onto the sensor surface was followed by means of acoustic (gravimetric) detection. Low PEN concentrations led to a high binding load of free antibodies to the surface and hence to high signals. The detection limit for PEN in low-fat milk was 2.2 μg/l.

The condensed overview of immunosensors used to determine antibiotic residues in milk is given in Table 5.

Biosensor Assay	Bio-Selective Element	Antibiotics	LOD	Linear Range	Ref.
Piezoelectric immunosensor	Polyclonal and monoclonal antibodies	PEN, AMP, group of penicillins	0.8 μg/l (PEN), 3.9 μg/l (AMP), 1.7 μg/l (group)	2.5–250 μg/l (PEN), 2.5–500 μg/l (AMP), 1–500 μg/l (group)	[61]
Magnetic relaxation Immune-nanosensor	Monoclonal anti-KAN	KAN	0.1 μg/l	1.5–25.2 μg/l	[60]
Electrochemical immunoassay	Polyclonal rabbit antibody (As167)	SAs	0.15 μg/l	0.6–64.2 μg/l	[49]
Amperometric immunoassay	Polyclonal sheep anti-TC antibody	DXC, OTC, TC, CTC	8.9 μg/l (TC), 1.2 μg/l (OTC), 66.8 μg/l (CTC), 0.7 μg/l (DXC)	17.8–189.6 μg/l (TC), 4.0–242.3 μg/l (OTC), 144.2–2001.9 μg/l (CTC), 2.6–234.9 μg/l (DXC)	[48]
Surface plasmon resonance assay	Polyclonal anti-FQ haptenized protein (FQ-BSA) antibody	ENRO, CIP, NOR	2.0 μg/l	n.a.	[40]
Surface plasmon resonance assay	Haptenized protein antibodies	ENRO, SPY, CAP	1.7 μg/l (ENRO), 2.1 μg/l (SPY), 1.1 μg/l (CAP)	n.a.	[41]
Waveguide interrogated optical sensor	Polyclonal rabbit antibody (As155)	SAs	0.5 μg/l	1.4–26.4 μg/l	[52]
Waveguide interrogated optical sensor	Polyclonal and monoclonal antibodies	SPY, CIP, AMP, OTC	0.5 μg/l (SPY), 1.3 μg/l (CIP), 3.1 μg/l (AMP), 34.2 μg/l (OTC)	1.4–26.4 μg/l (SPY), 4.4–70.3 μg/l (CIP), 7.1–100.0 μg/l (AMP), 56.8–193.3 μg/l (OTC)	[53]
Surface plasmon resonance assay	Monoclonal and polyclonal antibodies	NEO, GEN, KAN, STR, ENRO, CAP, SMZ	ppb levels	n.a.	[42]

Biosensor Assay	Bio-Selective Element	Antibiotics	LOD	Linear Range	Ref.
Surface plasmon resonance assay	Monoclonal anti-AMP antibody	AMP	2.5 μg/l	n.a.	[39]
Waveguide interrogated optical sensor	Antiserum receptors	SPY, CIP, OTC	Positive/negative detection: 100 μg/ml		[51]
Resonance-scattering immunosensor	Goat anti-rabbit PEN	PEN	0.78 μg/l	7.5–1700 μg/l	[59]
Impedance spectroscopy immunosensor	Polyclonal anti-CIP antibody	CIP	10 pg/ml	n.a.	[54]
Impedimetric flow immunosensor	Monoclonal anti-PEN	PEN	3.0×10^{-15} M	1.0×10^{-13}–1.0×10^{-8} M	[55]
Surface plasmon resonance assay	Qflex™ CAP antibody	CAP	0.05 μg/l	n.a.	[43]
Parallel affinity immunosensor array	Monoclonal antibodies	PEN, CLOX, CEFA, SDZ, SMT, STR, GEN, NEO, ERY, TYL	0.12–32 μg/l (near to MRL for PEN), for others far below the respective MRLs	3.3–41.3 μg/l (PEN), 0.29–3.63 μg/l (CLOX), 0.12–1.45 μg/l (CEFA), 3.49–43.5 μg/l (SDZ), 4.93–63.0 μg/l (SMT), 5.06–66.0 μg/l (STR), 12.1–141 μg/l (GEN), 31.8–427 μg/l (NEO), 0.36–4.70 μg/l (ERY), 0.95–12.6 μg/l (TYL)	[46]
Surface plasmon resonance assay	Monoclonal anti-dihydro STR antibodies	STR	20 μg/l	n.a.	[44]
Surface plasmon resonance assay	Anti-AMP antibody	Penicillins	5.9 μg/l (chemical pretreatment); 14.6 μg/l (enzymatic pretreatment)	n.a.	[38]
Chemiluminescence immunosensor	Monoclonal antibodies	SMZ, SDZ, STR, CLOX, AMP, PEN, CEFA, NEO, GEN, ERY, TYL, ENRO, TC	n.a.	20–320 μg/l (SMZ), 3.1–440 μg/l (SDZ), 21–608 μg/l (STR), 0.5–320 μg/l (CLOX), 1.7–1800 μg/l (AMP), 3.0–320μg/l (PEN), 0.8–797 μg/l (CEFA), 135–2300 μg/l (NEO), 6.8–540 μg/l (GEN), 0.1–45 μg/l (ERY), 0.6–120 μg/l (TYL), 0.5–6.3 μg/l (ENRO), 0.05–43 μg/l (TC)	[47]

Biosensor Assay	Bio-Selective Element	Antibiotics	LOD	Linear Range	Ref.
Amperometric immunosensor	Methylene blue and HRP-labelled PEN polyclonal antibody	PEN	1.82 nM	5.2–41.6 nM	[57]
Amperometric immunoassay	Anti-PEN-biotin-avidin-peroxidase complex	PEN	1.5×10^{-11} M	3.0×10^{-11}–3.0×10^{-4} M	[56]
Surface plasmon resonance assay	Qflex™ STR antibody	STR, dihydro STR	30 μg/l	n.a.	[45]
Surface acoustic wave assay	Monoclonal anti-PEN (clone 8.F. 223)	PEN	2.2 μg/l	2–6 μg/l	[62]
Immunoassay	Polyclonal rabbit antibody (As167)	SPY	0.13 μg/l	n.a.	[50]
Amperometric magneto nanosensor	Ab171	CIP	0.009 μg/l	0.043–7.38 μg/l	[58]

PEN—penicillin G; AMP—ampicillin; KAN—kanamycin; DXC—doxycycline; OTC—oxytetracycline; TC—tetracycline; CTC—chlortetracycline; ENRO—enrofloxacin; CIP—ciprofloxacin; NOR—norfloxacin; SPY—sulfapyridine; SAs—sulfonamides; CAP—chloramphenicol; NEO—neomycin; GEN—gentamycin; STR—streptomycin, SMZ—sulfamethazine; AMOX—amoxicillin; CLOX—cloxacillin; CEFL—cephalexin; CEFO—cefoperazone; CEFA—cefapirin; CIP—ciprofloxacin; SDZ—sulfadiazine; SMT—sulfamethazine; ERY—erythromycin; TYL—tylosin; DTC—doxytetracycline; HRP—horseradish peroxidase; As—antiserum; Ab—antibody; LOD—limit of detection; n.a.—data not available.

Table 5. Immunosensors for the detection of antibiotic residues in milk.

5. Aptasensors

For the detection of antibiotic residues in milk, aptamer-based biosensors (aptasensors) have been developed during last 5 years [63-77]. Aptamers can be considered as chemical or "synthetic" antibodies because of their in vitro production based on the systematic evolution of ligands by exponential enrichment (SELEX) [78]. The SELEX process enables the fabrication of aptamers also for non-immunogenic and toxic targets that cannot be produced by natural immune systems [79, 80]. The selection provides specific aptamers fold into well-defined three-dimensional shapes, which can recognize their target molecules with high affinity [78, 81]. In most studied biosensing systems, the dissociation constants of the aptamer-target molecule complexes are in the nanomolar range. In addition, aptamers are quite stable and are not affected by reasonable temperature or pH shifts; at optimal conditions they can restore their original conformation. Aptamers are smaller in size compared to antibodies enabling them to reach previously blocked or intracellular targets [80].

The immobilization of aptamers is carried out with the help of two major techniques [82]: (i) direct attachment of aptamers on a bio-coated sensor surface via suitable linkers; and (ii) non-covalent conjugation of aptamers to functionally activated surfaces. To facilitate direct immobilization, the aptamer must be functionalized by adding a terminal functional group such as biotin or amine [82]. Sometimes an extra linker is used to create flexibility between the aptamer and terminal functional group. To minimize the nonspecific binding caused by the linker, the linker usually consists of a string of thymidine [82, 83]. A serious disadvantage using aptasensors for milk analyses is the presence of milk proteins and fat, and the non-transparency of the samples, which hamper the application of optical detection methods [64, 68, 71]. Commonly pretreatment of milk samples is required [64, 68-71, 73, 74, 77].

For the detection of β-L antibiotics, several aptasensors have been proposed [64, 67, 69, 71]. Dapra et al. [67] developed polymer biosensor chips comprising a micro-fluidic system and immobilized aptamers, integrated with the measurement of electrochemical impedance. Polymers exhibit excellent properties to master the task of transducing a binding event between an analyte and a biological probe into a measurable signal. Polymers have been used in biosensing as alternatives to traditional electrode materials due to their inexpensive fabrication and simple functionalization [84]. In aptasensors proposed by Dapra et al., single-stranded DNA (ssDNA) aptamers functionalized with fluorescein amidite (FAM) were used [67]. With this sensor it was possible to detect ampicillin (AMP) concentrations below the established MRL (the detection range was 35 ng/l to 350 μg/l). Song et al. [69] proposed an aptasensor for sulfadimethoxine (SDMX), which consisted of FAM-modified ssDNA aptamers attached to coordinated polymer nanobelts (CPNBs). The detection limit for this aptasensor for SDMX in milk was 10 μg/l, which is 10 times below the allowed MRL in EU. However, the aptamer–antibiotic interaction was interfered by other components of raw milk (proteins, fat, $[K]^+$, $[Na]^+$), reported also by other authors [63, 70, 71, 85]. It has been proposed that changes of $[K]^+$ and $[Na]^+$ inactivate aptamers by preventing them to fold into correct structures, and accordingly at least 10-fold diluted matrix should be used for milk analyses [63, 85].

There are numerous aptasensors for the detection of chloramphenicol (CAP) [65, 68, 70], whose use is actually banned in food-producing animals and whose presence in milk is strictly prohibited. The regulations set the MRL for CAP in milk at 0.3 μg/l, which is the LOD with HPLC-MS [6]. Wu et al. [68] proposed a fluorescence-based aptasensor, using aptamer-conjugated magnetic nanoparticles (MNPs) for both CAP recognition and concentration. In the absence of target molecules (antibiotics), MNP-aptamer complex hybridizes to its complementary DNA (cDNA) modified with upconversion nanoparticles (UCNPs) to form the duplex structure giving a maximum fluorescent signal. Upon CAP addition, the aptamer preferentially binds with CAP and causes the dissociation of some cDNA, liberating some UCNPs-cDNA complexes, and leads to a decreased fluorescence signal on the surface of MNPs. Under optimal conditions, a linear CAP detection range from 0.01 to 1 μg/l was achieved. Alibolandi et al. [70] constructed an aptasensor for the detection of CAP, where aptamers were conjugated with Cd-Te quantum dots (QDs), which exhibit high resistance to photo-bleaching, stable fluorescence, high quantum yield, narrow and symmetric emission band, and broad adsorption spectra [86]. The detection limit of CAP with this aptasensor was 0.2 μg/l [70].

Various aptasensors for the detection of tetracyclines (TCs) in milk have been proposed [73-77, 85]. Jeong and Paeng [85] used two different enzyme-linked aptasensors (ELAA) based either on a ssDNA-aptamer or on an RNA-aptamer. A competitive assay with sequential mode detection was adapted for both systems. A similar system was developed by Kim et al. [73], who used ssDNA aptamers for the detection of oxytetracycline (OTC). In terms of specificity, detection limit, and dynamic range, the results obtained with ELAA in both the above-mentioned studies were not an improvement in comparison with the commonly used immunoassays (ELISA) [73]. For the detection of TC, colorimetric aptasensors have been proposed by He et al. [74, 75]. In these systems, aptamers were adsorbed onto the gold nanoparticles (AuNPs) by electrostatic interactions. To generate and stabilize the AuNPs, poly(diallyldimethylammonium) (PDDA) [75] and hexadecyltrimethylammonium bromide (CTAB) [74] were used. The detection of TCs was based on the aggregation phenomenon of functionalized AuNPs, which can be detected by the change in color of the solution from blue to wine red. According to the information presented, if AuNPs were stabilized with PDDA, nanoparticles aggregated in the presence of TC [75]. If CTAB was used, the aggregation of nanoparticles was not favored in the presence of TC and an opposite effect was detected [74]. These assays also required pretreatment of milk samples with acetic acid in order to remove Ca^{2+}. In both systems, the average recoveries of TC were in the range 81–112%. Compared to the PDDA assay, the CTAB assay had 2.7 times higher detection limit (20 μg/l and 54 μg/l, respectively). A label-free electrochemical method based on modified glassy carbon (GC) electrode and ssDNA-aptamers has been proposed by Zhang et al., also for the detection of TCs [76]. The advantage of this electrochemical aptasensor in comparison with the optical ones is the fact that the proposed system did not need any sample pretreatment. The linear detection range for TC was 0.1–100 μg/l. An ultrasensitive resonance scattering method for the detection of TC in milk was described by Luo et al. [77]. This biosensor was based on the competition of aptamers between nanogold and TC. SsDNA-aptamer coats the surface of nanogold particles through van der Waals and intermolecular forces and prevents the aggregation of nanogold particles. But in the presence of TC, the nanogold surface becomes naked, because of the high affinity between TC and aptamer, and the nanoparticles aggregate. The constructed biosensor could be used to detect trace levels of TC in milk samples with good sensitivity (the limit of detection was 22 μg/l).

The overview of aptasensors used to determine antibiotic residues in milk is given in Table 6.

Biosensor Assay	Antibiotic Residues	K_d	Assay Time	LOD	Linear Range	Comments	Ref.
Indirect competitive enzyme-linked aptamer assay	OTC	Su3: 4.7 nM	60 min + 30 min sample pretreatment	12.3 μg/l	n.a.	The 5′-biotin ssDNA-aptamer	[73]
Electrochemical microfluidic biosensor	AMP	13.4 nM	220 min + sample pretreatment	n.a.	100 pM-1 μM (AMP)	The 5′-amino modified ssDNA-aptamer	[67]

Biosensor Assay	Antibiotic Residues	K_d	Assay Time	LOD	Linear Range	Comments	Ref.
	KAN	78.8 nM		n.a.	10 nm-1 mM (KAN)		
Fluorescence nanoparticle bioassay	CAP	n.a.	110 min + 15 min sample pretreatment	0.01 μg/l	0.01–1 μg/l	The 5′-biotin ssDNA-aptamer	[68]
Fluorescence nanobelt bioassay	SDMX	Su13: 84 nM Su11: 150 nM	95 min + 50 min sample pretreatment	10 μg/l	10–100 μg/l	The 5′-fluorescein amidite labelled ssDNA-aptamer	[69]
Fluorescence-colorimetric nanoparticle bioassay	AMP	Su4: 9.4 nM Su17: 13.4 nM Su18: 9.8 nM	200 min + 50 min sample pretreatment	5 μg/l	5–50 μg/l	The 5′-fluorescein-amidite labelled ssDNA-aptamer	[64]
Colorimetric sensor	TCs	n.a.	25 min + sample pretreatment	45.8 nM	0.01–0.4 μM	5′-amino modified ssDNA-aptamer	[75]
Quantum dots and graphene electrochemical sensor	CAP	n.a.	25 min detection +sample pretreatment	0.2 μg/l	0.1–10 nM	5′-amino modified ssDNA-aptamer	[70]
Spectrophotometric assay	KAN	n.a.	100 min + 40 min sample pretreatment	1 nM	1–8 nM and 100–500 nM	5′-thiol-stranded ssDNA-aptamer	[71]
Indirect competitive chemiluminescent enzyme immunoassay	ENRO	Su17: 188 nM	65 min + 30 min sample pretreatment	2.26 μg/l	6.4–90.0 μg/l	ssDNA-aptamer	[63]
Ultrasensitive resonance scattering assay	TCs	n.a.	40 min + 40 min sample pretreatment	11.6 nM	Up to 250 nM	ssDNA-aptamer	[77]
Electrochemical assay	TCs	n.a.	n.a.	1 μg/l	0.1–100 μg/l	ssDNA-aptamer	[76]
Colorimetric nanoparticle sensor	TCs	n.a.	60 min + sample pretreatment	122 nM	0.01–0.5 μM	ssDNA-aptamer	[74]
Competitive enzyme-linked aptamer assay	TCs	Su76: 63 nM Su57: 770 pM	16 h + 160 min + sample pretreatment	45.7 μg/ml 16.8 μg/ml	3.2×10^{-4}-3.2×10^{-7} M 1.0×10^{-4}-1.0×10^{-7} M	3`-end biotin ssDNA-aptamer 5` dimethoxytrityl-	[85]

Biosensor Assay	Antibiotic Residues	K_d	Assay Time	LOD	Linear Range	Comments	Ref.
						biotin amidite RNA-aptamer	

CAP—chloramphenicol; AMP—ampicillin; TCs—tetracyclines; OTC—oxytetracycline; ENRO—enrofloxacin; KAN—kanamycin; SDMX—sulfadimethoxine; ssDNA—single-stranded DNA; n.a.—data not available; K_d—dissociation constant for the aptamer; LOD—limit of detection.

Assay time does not include aptamer and electrode preparation.

Table 6. Aptasensors for the detection of antibiotic residues in milk.

6. Molecularly Imprinted Polymer (MIP) sensors

A recent development in the biosensing of antibiotic residues in milk is the application of molecularly imprinted polymer sensors [87-93]. Molecular imprinting is a technique for the creation of synthetic materials containing specific receptor sites having high affinity toward the target molecule. Molecularly imprinted polymers (MIPs) are cross-linked organic structures containing pre-designed molecular recognition sites complementary in shape, size, and functional groups to the template molecule [94]. MIPs are effective alternatives for the natural receptors in biosensor assays.

For the detection of TCs, a photonic MIP sensor was developed by combining colloid crystal templating and molecular imprinting techniques [93]. Three structurally similar TCs (tetracycline, oxytetracycline, and chlortetracycline) were used as template molecules for MIPs synthesis. Target antibiotic molecules generated optical changes in the Bragg diffraction peak, detected directly using fiber optic spectroscopy. No labelling or sample pretreatment was necessary. The MIPs prepared enabled to discriminate between the very similar TCs, indicating that the cooperative effect of shape, size, and interaction sites of the formed binding areas plays a critical role in the selective molecular recognition process of MIPs. Hydrogen bonding is suggested to be the main interaction responsible for the retention of TCs in MIPs. Because hydroxyl groups form hydrogen bonds with carboxylic groups of the polymer, the difference in the amount of OH^- groups along with the molecular structure of particular TCs result detectable variations in retention [93].

A voltammetric MIP sensor for the detection of sulfadiazine (SDZ) based on carbon paste electrode modified with SDZ-MIP and measuring the potential change due to the binding of antibiotics was proposed by Sadeghi and Motaharian [92]. However, the selectivity of the MIP sensor was rather poor, as sulfonamides including two aromatic cycles generated a difference in the measured currents less than 20% in the case of SDZ and sulfapyridine, and around 30% in the case of benzenesulfonamide and 4-methyl-benzenesulfonamide. The milk matrix did not interfere with the measurements.

An electrochemical MIP sensor was fabricated for the detection of erythromycin (ERY) by decorating a gold electrode with chitosan-platinum nanoparticles/graphene-gold nanoparti-

cles double-layer nanocomposite [91]. The MIPs were prepared using $HAuCl_4$ and 2-mercaptonicotinic acid, and ERY as a template. The selectivity of the sensor was evaluated toward four different antibiotics (ERY, kanamycin, neomycin, and spiramycin). The response of the MIP electrode to ERY was approximately 80% higher than toward the other studied compounds, revealing the good selectivity of the MIP sensor. The limit of ERY detection in milk was around 75 μg/l [91].

MIP sensors have also been developed for the detection of different aminoglycosides. For tobramycin (TOB), an MIP-based pyrrole glassy carbon electrode [89] and a quartz crystal microbalance nanosensor [88] have been proposed. These MIP sensors were very sensitive, with the detection limits in milk under 1 μg/l. The selectivity and stability of these sensors were also excellent. Liu et al. [87] proposed a method for the detection of streptomycin (STR) residues in milk, based on nanogold-encapsulated poly(*o*-phenylenediamine) shell on magnetic iron oxide core. The assay was carried out in a competitive-type mode between the target molecule and glucose oxidase-labelled STR. The sensor response was based on the catalytic oxidation of glucose by glucose oxidase, amplifying the signal and improving the sensitivity. The application of magnetic beads facilitates the construction of the sensors and enables the concentration of samples. In milk, the detection limit for STR of this system was 1 μg/l [87]. For the detection of neomycin (NEO), a multilayer sensor system of gold electrode, composite material (chitosan-silver nanoparticles/graphene-multiwalled carbon nanotubes), and poly(pyrrole)-based MIP were used [90]. The selectivity of this sensor was good toward NEO, and in milk the detection limit was 6 μg/l.

The MIP sensors, which have been developed, are very sensitive with low detection limits. However, the linear ranges of detection of these sensors tend to be below the allowed MRL values, so complicating their practical applications. The condensed overview of MIP sensors used to determine antibiotic residues in milk is given in Table 7.

Sensor Assay	Antibiotic Residues	Stability of the MIP	Monomer	LOD	Linear Range	Assay/Recovery	Ref.
Quartz crystal microbalance nanosensor	TOB	After 45 days, the value of mass shift was approx. 98% of the initial	2-hydroxyethyl methacrylate and N-methacryloyl-L-glutamic acid (ethylenglycol dimethacrylate as a linker)	5.7×10^{-12} M	1.7×10^{-11}–1.5×10^{-10} M	Gold electrode, recovery in milk 95–101%	[88]
Electrochemical assay	NEO	After 2 weeks at 4°C the current response decreased by about 7.3%	Pyrrole	7.6×10^{-9} M	9×10^{-9}–7×10^{-6} M	Gold electrodes decorated with chitosan-silver nanoparticles/graphene-multiwalled carbon nanotubes	[90]

Sensor Assay	Antibiotic Residues	Stability of the MIP	Monomer	LOD	Linear Range	Assay/Recovery	Ref.
						composites, recovery in milk 98–103%	
Electrochemical assay	TOB	After 45 days, the peak current was approx. 96%	Pyrrole	1.4×10^{-10} M	5×10^{-10}–$1x10^{-8}$ M n.a.	Glassy carbon electrode, recovery in milk 88–100%	[89]
Voltammetric assay	SDZ	At ambient conditions for more than 18 weeks without significant change in the response	Ethylene glycol dimethacrylate and 2-azobisiso-butyronitrile	1.4×10^{-7} M	2×10^{-7}–1×10^{-4} M	Carbon paste electrode, recovery in milk 97–104%	[92]
Electrochemical assay	STR	After 25 days at 4°C the response decreased by 10%	*o*-phenylene-diamine	10 pg/ml	0.05–20 μg/l	Gold-promoted magnetic nanospheres, recovery in milk 94–113%	[87]
Electrochemical assay	ERY	After stored at 4°C for 2 weeks the current response decreased by 9%	2-mercaptoni-cotinicacid and $HAuCl_4$	2×10^{-8} M	7×10^{-8}–9×10^{-5} M	Chitosan-platinum nanoparticles/ graphene-gold nanoparticles composites, recovery in milk 96–101%	[91]
Optical spectrometric assay	TC, OTC, CTC	n.a.	Acrylamide (N,N′-methylene bisacrylamide as linker)	n.a.	0.05–20 μg/l		[93]

TOB—tobramycin; NEO—neomycin; SDZ—sulfadiazine; STR—streptomycin; ERY—erythromycin; TC—tetracycline; OTC—oxytetracycline; CTC—chlortetracycline; LOD—limit of detection; n.a.—data not available; MIP—molecularly imprinted polymers

Table 7. Molecularly imprinted polymer sensors for the detection of antibiotic residues in milk.

7. Conclusions

Due to the urgent need for on-site analysis of milk quality and safety, the research activity for the development of biosensors for the detection of antibiotic residues in milk has been very high during the last years. Most studies are focusing on the detection of antibiotics, which are most commonly used for the treatment of food-producing animals: β-lactams, tetracyclines,

sulphonamides, and aminoglycosides. Biosensors proposed for these antibiotics often exhibit detection limits below or equal to the allowed maximum residue levels in milk. There are almost a hundred studies within the last decade reporting about biosensors for the detection of different antibiotic residues in milk; unfortunately, we could not find any studies dealing with biosensing of colistin or trimethoprim.

In most studies, milk samples spiked with a selected antibiotic or antibiotics have been used for the "proof of concept" and validation of the proposed technology. Analyses of the milk of animals, who are undergoing antibiotic treatment, are scarce, although these real samples can be a key factor to indicate the applicability of biosensor technology for practical analyses, as these "natural" samples can contain in addition different metabolites of antibiotic compounds. Some complex biosensing systems require additional pretreatment of milk samples to remove fat and proteins—the implication of these technologies for on-site analyses also seems problematic. The average detection time (excluding pretreatment) is 30–40 min or even up to 2 hours in case a longer incubation period for bio-recognition is required.

The biggest problem in biosensing is the stability of compounds, used for bio-recognition and the life-time of sensors. In terms of stability, the most prospective are MIP biosensors, where the biological material is used only as a template, and aptasensors. Concerning the regeneration of biosensor systems, there is very little data available. Usually, the full dissociation of complexes, formed during the bio-recognition, takes 30–40 min. In terms of rapid analyses, single use biosensors have a great advantage.

In analytical developments, reliability and robustness are the keywords of future trends. We hope that along with automation and independent action, these features enable biosensors to become an essential tool for on-site analysis of milk and other substances.

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References

[1] E. L. Miller. The penicillins: A review and update. Journal of Midwifery & Women's Health 2002; 47, 426–434.

[2] A. D. Dayan. Allergy to antimicrobial residues in food: Assessment of the risk to man. Veterinary Microbiology 1993; 35, 213–226.

[3] B. Mole. MRSA: Farming up trouble. Nature 2013; 499, 398–400.

[4] Sales of veterinary antimicrobial agents in 26 EU/EEA countries in 2012. 4th ESVAC Report, European Medicines Agency 2014.

[5] Chemical Analysis of Antibiotic Residues in Food, Edited by Jian Wang, James D. MacNeil, and Jack F. Kay (Wiley, Hoboken, 2012).

[6] Commission Regulation (EU) No. 37/2010, ec.europa.eu/health/files/mrl/mrl_20101212_consol.pdf 2010

[7] R. Babington, S. Matas, M. P. Marco, and R. Galve. Current bioanalytical methods for detection of penicillins. Analytical and Bioanalytical Chemistry 2012; 403, 1549–1556.

[8] C. Chafer-Pericas, A. Maquieira, and R. Puchades. Fast screening methods to detect antibiotic residues in food samples. Trends in Analytical Chemistry 2010; 29, 1038–1049.

[9] M. C. Beltran, M. I. Berruga, A. Molina, R. L. Althaus, and M. P. Molina. Performance of current microbial tests for screening antibiotics in sheep and goat milk. International Dairy Journal 2015; 41, 13–15.

[10] L. Kantiani, M. Farre, and B. Damia. Analytical methodologies for the detection of [beta]-lactam antibiotics in milk and feed samples. Trends in Analytical Chemistry 2009; 28, 729–744.

[11] Chemical Analysis of Antibiotic Residues in Food, Edited by J. Wang, J. D. MacNeil, and J. F. Kay (Wiley, Hoboken, 2012).

[12] ThermoFisher; Rapid Antibiotic Residue Testing in Milk, www.thermofisher.com.au/show.aspx?page=/ContentAUS/Scientific/Microbiology-Products/Microbial-Safety-Testing/Rapid-Antibiotic-Residue-Testing-Milk/Rapid-Antibiotic-Residue-Testing-Milk.html 2015

[13] DSM: Reliable Antibiotic Residue Tests for Milk, www.dsm.com/content/markets/foodandbeverages/en_US/solutions/responsible-food-delivery/reliable-antibiotic-residue-tests.html?wt.srch=1&wt.mc_id=GA-Branding 2015

[14] T. F. McGrath, C. T. Elliott, and T. L. Fodey. Biosensors for the analysis of microbiological and chemical contaminants in food. Analytical and Bioanalytical Chemistry 2012; 403, 75–92.

[15] K. Narsaiah, S. N. Jha, R. Bhardwaj, R. Sharma, and R. Kumar. Optical biosensors for food quality and safety assurance – A review. Journal of Food Science and Technology 2012; 49, 383–406.

[16] K. Reder-Christ and G. Bendas. Biosensor applications in the field of antibiotic research – A review of recent developments. Sensors 2011; 11, 9450–9466.

[17] G. Cacciatore, M. Petz, S. Rachid, R. Hakenbeck, and A. A. Bergwerff. Development of an optical biosensor assay for detection of beta-lactam antibiotics in milk using the penicillin-binding protein 2x*. Analytica Chimica Acta 2004; 520, 105–115.

[18] E. Gustavsson, P. Bjurling, J. Degelaen, and A. Sternesjö. Analysis of beta-lactam antibiotics using a microbial receptor protein-based biosensor assay. Food and Agricultural Immunology 2002; 14, 121–131.

[19] A. Sternesjö and E. Gustavsson. Biosensor analysis of beta-lactams in milk using the carboxypeptidase activity of a bacterial penicillin binding protein. Journal of AOAC International 2006; 89, 832–837.

[20] E. Gustavsson, J. Degelaen, P. Bjurling, and A. Sternesjö. Determination of beta-lactams in milk using a surface plasmon resonance-based biosensor. Journal of Agricultural and Food Chemistry 2004; 52, 2791–2796.

[21] J. Lamar and M. Petz. Development of a receptor-based microplate assay for the detection of beta-lactam antibiotics in different food matrices. Analytica Chimica Acta 2007; 586, 296–303.

[22] S. J. Setford, R. M. Van Es, Y. J. Blankwater, and S. Kröger. Receptor binding protein amperometric affinity sensor for rapid beta-lactam quantification in milk. Analytica Chimica Acta 1999; 398, 13–22.

[23] E. Gustavsson, P. Bjurling, and A. Sternesjö. Biosensor analysis of penicillin G in milk based on the inhibition of carboxypeptidase activity. Analytica Chimica Acta 2002; 468, 153–159.

[24] S. M. Drawz and R. A. Bonomo. Three decades of beta-lactamase inhibitors. Clinical Microbiology Reviews 2010; 23, 160–201.

[25] B. Chen, M. Ma, and X. Su. An amperometric penicillin biosensor with enhanced sensitivity based on co-immobilization of carbon nanotubes, hematein, and beta-lactamase on glassy carbon electrode. Analytica Chimica Acta 2010; 674, 89–95.

[26] Y. Wu, L. Tang, L. Huang, Z. Han, J. Wang, and H. Pan. A low detection limit penicillin biosensor based on single graphene nanosheets preadsorbed with hematein/ionic liquids/penicillinase. Materials Science and Engineering: C 2014; 39, 92–99.

[27] F. Ismail, S. B. Adeloju, and A. N. Moline. Fabrication of a single layer and bilayer potentiometric biosensors for penicillin by galvanostatic entrapment of penicillinase into polypyrrole films. Electroanalysis 2014; 26, 2607–2618.

[28] S. Zhou, Y. Zhao, M. Mecklenburg, D. Yang, and B. Xie. A novel thermometric biosensor for fast surveillance of beta-lactamase activity in milk. Biosensors and Bioelectronics 2013; 49, 99–104.

[29] L. M. Goncalves, W. F. A. Callera, M. D. P. T. Sotomayor, and P. R. Bueno. Penicillinase-based amperometric biosensor for penicillin G. Electrochemistry Communications 2014; 38, 131–133.

[30] F. Ismail and S. B. Adeloju. Galvanostatic entrapment of penicillinase into polytyramine films and its utilization for the potentiometric determination of penicillin. Sensors 2010; 10, 2851–2868.

[31] T. Rinken and R. Jaaniso. On-line system, method of its calibration and simultaneous detection of antibiotic residues and their concentration in milk, 23-10-2013, EP2529217 B1.

[32] M. Gamella, S. Campuzano, F. Conzuelo, M. Esteban-Torres, B. Las Rivas, A. J. Reviejo, R. Muṇoz, and J. M. Pingarron. An amperometric affinity penicillin-binding protein magnetosensor for the detection of beta-lactam antibiotics in milk. Analyst 2013; 138, 2013–2022.

[33] F. Conzuelo, V. Ruiz-Valdepenas Montiel, S. Campuzano, M. Gamella, R. M. Torrente-Rodriguez, A. J. Reviejo, and J. M. Pingarron. Rapid screening of multiple antibiotic residues in milk using disposable amperometric magnetosensors. Analytica Chimica Acta 2014; 820, 32–38.

[34] B. G. Healey and D. R. Walt. Improved fiber-optic chemical sensor for penicillin. Analytical Chemistry 1995; 67, 4471–4476.

[35] A. M. Ferrini, V. Mannoni, G. Carpico, and G. E. Pellegrini. Detection and identification of beta-lactam residues in milk using a hybrid biosensor. Journal of Agricultural and Food Chemistry 2008; 56, 784–788.

[36] S. Das, N. Kumar, R. H. Vishweswaraiah, L. Haldar, M. Gaare, V. K. Singh, and A. K. Puniya. Microbial based assay for specific detection of beta-lactam group of antibiotics in milk. Journal of Food Science and Technology 2014; 51, 1161–1166.

[37] G. E. Pellegrini, G. Carpico, and E. Coni. Electrochemical sensor for the detection and presumptive identification of quinolone and tetracycline residues in milk. Analytica Chimica Acta 2004; 520, 13–18.

[38] V. Gaudin, J. Fontaine, and P. Maris. Screening of penicillin residues in milk by a surface plasmon resonance-based biosensor assay: Comparison of chemical and enzymatic sample pre-treatment. Analytica Chimica Acta 2001; 436, 191–198.

[39] Z. F. Zhang, J. Liu, K. X. Xu, and T. Shi. Development of a miniature optical biosensor-based inhibition immunoassay for detection of ampicillin residues in milk. Nanotechnology and Precision Engineering 2009; 7, 483–489.

[40] F. Fernandez, D. G. Pinacho, F. Sanchez-Baeza, and M. P. Marco. Portable surface plasmon resonance immunosensor for the detection of fluoroquinolone antibiotic residues in milk. Journal of Agricultural and Food Chemistry 2011; 59, 5036–5043.

[41] F. Fernandez, K. Hegnerova, M. Piliarik, F. Sanchez-Baeza, J. Homola, and M. P. Marco. A label-free and portable multichannel surface plasmon resonance immunosensor for on site analysis of antibiotics in milk samples. Biosensors and Bioelectronics 2010; 26, 1231–1238.

[42] S. R. Raz, M. G. E. G. Bremer, W. Haasnoot, and W. Norde. Label-free and multiplex detection of antibiotic residues in milk using imaging surface plasmon resonance-based immunosensor. Analytical Chemistry 2009; 81, 7743–7749.

[43] J. Ferguson, A. Baxter, P. Young, G. Kennedy, C. Elliott, S. Weigel, R. Gatermann, H. Ashwin, S. Stead, and M. Sharman. Detection of chloramphenicol and chloramphenicol glucuronide residues in poultry muscle, honey, prawn and milk using a surface plasmon resonance biosensor and Qflex kit chloramphenicol. Analytica Chimica Acta 2005; 529, 109–113.

[44] W. Haasnoot, E. E. M. G. Loomans, G. Cazemier, R. Dietrich, R. Verheijen, A. A. Bergwerff, and R. W. Stephany. Direct versus competitive biosensor immunoassays for the detection of (dihydro)streptomycin residues in milk. Food and Agricultural Immunology 2002; 14, 15–27.

[45] J. P. Ferguson, G. A. Baxter, J. D. G. McEvoy, S. Stead, E. Rawlings, and M. Sharman. Detection of streptomycin and dihydrostreptomycin residues in milk, honey and meat samples using an optical biosensor. Analyst 2002; 127, 951–956.

[46] B. G. Knecht, A. Strasser, R. Dietrich, E. Märtlbauer, R. Niessner, and M. G. Weller. Automated microarray system for the simultaneous detection of antibiotics in milk. Analytical Chemistry 2004; 76, 646–654.

[47] K. Kloth, M. Rye-Johnsen, A. Didier, R. Dietrich, E. Märtlbauer, R. Niessner, and M. Seidel. A regenerable immunochip for the rapid determination of 13 different antibiotics in raw milk. Analyst 2009; 134, 1433–1439.

[48] F. Conzuelo, M. Gamella, S. Campuzano, A. J. Reviejo, and J. M. Pingarron. Disposable amperometric magneto-immunosensor for direct detection of tetracyclines antibiotics residues in milk. Analytica Chimica Acta 2012; 737, 29–36.

[49] F. Conzuelo, M. Gamella, S. Campuzano, G. Pinacho, A. J. Reviejo, M. P. Marco, and J. M. Pingarron. Disposable and integrated amperometric immunosensor for direct determination of sulfonamide antibiotics in milk. Biosensors and Bioelectronics 2012; 36, 81–88.

[50] F. Conzuelo, L. Stratmann, S. Grützke, J. M. Pingarron, and W. Schuhmann. Detection and quantification of sulfonamide antibiotic residues in milk using scanning electrochemical microscopy. Electroanalysis 2014; 26, 481–487.

[51] G. Suarez, Y. H. Jin, J. Auerswald, S. Berchtold, H. F. Knapp, J. M. Diserens, Y. Leterrier, J. A. E. Manson, and G. Voirin. Lab-on-a-chip for multiplexed biosensing of residual antibiotics in milk. Lab on a Chip – Miniaturisation for Chemistry and Biology 2009; 9, 1625–1630.

[52] J. Adrian, S. Pasche, J. M. Diserens, F. J. Sanchez-Baeza, H. Gao, M. P. Marco, and G. Voirin. Waveguide interrogated optical immunosensor (WIOS) for detection of sulfonamide antibiotics in milk. Biosensors and Bioelectronics 2009; 24, 3340–3346.

[53] J. Adrian, S. Pasche, G. Voirin, J. Adrian, D. G. Pinacho, H. Font, F. J. Sanchez-Baeza, M. P. Marco, J. M. Diserens, and B. Granier. Wavelength-interrogated optical biosensor for multi-analyte screening of sulfonamide, fluoroquinolone, beta-lactam and tetracycline antibiotics in milk. Trends in Analytical Chemistry 2009; 28, 769–777.

[54] R. E. Ionescu, N. Jaffrezic-Renault, L. Bouffier, C. Gondran, S. Cosnier, D. G. Pinacho, M. P. Marco, F. J. Sanchez-Baeza, T. Healy, and C. Martelet. Impedimetric immunosensor for the specific label free detection of ciprofloxacin antibiotic. Biosensors and Bioelectronics 2007; 23, 549–555.

[55] P. Thavarungkul, S. Dawan, P. Kanatharana, and P. Asawatreratanakul. Detecting penicillin G in milk with impedimetric label-free immunosensor. Biosensors and Bioelectronics 2007; 23, 688–694.

[56] G. Merola, E. Martini, M. Tomassetti, and L. Campanella. Simple and suitable immunosensor for beta-lactam antibiotics analysis in real matrixes: Milk, serum, urine. Journal of Pharmaceutical and Biomedical Analysis 2014; 106, 186-196.

[57] H. Wu, S. Fan, W. Zhang, H. Chen, L. Peng, X. Jin, J. Ma, and H. Zhang. Amperometric immunosensor based on covalent immobilization of new methylene blue and penicillin polyclonal antibody for determination of penicillin G in milk. Analytical Methods 2014; 6, 497–502.

[58] D. G. Pinacho, F. Sanchez-Baeza, M. I. Pividori, and M. P. Marco. Electrochemical detection of fluoroquinolone antibiotics in milk using a magneto immunosensor. Sensors (Switzerland) 2014; 14, 15965–15980.

[59] Z. Jiang, Y. Li, A. Liang, and A. Qin. A sensitive and selective immuno-nanogold resonance-scattering spectral method for the determination of trace penicillin G. Luminescence 2008; 23, 157–162.

[60] Y. P. Chen, M. q. Zou, C. Qi, M. X. Xie, D. N. Wang, Y. F. Wang, Q. Xue, J. F. Li, and Y. Chen. Immunosensor based on magnetic relaxation switch and biotin-streptavidin system for the detection of Kanamycin in milk. Biosensors and Bioelectronics 2013; 39, 112–117.

[61] N. A. Karaseva and T. N. Ermolaeva. Piezoelectric immunosensors for the detection of individual antibiotics and the total content of penicillin antibiotics in foodstuffs. Talanta 2014; 120, 312–317.

[62] F. J. Gruhl and K. Länge. Surface acoustic wave (SAW) biosensor for rapid and label-free detection of penicillin G in milk. Food Analytical Methods 2014; 7, 430–437.

[63] H. Ni, S. Zhang, X. Ding, T. Mi, Z. Wang, and M. Liu. Determination of enrofloxacin in bovine milk by a novel single-stranded DNA aptamer chemiluminescent enzyme immunoassay. Analytical Letters 2014; 47, 2844–2856.

[64] K. M. Song, E. Jeong, W. Jeon, M. Cho, and C. Ban. Aptasensor for ampicillin using gold nanoparticle based dual fluorescence-colorimetric methods. Analytical and Bioanalytical Chemistry 2012; 402, 2153–2161.

[65] S. Pilehvar, J. Mehta, F. Dardenne, J. Robbens, R. Blust, and K. De Wael. Aptasensing of chloramphenicol in the presence of its analogues: Reaching the maximum residue limit. Analytical Chemistry 2012; 84, 6753–6758.

[66] A. N. Berlina, N. A. Taranova, A. V. Zherdev, Y. Y. Vengerov, and B. B. Dzantiev. Quantum dot-based lateral flow immunoassay for detection of chloramphenicol in milk. Analytical and Bioanalytical Chemistry 2013; 405, 4997–5000.

[67] J. Dapra, L. H. Lauridsen, A. T. Nielsen, and N. Rozlosnik. Comparative study on aptamers as recognition elements for antibiotics in a label-free all-polymer biosensor. Biosensors and Bioelectronics 2013; 43, 315–320.

[68] S. Wu, H. Zhang, Z. Shi, N. Duan, C. Fang, S. Dai, and Z. Wang. Aptamer-based fluorescence biosensor for chloramphenicol determination using upconversion nanoparticles. Food Control 2015; 50, 597–604.

[69] K. M. Song, E. Jeong, W. Jeon, H. Jo, and C. Ban. A coordination polymer nanobelt (CPNB)-based aptasensor for sulfadimethoxine. Biosensors and Bioelectronics 2012; 33, 113–119.

[70] M. Alibolandi, F. Hadizadeh, F. Vajhedin, K. Abnous, and M. Ramezani. Design and fabrication of an aptasensor for chloramphenicol based on energy transfer of CdTe quantum dots to graphene oxide sheet. Materials Science and Engineering C 2015; 48, 611–619.

[71] N. Zhou, J. Zhang, and Y. Tian. Aptamer-based spectrophotometric detection of kanamycin in milk. Analytical Methods 2014; 6, 1569–1574.

[72] S. Jeong and I. Rhee Paeng. Sensitivity and selectivity on aptamer-based assay: The determination of tetracycline residue in bovine milk. The Scientific World Journal 2012; 2012, 159456.

[73] C. H. Kim, L. P. Lee, J. R. Min, M. W. Lim, and S. H. Jeong. An indirect competitive assay-based aptasensor for detection of oxytetracycline in milk. Biosensors and Bioelectronics 2014; 51, 426–430.

[74] L. He, Y. Luo, W. Zhi, Y. Wu, and P. Zhou. A colorimetric aptamer biosensor based on gold nanoparticles for the ultrasensitive and specific detection of tetracycline in milk. Australian Journal of Chemistry 2013; 66, 485–490.

[75] L. He, Y. Luo, W. Zhi, and P. Zhou. Colorimetric sensing of tetracyclines in milk based on the assembly of cationic conjugated polymer-aggregated gold nanoparticles. Food Analytical Methods 2013; 6, 1704–1711.

[76] J. Zhang, B. Zhang, Y. Wu, S. Jia, T. Fan, Z. Zhang, and C. Zhang. Fast determination of the tetracyclines in milk samples by the aptamer biosensor. Analyst 2010; 135, 2706–2710.

[77] Y. Luo, L. He, S. Zhan, Y. Wu, L. Liu, W. Zhi, and P. Zhou. Ultrasensitive resonance scattering (RS) spectral detection for trace tetracycline in milk using aptamer-coated nanogold (ACNG) as a catalyst. Journal of Agricultural and Food Chemistry 2014; 62, 1032–1037.

[78] M. Famulok and G. Mayer. Aptamer modules as sensors and detectors. Accounts of Chemical Research 2011; 44, 1349–1358.

[79] W. Zhou, P. J. Jimmy Huang, J. Ding, and J. Liu. Aptamer-based biosensors for biomedical diagnostics. Analyst 2014; 139, 2627–2640.

[80] S. Y. Toh, M. Citartan, S. C. B. Gopinath, and T. H. Tang. Aptamers as a replacement for antibodies in enzyme-linked immunosorbent assay. Biosensors and Bioelectronics 2015; 64, 392–403.

[81] D. Shangguan, Z. Tang, P. Mallikaratchy, Z. Xiao, and W. Tan. Optimization and modifications of aptamers selected from live cancer cell lines. ChemBioChem 2007; 8, 603–606.

[82] S. Balamurugan, A. Obubuafo, S. A. Soper, and D. A. Spivak. Surface immobilization methods for aptamer diagnostic applications. Analytical and Bioanalytical Chemistry 2008; 390, 1009–1021.

[83] S. Centi, S. Tombelli, M. Minunni, and M. Mascini. Aptamer-based detection of plasma proteins by an electrochemical assay coupled to magnetic beads. Analytical Chemistry 2007; 79, 1466–1473.

[84] W. Xiuyun and U. Shunichi. State of the Art in Biosensors – General Aspects, Edited by Toonika Rinken (InTech, Rijeka, 2013), Chap. 3, p. 67.

[85] S. Jeong and I. Rhee Paeng. Sensitivity and selectivity on aptamer-based assay: The determination of tetracycline residue in bovine milk. The Scientific World Journal 2012; 2012, 159456.

[86] M. F. Frasco and N. Chaniotakis. Semiconductor quantum dots in chemical sensors and biosensors. Sensors 2009; 9, 7266–7286.

[87] B. Liu, D. Tang, B. Zhang, X. Que, H. Yang, and G. Chen. Au(III)-promoted magnetic molecularly imprinted polymer nanospheres for electrochemical determination of streptomycin residues in food. Biosensors and Bioelectronics 2013; 41, 551–556.

[88] M. L. Yola, L. Uzun, N. Özaltin, and A. Denizli. Development of molecular imprinted nanosensor for determination of tobramycin in pharmaceuticals and foods. Talanta 2014; 120, 318–324.

[89] V. K. Gupta, M. L. Yola, N. Özaltin, N. tar, Z. Üstündag, and L. Uzun. Molecular imprinted polypyrrole modified glassy carbon electrode for the determination of tobramycin. Electrochimica Acta 2013; 112, 37–43.

[90] W. Lian, S. Liu, J. Yu, J. Li, M. Cui, W. Xu, and J. Huang. Electrochemical sensor using neomycin-imprinted film as recognition element based on chitosan-silver nanoparticles/graphene-multiwalled carbon nanotubes composites modified electrode. Biosensors and Bioelectronics 2013; 44, 70–76.

[91] W. Lian, S. Liu, J. Yu, X. Xing, J. Li, M. Cui, and J. Huang. Electrochemical sensor based on gold nanoparticles fabricated molecularly imprinted polymer film at chitosan-platinum nanoparticles/graphene-gold nanoparticles double nanocomposites modified electrode for detection of erythromycin. Biosensors and Bioelectronics 2012; 38, 163–169.

[92] S. Sadeghi and A. Motaharian. Voltammetric sensor based on carbon paste electrode modified with molecular imprinted polymer for determination of sulfadiazine in milk and human serum. Materials Science and Engineering: C 2013; 33, 4884–4891.

[93] L. Q. Wang, F. Y. Lin, and L. P. Yu. A molecularly imprinted photonic polymer sensor with high selectivity for tetracyclines analysis in food. Analyst 2012; 137, 3502–3509.

[94] E. Turiel and A. Martin-Esteban. Molecularly imprinted polymers for sample preparation: A review. Analytica Chimica Acta 2010; 668, 87–99.