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TECHNICAL NOTE

Reflectometric interference spectroscopy (RIfS) as a new tool to measure in the complex matrix milk at low analyte concentration

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Abstract Measurements in complex matrices like milk still present a challenge in biosensor development. This is especially important when using a label-free detection method or when measuring low analyte concentrations. The direct optical method reflectometric interference spectroscopy (RIfS) was used for investigating matrix effects in immunoassay development. Furthermore, approaches to reduce these effects have been established. As a model system, the hormone testosterone has been chosen because this immunoassay has been well characterized in buffer. In a first step, the immunoassay for the detection of testosterone in buffer was improved beyond former published results. Therefore, the sensor surface was optimized, resulting in a fivefold lower limit of detection (70.2 ng L^{-1}) and limit of quantification (130.0 ng L^{-1}). Additionally, the assay time could be reduced to 15 min. Consequently, we used this improved assay to investigate matrix effects of whole pasteurized bovine milk. To minimize these effects, the surface chemistry was adapted and a suitable evaluation method was established, reducing the effects of Tyndall scattering and nonspecific binding to the sensor surface. These improvements allow for very reliable quantitative measurements in milk. The assay developed required no sample pretreatment and allowed for the regeneration of the sensor surface so that calibration could be performed on one chip. The calibration in milk (3.5% fat) resulted in a limit of detection of 94.4 ng L^{-1} and a limit of quantification of 229.3 ng L^{-1}. Furthermore, recovery rates between 70% and 120% could be obtained. Thus, for the first time, an analyte in the matrix milk was successfully quantified with RIfS at low concentrations.

Keywords Reflectometric interference spectroscopy (RIfS) · Milk analysis · Matrix effects · Testosterone · Label-free biosensor · Environmental analysis

Introduction

The analysis of undesired substances in different types of matrices like environmental or food samples has gained increasing importance in the last years. Pollutants such as polychlorinated biphenyls [1, 2], pesticides [3–5], pharmaceutical residues [6], hormones [7], or endocrine disrupting compounds [8] are only a few examples of the many contaminants found in the environment and therefore in the food chain. Some contaminants also occur naturally in the food supply like mycotoxins [9, 10], phytoestrogens [11], or *Staphylococcus aureus* and its enterotoxins [12, 13].

Biosensors have demonstrated a great potential not only in environmental analysis but also in food quality monitoring [14–16]. Advantages compared with conventional methods are due to their simplicity, their portability, their potential for miniaturization, and their fast response time, which lead to the opportunity for on-site measurements.

Milk is an important agricultural product and is part of the everyday nutrition of humans. Besides being an essential food commodity itself, it is also the basis of many food products like butter, cheese, or yogurt. Milk is prone

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to contamination by a multitude of substances like environmental pollutants [17, 18], microorganisms [19], or hormonally active substances [20]. In order to protect consumer's health and prevent disease development, it is vital to develop reliable tools for detecting these contaminants in milk.

However, milk is a complex matrix consisting of a variety of compounds such as proteins, fat, minerals, vitamins, and sugar, which can cause matrix effects. Matrix effects are defined as induced deviation from theoretically predicted results due to the constitution of the sample matrix [21]. Especially direct optical detection methods such as surface plasmon resonance (SPR), reflectometric interference spectroscopy (RIfS), or integrated optical devices are influenced by these effects. Nevertheless, it is desirable to use them because by avoiding labeling steps, these methods are fast and cost-effective and, furthermore, no alteration of the monitored binding event has to be feared. In contrast to RIfS, SPR has already been used as a detection method for milk measurements [22, 23]. Yet the time-resolved sensing method RIfS possesses some advantages such as its temperature independence or its simple instrumental setup.

RIfS is based on white light interference at thin solid films. The binding of biomolecules to the sensitive layer is monitored by changes of the optical thickness. Therefore, for this detection method, the main disturbing matrix effects of milk are Tyndall scattering and nonspecific binding of milk components to the sensor surface.

Before developing assays against milk contaminants, the matrix effects of milk have to be investigated and approaches on how to deal with them have to be found. Therefore, a model assay was needed which allows for very reliable measurements in buffer. With this in mind, an immunoassay for the detection of the hormone testosterone was used.

In the last years, some optical and electrochemical immunoassays for monitoring of the hormone testosterone have been reported [24–27]. We previously described the development of an immunoassay for environmental monitoring of testosterone in real water samples [28]. With the used biosensor setup, no sample pretreatment or sample pre-concentration is necessary. This is a great benefit compared with the commonly used analytical methods such as GC-MS, utilized to quantify testosterone in water analysis. Before the immunoassay was adapted on the River Analyser (RIANA), whose detection principle is based on the total internal reflectance fluorescence, a biosensor based on RIfS was established because RIfS allows for good characterization of the investigated immunochemistry [29].

We further optimized this RIfS-based assay with regard to surface chemistry as well as assay conditions to reduce the assay time and obtain better validation parameters such as limit of detection (LOD) and limit of quantification (LOQ) in this work.

Afterwards, this newly improved and well-characterized immunoassay has been transferred to the more complex matrix: pasteurized whole milk. To eliminate the matrix effects of milk as much as possible, the sensor surface has been optimized and a suitable detection method established, reducing the effect of Tyndall scattering and nonspecific binding to the sensor surface.

In this work, the focus lies therefore on the successful adaption of the testosterone immunoassay in buffer to pasteurized whole milk without any sample pretreatment, with as less as possible matrix effects and with an output of knowledge on how to handle this complex matrix.

Experimental

Materials

Common chemicals of analytical grade were purchased from Sigma-Aldrich (Deisenhofen, Germany) or Merck (Darmstadt, Germany). The steroidal hormone testosterone (17β-hydroxy-4-androsten-3-one; molecular formula, $C_{19}H_{28}O_2$; molecular weight, 288.42) was purchased from Sigma-Aldrich. The monoclonal IgG1 antibody to testosterone (clone 7003, host mouse, isotype IgG1, immunogen: T-3-CMO-BSA) was purchased from Acris Antibodies (Hiddenhausen, Germany).

The testosterone derivative, testosterone-3-(*O*-carboxymethyl)oxime (17β-hydroxy-4-androsten-3-one-3-(*O*-carboxymethyl)oxime molecular formula, $C_{21}H_{31}NO_4$; molecular weight, 361.48) used for surface chemistry was purchased from Sigma-Aldrich. 3-Glycidyloxypropyltrimethoxysilane (GOPTS) was purchased from Fluka (Neu-Ulm, Germany). Di-amino-poly(ethylene glycol) (DAPEG) and methoxy-poly(ethylene glycol) amine (MPEG), both with molecular masses of 2,000 Da, were purchased from Rapp Polymere (Tübingen, Germany). Aminodextran (AMD) with 100-kDa molecular weight was purchased from Innovent e.V. (Jena, Germany).

Phosphate-buffered saline (PBS, pH 7.4, comprising 150 mM sodium chloride and 10 nM potassium phosphate monobasic) was used as the buffer solution. Regeneration of the biosensor surface was carried out with a solution of 0.5% sodium dodecyl sulfate in Milli-Q water (pH 2).

Biosensor setup

The biosensor utilized is based on the label-free and time-resolved method RIfS [30] using multiple reflections at thin layers. Therefore, light from a halogen white light source is guided to the transduction layer via an optical fiber and is

partially reflected at each interface. The beams superimpose and build a characteristic interference spectrum which is detected using a diode array spectrometer (Spekol-1100, Analytik, Jena, Germany). The liquid handling system consists of a commercial flow system (Automated Sample Injection Analyser, ASIA and autosampler; Ismatec, Wertheim-Mondfeld, Germany). A detailed description of the RIfS setup can be found elsewhere [31, 32].

Immobilization

The glass transducers were modified in accordance with the immobilization protocols described by Piehler et al. [33] and Mehne et al. [34]. After cleaning, activation, and silanization of the sensor surface, a polymer layer was immobilized to reduce the nonspecific binding of antibodies as well as milk ingredients to the surface during the measurements. In this work, different polymers have been used.

For the immobilization of DAPEG, 30 μL of a 4 g L^{-1} DAPEG–methylene chloride solution was pipetted onto the surfaces. They were transferred into an oven and left there overnight at 70 °C. Afterwards, they were rinsed with Milli-Q water and dried in a nitrogen stream.

For the preparation of PEG layers with a lower amount of functional groups, a solution containing an overall PEG concentration of 4 g L^{-1} was prepared. Therefore, the percent by volume ratio (5% DAPEG/95% MPEG) was adjusted, dissolved in methylene chloride, vortexed, and immobilized on the sensor surface as mentioned above.

For the immobilization of AMD, 10 μL of a 0.2 mg μL^{-1} AMD-Milli-Q water solution was pipetted onto the surface. Each surface was covered with another freshly silanized surface and left to react for up to 24 h in a saturated Milli-Q-water atmosphere. Afterwards, they were rinsed with Milli-Q water und dried in a nitrogen flow.

Testosterone-3-(*O*-carboxymethyl)oxime was immobilized onto the different polymer layers by active ester chemistry: testosterone-3-(*O*-carboxymethyl)oxime (1 mg, 2.8 mmol) and *N*,*N*'-diisopropylcarbodiimide (1,5 μL, approx. 10 μmol) were mixed with dry dimethylformamide (DMF, 10 μL) followed by pipetting 10 μL onto a slide. After covering each slide with another, they were left to react overnight in a saturated DMF atmosphere, rinsed with DMF and Milli-Q water, and then dried in a nitrogen stream.

Immunochemistry

The biosensor used takes advantage of a binding inhibition assay in heterogeneous phase that requires high-affinity antibodies directed against specific analytes and analyte derivatives that can be covalently bound to a sensor surface. In our assay, anti-testosterone antibodies are used. The sample containing testosterone is incubated with this specific antibody for 20 min, thus guaranteeing well-defined conditions for the reaction. For that purpose, 100 μL of the antibody stock solution is mixed with 900 μL of the sample. Afterwards, the sample is pumped over the sensor surface. Only the antibody molecules with free binding sites can bind to the immobilized testosterone derivatives on the surface. To yield quantitative results with the binding inhibition assay, the binding of the antibody to the surface has to be mass transport-limited. To ensure this, the number of binding sites on the surface must be much higher than the amount of antibodies used in the measurement. Therefore, small amounts of antibody are used and a huge excess of analyte derivative is immobilized on the surface. This has been demonstrated by additional RIfS measurements as already described in literature [35] and surface evaluation experiments [36, 37]. The general assay procedure is shown in Fig. 1.

Assay procedure and data evaluation

For the measurements, we used the described commercial available monoclonal mouse IgG1 antibody and the suitable analyte derivative (testosterone-3-(*O*-carboxymethyl)oxime) immobilized on the sensor surface.

For a calibration, routine testosterone standards were prepared in buffer or milk (UHT milk, 3.5% fat). For a measurement, 900 μL of a testosterone standard was mixed with 100 μL of an antibody stock solution containing the antibody and ovalbumin (OVA) in PBS. After incubation for 20 min, the mixture was measured using the biosensor setup.

For measurements in buffer, the common evaluation technique was applied, which means that the binding curve which can be obtained while the sample is pumped over the sensor surface was evaluated. Because of Tyndall scattering, that is not possible for measurements in milk. Instead, the height of the signal at a certain time of the dissociation

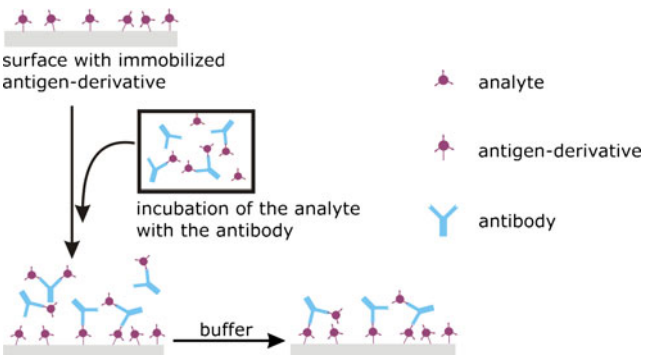

Fig. 1 Principle of the binding inhibition test

phase was evaluated. On the basis of nonspecific binding of milk ingredients, three measurements of pure milk samples had to be performed before the calibration in milk could be started. The mean value of the signal heights at this certain time of the dissociation phase had to be deducted from those received for the calibration measurements.

For each concentration step and the blank measurements, which all were measured threefold, the mean value and the standard deviation (SD) for the replica were calculated. The mean value of the blanks was set to 100% and all spiked sample measurements were normalized to it. To fit the data set, a logistic fit function [38] with four free parameters (A_1, A_2, x_0, and p) was used according to

$$y = \frac{A_1 - A_2}{1 + (x/x_0)^p} + A_2$$

A_1 is the upper and A_2 the lower asymptote. The range between them is defined as the dynamic signal range. The working range is often given by the 10–90% block of the dynamic signal range. The inflection point is given by the variable x_0. It represents the analyte concentration which corresponds to a decrease of 50% of the dynamic signal range (IC_{50}). The slope of the tangent in this point is given by the parameter p.

In compliance with the IUPAC rules [39], the LOD is calculated as three times the SD of the blank measurements (SD_{blanks}). The LOQ is calculated as ten times the SD of the blank measurements (SD_{blanks}).

Results and discussion

Assay optimization in buffer

The previously described immunoassay for the detection of testosterone in water was further developed and optimized regarding optimal surface properties and assay time. Binding of anti-testosterone to immobilized testosterone derivative results in a significant larger change of the optical thickness than the binding of testosterone to immobilized anti-testosterone. Therefore, a binding inhibition assay instead of a direct assay was used. A prerequisite of the binding inhibition assay is that antibody binding to the surface has to be mass transport-limited. Thus, it is important that a huge excess of analyte derivative, in this case testosterone-3-(*O*-carboxymethyl)oxime, is immobilized onto the surface. This necessitates a suitable surface coating that provides a high number of binding sites for the immobilization of the testosterone derivative while suppressing nonspecific adsorption. For optimization of the testosterone immunoassay in buffer, two different polymers have been used: DAPEG and AMD. Comparing these two polymers when immobilized onto a planar surface, DAPEG forms a two-dimensional planar structure onto which one monolayer of proteins can be immobilized (5–6 ng mm^{-2}). In contrast, AMD forms a three-dimensional matrix with a huge number of binding sites. About 20 ng mm^{-2} proteins can be immobilized [34]. However, one advantage of PEG layers is the much better prevention of nonspecific binding of proteins to the modified sensor surface.

By monitoring the interaction of anti-testosterone with the sensor surface neither for the DAPEG–testosterone

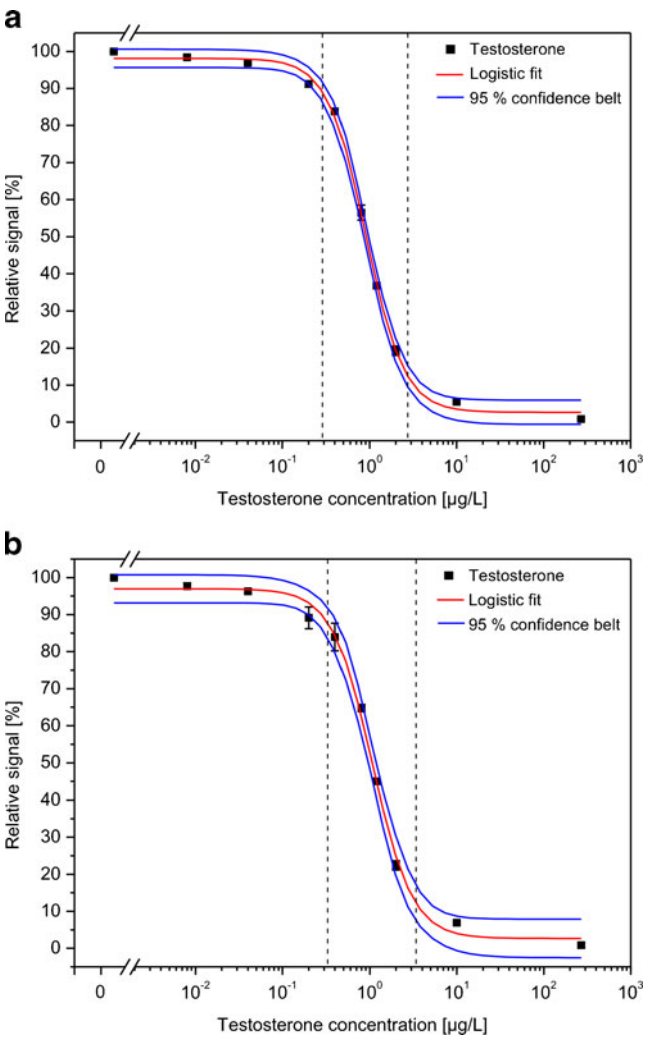

Fig. 2 **a** Calibration plot (logistic fit) and 95% confidence belt measured on a DAPEG–testosterone-3-(*O*-carboxymethyl)oxime-modified transducer from 0.008 to 270 µg L^{-1} in buffer (threefold measurements, nine steps). The antibody concentration was 0.4 mg L^{-1} in each sample. The working range is 0.29–2.75 µg L^{-1} (*dotted lines*). The data are given by the mean value with SD. **b** Calibration plot (logistic fit) and 95% confidence belt measured on a AMD-testosterone-3-(*O*-carboxymethyl)oxime-modified transducer from 0.008 to 270 µg L^{-1} in buffer (threefold measurements, nine steps). The antibody concentration was 0.4 mg L^{-1} in each sample. The working range is 0.33–3.40 µg L^{-1} (*dotted lines*). The data are given by the mean value with SD

derivative nor for the AMD-testosterone derivative-coated surface, unspecific binding of proteins such as ovalbumin (OVA) or bovine serum albumin (BSA) could be observed. Furthermore, measurements showed that for the used antibody concentration of 0.4 mg/L per sample, the amount of testosterone derivatives provided on a DAPEG–testosterone derivative surface is sufficient to guarantee mass transport-limited conditions. Therefore, no difference in the signal behavior could be monitored. Thus, for measurements performed in buffer, the biopolymer did not affect the sensitivity of the assay. This fact is also reflected in the calibration curves described in the "Calibration in buffer."

Because this binding inhibition immunoassay ought to be transferred to the matrix milk, the cross-reactivity of the hormone progesterone, which can be found in milk, was under examination. The cross-reactivity of progesterone toward anti-testosterone amounts to 4.68% [40] and can therefore be neglected.

The assay time including the washing steps, injection of the sample, and regeneration of the surface was reduced to 15 min by optimizing the flushing volume of the washing and regeneration steps and by increasing the speed of the pump.

Calibration in buffer

One calibration was performed using a DAPEG–testosterone derivative-coated surface and one using an AMD-testosterone derivative-coated surface. The prepared transducers were calibrated with testosterone from 0.008 to 270.0 μg L^{-1} in nine steps using 0.4 mg L^{-1} anti-testosterone per sample. Calibration using a DAPEG–testosterone derivative-coated surface resulted in a limit of detection of 70.2 ng L^{-1} and a limit of quantification of 130.0 ng L^{-1}. The data set and the logistic fit function were plotted in a semi-logarithmic graph, as shown in Fig. 2a. Additionally, the parameters of the logistic fit and the validation parameters are summarized in Table 1. Calibration using an AMD–testosterone derivative-coated surface resulted in a limit of detection of 75.2 ng L^{-1} and a limit of quantification of 142.1 ng L^{-1}. The resulting calibration curve is shown in Fig. 2b; the validation parameters and the parameters of the logistic fit can be found in Table 1.

Accordingly, it could be demonstrated that despite considerably reducing the assay time, better validation parameters (by a factor of 5–6 better) could be achieved in comparison to the LOD of 405.5 ng L^{-1} and the LOQ of 808.7 ng L^{-1} obtained and published recently [28].

Assay optimization in milk

For measurements in buffer, DAPEG–testosterone derivative and AMD–testosterone derivative-coated surfaces were used. However, milk is a much more complex matrix, which can interact with the sensor surface. The so far described surfaces can be utilized, but do not allow for reliable reproducible measurements with low SDs. Therefore, modified PEG layers were tested using a mixture of DAPEG and MPEG in different proportions. Measurements with these modified PEG–testosterone derivative-coated surfaces are possible without any loss of signal for the investigated testosterone/anti-testosterone system. A polymer layer with 5% DAPEG and 95% MPEG meets the requirements the best. Therefore, this polymer layer was used for measurements and for the calibration of testosterone in whole pasteurized milk.

Concerning long-term stability, the described surface allows for over 100 measurements on a chip despite the complex matrix. The variation of the analytical signal with the number of measurement is presented in the Electronic supplementary material (ESM) Fig. S1.

Typical measurement cycles in pasteurized whole bovine milk with and without addition of anti-testosterone are shown in Fig. 3. While pumping the samples over the

Table 1 Parameters of the logistic fit function (A_1, A_2, x_0, p) including errors (S.D.A_1, S.D.A_2, S.D.x_0, S.D.p) and validation parameters (LOD, LOQ, working range) for all three calibration curves (buffer and milk)

Matrix	Water		Milk
Surface modification	DAPEG–testosterone-3-(*O*-carboxymethyl)oxime	AMD–testosterone-3-(*O*-carboxymethyl)oxime	(5% DAPEG/95% MPEG)–testosterone-3-(*O*-carboxy-methyl)oxim
A_1 (%)	98.1±1.0	97.0±1.5	99.2±0.6
A_2 (%)	2.7±1.3	2.7±2.1	1.6±0.9
x_0 (μg L^{-1})	0.91±0.03	1.07±0.06	1.84±0.05
p	1.98±0.12	1.90±0.19	1.40±0.05
LOD (ng L^{-1})	70.2	75.2	94.4
LOQ (ng L^{-1})	130.0	142.1	229.3
Working range (μg L^{-1})	0.29–2.75	0.33–3.40	0.38–8.86

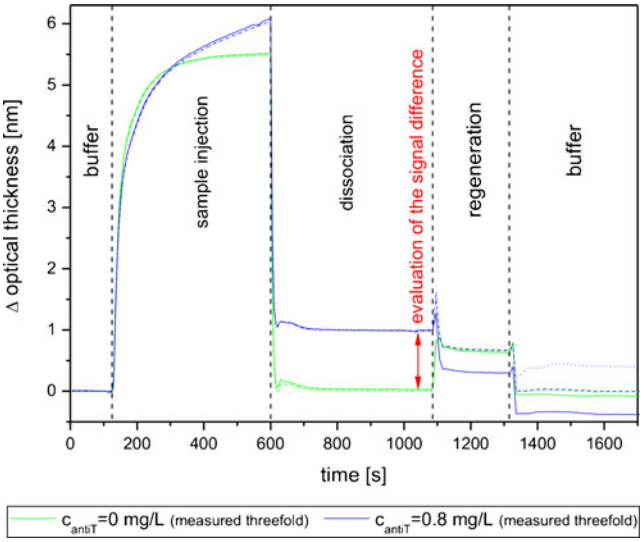

Fig. 3 Typical binding curves in pasteurized whole milk obtained with RIfS. After 125 s baseline, the milk samples were pumped over the sensor surface for 475 s. Subsequently, after 486 s buffer pumping over the transducer, it was regenerated (230 s) followed by 384 s baseline

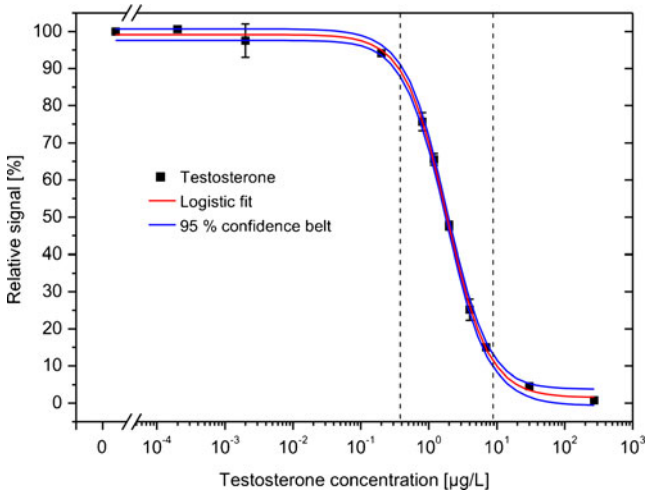

Fig. 4 Calibration plot (logistic fit) and 95% confidence belt measured in milk from 0.2 to 270 µg L^{-1} (threefold measurements, nine steps). The antibody concentration was 0.8 mg L^{-1} in each sample. The working range is 0.38–8.86 µg L^{-1} (*dotted lines*). The data are given by the mean value with SD

surface, a huge change of the optical thickness can be observed. This is due to the described matrix effects such as Tyndall scattering and to the refractive index of milk. Since these effects overlap the signal obtained by the specific binding of anti-testosterone to the testosterone derivative, it is more practical to evaluate the signal at a certain period during the dissociation time in contrast to measurements in rather convenient matrices. If only measuring milk without the addition of anti-testosterone, the signal during dissociation phase should be at baseline again. The more susceptible the sensor surface for nonspecific binding, the higher the signal during dissociation phase. The used sensor surface is rather good concerning the fact that only 0.3 nm signal height during dissociation phase can be noticed (whole pasteurized milk, 3.5% fat). Therefore, the nonspecific binding of milk components to the optimized sensor surface is rather slight. Due to this good result, no blocking agents have been needed. Furthermore, the nonspecific binding to the sensor surface is very similar in each measurement; therefore, the measurements are very comparable.

To be independent from the effect of nonspecific binding to the sensor surface and to be able to compare measurements of milk samples differing in their milk composition, it would be best to determine the relative signal height instead of the absolute signal height at a certain time during the dissociation phase. To obtain the relative signal height, the mean value of the threefold measurement without antibody has to be deducted from those received with antibody.

With these optimized sensor surface and evaluation method, it would be possible to measure in whole undiluted milk independently from its composition. However, in these measurements, the milk samples have been diluted by a factor of 9:1 simply because the anti-testosterone had to be added to the sample.

Calibration in milk

The prepared transducer was calibrated with testosterone from 0.0002 to 270 µg L^{-1} in ten steps using 0.8 mg L^{-1} anti-testosterone per sample. In Fig. 4, the data set and the logistic fit function are shown. The obtained parameters are

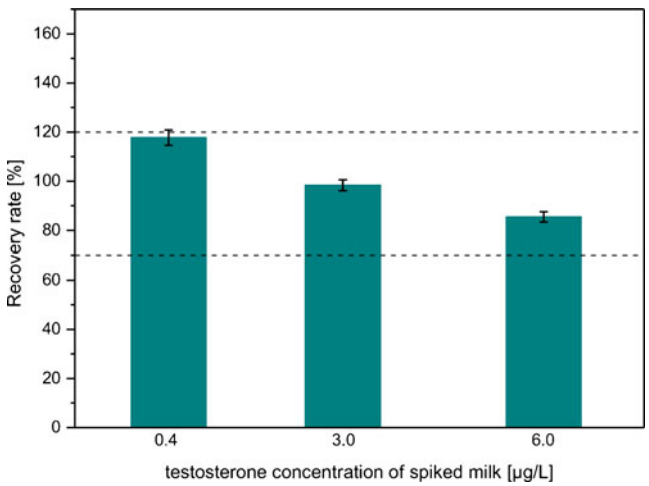

Fig. 5 Mean recovery rates with error bars (triplicate determination) for testosterone in milk (UHT, 3.5% fat) at three spiking levels. Recovery rates should lie between 70% and 120% (*dotted lines*)

summarized in Table 1. The slope of the calibration curve in milk is significantly lower than in buffer, resulting in a working range which is much wider. The reason for that cannot be enlightened completely within the scope of this work. In fact, antibodies behave partially differently in different matrices. The LOD of 94.4 ng L^{-1} and the LOQ of 229.3 ng L^{-1} are only slightly higher than in the simpler matrix buffer.

To ensure the quality of the measured calibration, recovery rates were determined. The whole pasteurized milk was spiked with three different levels (0.4, 3, and 6 μg L^{-1} of added testosterone). The results obtained from triplicate measurements were evaluated using the calibration plot and the corresponding logistic fit. As recommended by the AOAC International, all recoveries lie between 70% and 120%. Mean recovery values of 117.7±3.1%, 98.4±2.2%, and 85.5±2.1% were obtained for spiking with 0.4, 3, and 6 μg L^{-1}, respectively. Therefore, all spiking levels resulted in good recoveries with small errors for triplicate measurements. The results are shown in Fig. 5, where the upper and lower limits in the 70–120% range are shown as dashed lines.

Conclusions

The aim of this work was to find a practical solution to measure in the complex matrix milk with the label-free and time-resolved detection method RIfS. To investigate and eliminate matrix effects occurring in RIfS measurements, a model immunoassay was used. As model analyte, the hormone testosterone has been chosen because the corresponding assay allows for very reliable measurements in buffer.

Therefore, this immunoassay has been further improved in buffer regarding optimal surface properties and assay time. Compared with previously described results [28], improved detection and quantification limits (by a factor of 5) could be achieved. Calibration using a DAPEG–testosterone derivative-coated surface resulted in a LOD of 70.2 ng L^{-1} and a LOQ of 130.0 ng L^{-1}. Calibration using an AMD–testosterone derivative-coated surface resulted in a LOD of 75.2 ng L^{-1} and a LOQ of 142.1 ng L^{-1}. Consequently, by comparing these two different surfaces, we were also able to show that for this antigen–antibody system, the biopolymers used did not affect the sensitivity of the assay in buffer.

We used this improved assay to investigate the matrix effects of milk. The most reliable measurements could be achieved with a polymer layer consisting of 5% DAPEG and 95% MPEG. Also, an evaluation method was established by which matrix effects are eliminated as much as possible. Due to these optimizations, reproducible quantitative measurements without any sample pre-preparation could be obtained.

The sensor could be calibrated in milk with a LOD of 94.4 ng L^{-1} and a LOQ of 229.3 ng L^{-1}. These validation parameters are only slightly higher than in buffer. Furthermore, good recovery rates verified the high-precession of the calibration. These successful measurements in milk will lead to the establishment of new biosensor assays for different contaminants of milk like hormonally active substances, staphylococcal enterotoxins, or polychlorinated biphenyls by means of direct optical detection such as RIfS.

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