Analytical Methods

PAPER



Cite this: *Anal. Methods*, 2018, **10**, 5773

# Three steps improving the sensitivity of sulfonamide immunodetection in milk†

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Three approaches improving the sensitivity of previously established immunoassays for broad detection of sulfonamides (SAs) in milk are described. A novel bis-SA hapten, P2S (*N*,*N*′-1,4-phenylenebis(4-aminobenzenesulfonamide)), was synthesized for development of a heterologous format of a direct competitive ELISA. Three types of conjugates were compared as heterologous coating antigens. Among them, glutaraldehyde-linked Gel–P2S was found to be the one providing a 2–4-fold increase in assay sensitivity. The second step of improvements was based on adaptation of analysis for a chemiluminescent detection system. The developed chemiluminescent immunoassay (CLIA) allowed one order of reduction of the coating antigen concentration that improved the sensitivity of detection of a broad range of SAs in PBST by two-fold. Application of a matrix-imitation approach was effective for analyzing whole-milk samples without complex pretreatment or dilution. Two simulators, skimmed milk powder reagent and casein, were selected as equivalents of the matrix effect of raw milk. Using standard curves based on these simulators, SA detection in whole milk using the CLIA had 3–6-fold higher sensitivity in comparison with ELISA, which was exemplified with sulfamethoxazole (SMX, $IC_{50}$ = 0.15–0.3 *vs.* 0.91–0.98 ng $mL^{-1}$). The resultant sensitivity improvement of the developed CLIA permitted detectability of 19 SAs in milk at their maximum residue level (25 μg $kg^{-1}$) and below. The recovery of 0.1–2.0 ng $mL^{-1}$ of SMX in whole milk obtained using skimmed milk- and casein-based standard curves in ELISA was 86–108% and 102–133%, respectively; the analogic values for CLIA were 65–109% and 89–134%, respectively.

Received 28th August 2018
Accepted 6th November 2018

DOI: 10.1039/c8ay01904e

rsc.li/methods

## 1. Introduction

Sulfonamides (SAs) were one of the first synthetic anti-infective agents, and their antibacterial activity was discovered in 1934.[1] Since then, numerous representatives of this class of compounds have been developed and used actively in medical and veterinary fields to combat microbial infections and for other purposes.

At present, consumption of antibacterial SAs for food-producing animals prevails over its usage in human medicine by almost sevenfold (826.3 *vs.* 121.5 tonnes in the European Union (EU)).[2] The share of SAs for food-producing species among the other classes of antimicrobial drugs was 11.8% in 2015 according to sales data aggregated by 30 European countries.[3]

Besides antibacterial activity, sulfa drugs are known to possess diuretic, hypoglycemic, anti-obesity, anti-thyroid, and anti-neuropathic-pain activities.[4] Moreover, applications against cancer, glaucoma, dandruff and inflammation[5,6] make them a valuable and widespread class of compounds.

The downside of such popularity and mass consumption is the spreading of environmental pollution and the formation of natural resistance mechanisms that eventually result in drug inefficiency.[7,8] Investigations of water pollution with antibiotics conducted in different geographic regions have shown a high occurrence of SAs and the world-wide prevalence of this problem.[9–12] The limitations established for the residual concentrations of antibiotics, in particular SAs in foodstuffs, is one of the measures aimed at counteracting the expansion of microbial resistance. Maximum-residue levels (MRLs) for SAs are set in EU at 100 μg $kg^{-1}$ for all kinds of foodstuffs.[13] However, the contamination of milk with SA residues in several countries should not be more than 25- and 10-μg $kg^{-1}$ level.[14,15] The list of applicable SAs is not always specified and can include a great variety of representatives from this group of compounds. Hence, use of sensitive methods with group determination capable to reveal any type of SA residue in the sample is rational.

Recognition of structurally similar compounds may be realized using antibodies against generic hapten epitopes.[16–18] Furthermore, such methods of immunodetection are useful as effective screening tools for simultaneous testing of multiple samples. Identification of certain analytes is hampered because

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c8ay01904e

antibody cross-reactivity is not equal for all analytes and their detectability is determined mostly by the specificity and sensitivity of the test applied.

In our previous investigation, several formats of enzyme immunoassays were compared for the sensitivity of SA determination, and their resistance to the matrix effect examined.[19] The matrix effect of milk was shown to be difficult to eliminate using the dilution method. Eleven SAs could be detected at <100 μg $kg^{-1}$ (EU MRL) and very few analytes in milk could be revealed at 25 μg $kg^{-1}$ (Russian MRL).

In the present study, we investigated several mechanisms for improvement of enzyme-linked immunosorbent assay (ELISA) sensitivity and the resultant extension of several detectable SA representatives in milk. The first approach was based on modification of antibody–antigen interactions using the effect of a novel heterologous hapten. The second approach was directed on enhancement of a signal using a chemiluminescence detection system. The third point of sensitivity improvement was associated with matrix imitation and simplification of sample pretreatment.

# 2. Methods

## 2.1 Chemicals

Sulfanilamide (SAM), sulfacetamide (SAC), sulfaguanidine (SGN), asulam (ASU), sulfanylic acid (SAA), sulfisoxazole (SIZ), sulfamethoxazole (SMX), sulfaethidole (SET), sulfamethizole (SMT), sulfathiazole (STZ), phthalylsulfathiazole (PST), sulfanitran (SNT), sulfapyridine (SPY), sulfasalazine (SSZ), sulfachloropyridazine (SCP), sulfamethoxypyridazine (SMP), sulfadiazine (SDZ), sulfamerazine (SMR), sulfamethazine (SMZ), sulfadimethoxine (SDM), sulfamonomethoxine (SMM), sulfadoxine (SDX), sulfalene (SLE) and sulfaquinoxaline (SQX) were purchased from Chimmed (Moscow, Russia). The SA derivative 4-(4-(4-aminophenylsulfonamido)phenyl)butanoic acid (PB) and anti-PB monoclonal antibody 4D11 were kindly provided by Professor Zhanhui Wang.[20] Rabbit anti-mouse immunoglobulin G conjugated with horseradish peroxidase (RAM–HRP) was from Imtek (Moscow, Russia). Skimmed milk powder was from Fluka (Geneva, Switzerland). Bovine serum albumin (BSA) and HRP were from Sigma-Aldrich (Saint Louis, MO, USA). Bovine fetuin (Fet) and glutaraldehyde (GA) were from Serva (Heidelberg, Germany). Gelatin (Gel) was purchased from Bio-Rad Laboratories (Hercules, CA, USA).

Coating buffer was carbonate–bicarbonate buffer (CBB; 0.05 M; pH 9.5). Phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T) was used for washing and sample dilution. Two-component TMB substrate was from Bioservice (Moscow, Russia). The two-component luminol substrate Luminata™ Crescendo was from Millipore (Billerica, MA, USA). Other chemicals were of analytical grade.

## 2.2 Hapten synthesis

Hapten P2S (*N*,*N*′-1,4-phenylenebis(4-aminobenzenesulfonamide)) was synthesized according to the scheme shown in Fig. 1. Thin-layer chromatography (TLC) was run on Silufol plates and checked at 254 nm. All compounds were TLC-grade. Mass spectroscopy was done on a INCOS500 system. $^1$H-NMR spectra were recorded on Bruker system (DRX500) at 500 MHz. Liquid chromatography-mass spectroscopy (LC-MS) was done on a LC-MSD-Trap-SL system on a column (Reprosi-Pur Basic C18, 250 × 4.6 mm, 5 μm) with a gradient of 0.01% TFA (A) and acetonitrile 5–100% acetonitrile (B) for 20 min.

**4-*N*-Methyl-carbamoyl-sulfonamide-chloride (3).** 4-*N*-Methyl-carbamoyl-sulfonamide-chloride (**3**) was synthesized by a well-known procedure.[21]

**Diethyl [1,4-phenylenebis(iminosulfonyl-4,1-phenylene)] biscarbamate (4).** Compound (**3**) (1.03 g, 4.0 mmol) was added

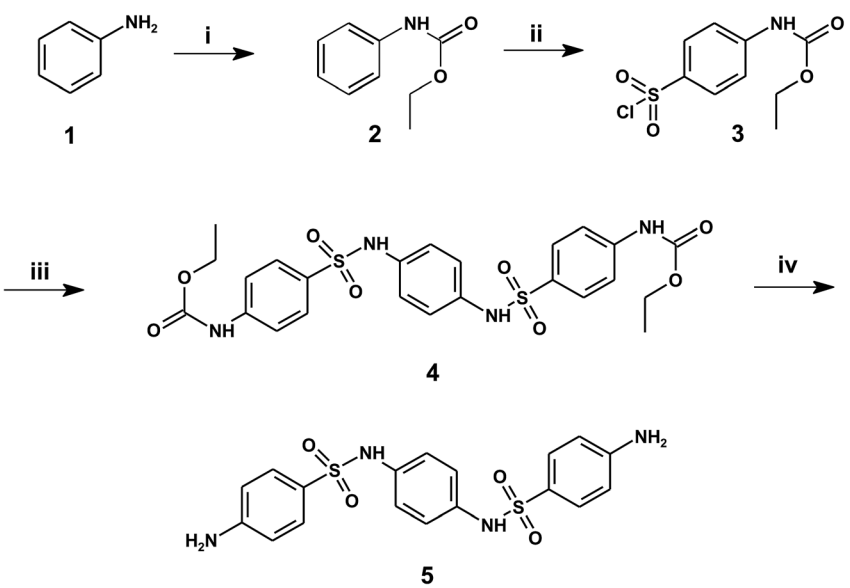



Fig. 1 Scheme of bis-hapten P2S synthesis. (i) – $C_3H_5ClO_2$, Py, 0 °C r.t. 6 h; (ii) – OH–$SO_2Cl$, 70 °C, 8 h; (iii) – *p*-amino-aniline, Py, 80 °C, 15 min; (iv) – NaOH, EtOH, reflux, 30 min.

portion-wise to a solution of 0.21 g (2.0 mmol) of *p*-aminoaniline in 10 mL of dry pyridine. This reaction mixture was stirred at 80 °C for 15 min and poured into an ice solution of dilute HCl. Precipitate (**4**) was filtered off, washed with cold water and dried. Yield 1.04 g (94.5%). Mass-spectra: *m*/*z* (%): 562 (20, $M^+$), 334 (60), 288 (20), 228 (100).

***N*,*N*′-1,4-Phenylenebis(4-aminobenzenesulfonamide) (5).** A mixture of 0.56 g of carbamate (**4**), 1.5 mL of ethanol and 3.5 mL of 40% NaOH was boiled for 30 min. The reaction mixture was cooled and acidified by concentrated HCl until the pH was ≤3.0. The precipitate was filtered off, washed with cold water, and crystallized from a mixture of ethanol : water (9 : 1). Crystals were separated and dried, with a melting point (mp) of 272–273 °C, which corresponded to that shown in ref. 22 (272 °C).

$^1$H-NMR (dmso-d6, 500 MHz, *J* Hz): 5.95 (4H, wide s); 6.49 (4H, d, *J* 8.5); 6.86 (4H, s); 7.24 (4H, d, *J* 8.5); 9.56 (2H, s); mass spectra: *m*/*z* (%): 418 (6, $M^+$), 263 (30), 156 (90). LCMS – RT 13.2, UV 220 – 96.57%, TIC – 100%, found $(M + H)^+$ 419.2, calculated for $C_{18}H_{18}N_4O_4S_2$ – 418.1.

### 2.3 Preparation of conjugated antigens based on P2S

**Fet(pi)–P2S, Gel(pi)–P2S.** Water (1.5 mL) solutions of glycoproteins containing 16 mg of bovine fetuin (320 nmol) and 24 mg of Gel (150 nmol) were oxidized using a 100-fold molar excess of sodium periodate from a 26 mg $mL^{-1}$ solution. The solutions were combined, stirred for 20 min and then dialyzed against water overnight. The next day, the periodate-oxidized glycoproteins were divided into equal-volume portions. Each portion was added to P2S solution in CBB and stirred for 2 h using a magnetic stirrer. Fet : P2S ratios were taken as 1 : 50 and 1 : 100, and Gel–P2S were 1 : 10, 1 : 30, and 1 : 100. To reduce the Schiff bases formed, sodium borohydride (0.1 mL from 2 mg $mL^{-1}$) was supplemented and maintained for 2 h with occasional stirring.

**BSA–P2S(edc); Gel–P2S(edc).** To activate the carboxylate residues in proteins, 30 mg of EDC powder was added to water solutions of BSA (16 mg in 1.0 mL) and Gel (24 mg in 1.5 mL) and mixed for 10 min. Then, P2S (10 mg $mL^{-1}$ solution in DMF) in 50- and 100-fold molar excess over BSA and 10-, 30-, and 100-fold molar excess over Gel was added and stirred for 3 h at room temperature.

**BSA–P2S(ga); Gel–P2S(ga).** The reaction mixtures were formulated keeping the molar proportions between BSA and P2S as 1 to 100. For Gel and P2S, they were taken as 1 to 10 and 1 to 30, respectively. Then, 4 μL or 40 μL of a freshly prepared 2.5% solution of glutaraldehyde was added to each mixture and stirred for 2 h. Similarly, as described above, a reduction amination was completed by adding sodium borohydride solution.

All of the resultant conjugates were purified from unreacted substances by exhausting dialysis against three changes of 5 L of water.

### 2.4 Procedures for direct competitive enzyme-linked immunosorbent assays with optical and chemiluminescent detection (DC-ELISA and CLIA)

Previously, an indirect competitive ELISA format (IC-ELISA) was developed. It was used for evaluation of new hapten-based coating antigens and optimization of parameters according a classical procedure.[19]

First, monoclonal antibody (mAb) binding was tested in checkerboard titration mode. Each protein–hapten conjugate from the panel was prepared at a range of concentrations in CBB and coated onto 96-well polystyrene plates (Costar; Corning, Corning, NY, USA) overnight at 4 °C. The plates were washed thrice with PBS-T. The interaction with mAbs serially diluted in 1% BSA–PBS-T was allowed to proceed for 1 h at 25 °C. After washing, the bound antibodies were detected using RAM–HRP in 1% BSA–PBS-T (1 h, 37 °C) and then with a TMB-containing substrate mixture. The enzymatic reaction was stopped after 30 min with 100 μL of 1 M sulfuric acid per well, and the absorbance was measured at 450 nm using a Stat Fax 2100 reader (Awareness Technologies, Westport, CT, USA). Several pairs of immunoreagents were used at concentrations that permitted an absorbance ($B_0$) level of ∼1.0; these were selected and analyzed in a competitive assay.

For this purpose, standard solutions of haptens in a series of concentrations or SA analytes were added at 100 μL per well and incubated together with the mAbs. The more prominent inhibition of immunobinding ($B/B_0$) caused by the corresponding immunizing hapten served as a criterion to evaluate assay optimization. The concentrations of half-maximal binding ($IC_{50}$) were used as comparative indices.

Similarly, a checkerboard titration method allowed optimization of the direct-assay formats, whereby mAbs were adsorbed onto polystyrene and bound to the selected haptens labeled with HRP.

### 2.5 Specificity examination

Numerous representatives of a SA family were prepared as individual standard solutions (0.01–10 000 ng $mL^{-1}$) and analyzed in each assay format. The inhibitory activity of each concentration of analyte was expressed as relative antibody binding ($B/B_0 \times 100$) and this dependence was presented in the form of sigmoid standard curves using OriginPro 8.0 (OriginLab, Northampton, MA, USA).

For assessment of the specificity of the developed assay formats, their cross-reactivity profiles were compared. The $IC_{50}$ values for each analyte were calculated from the standard curve, and cross-reactivity was expressed by the ratio $IC_{50}$ main analyte/$IC_{50}$ analog × 100%.

### 2.6 Preparation of milk samples and matrix imitators

Samples of raw milk were centrifuged at 4000 × *g* for 10 min. Then, an aliquot free of the upper lipid layer was supplemented with 20× PBST concentrate, stirred thoroughly and analyzed in CLIA. To find an adequate concentration of the imitator, solutions of skimmed milk reagent (SM, 5–20%) and casein (1–5%) with different concentrations were prepared in PBST. For this, SM powder or casein was weighed, dissolved with warm distilled water, and made up to the calculated volume. After complete solvation, 20× PBST was added, and stirred solutions were analyzed for conformity parallel with pretreated milk samples.

# 3. Results and discussion

## 3.1 Preparation of hapten and coating conjugates

Synthesized P2S (**5**) represented a symmetrical structure of two SA moieties joined by an aromatic linker. The design of the bis-sulfonamide hapten was chosen to provide reliable links with carriers exposing common SA molecule moiety on the protein surface.

A rational approach to synthetic procedures is shown in Fig. 1. Ethylcarbamate, as protection of aromatic amino groups, was chosen because it was stable in the harsh conditions of synthesis of sulfonyl chloride[21] and gave high yields for compounds **3** and **4**. The electron-impact mass spectrum of **5** showed a parent peak at 418 with low intensity (Fig. S1†). Peaks at 263 and 156 in this spectrum were fragments of bis-sulfonamide **5**. LC-MS revealed good purity of **5** (96.57%) and was in accordance with the main peak to the calculated monoisotopic mass of **5**. The $^1$H-NMR spectrum was in best agreement with a proposed structure of bis-sulfonamide **5** (Fig. S2†). Both doublets at 6.49 and 7.24 showed simple aromatic *para*-substitution. The intensity of the peaks at 5.95 and 9.56 were in agreement with the intensity of the signals from four protons of aromatic amino groups and two protons of SA groups.

We attempted several coupling methods for preparation of heterologous coating antigens using P2S. Glycoproteins, Fet and Gel were oxidized using sodium periodate to convert carbohydrate glycols to aldehydes. The latter were ready to interact with hapten amine groups in a reductive amination reaction. The other zero-length crosslinking was conducted using a water-soluble carbodiimide-mediated reaction, and provided hapten attachment in the location of available carboxyl groups on the protein. A third group of conjugates was prepared by coupling hapten amines with the amines of proteins using glutaraldehyde as a crosslinking agent. Spectral characteristics confirmed conjugate formation. They illustrated the growth of characteristic peaks when increasing hapten excess at synthesis (Fig. 2A and B). Coupling was more effective at a higher molar ratio of glutaraldehyde to hapten (1.0 *vs.* 0.1) (Fig. 2C).

## 3.2 Selection of coating conjugates

Prepared conjugates based on Fet, BSA and Gel bearing different hapten loads were examined as coating antigens, and the one which provided the highest sensitivity was selected from each group. Gel-conjugates were prepared using a lower hapten excess at synthesis, and demonstrated better assay parameters in comparison with those for Fet- and BSA-based conjugates with a higher hapten : carrier ratio. This observation corresponds with our previous experience.[23] Thus, several IC-ELISAs were conducted to reveal the advantages of P2S hapten, its preferential presentation on the protein carrier due to conjugation, and to determine preliminarily the specificity level (Table S1†).

A panel of SA representatives was taken at 10 and 0.1 μg mL$^{-1}$ and used as competitors in IC-ELISAs based on studied conjugates. The glutaraldehyde conjugate provided better sensitivity characteristics among Gel–P2S antigens as well as the previously established coating antigen partner Gel–PBx10.[19,24] Thus, Gel–P2S × 30(ga) was selected for subsequent detailed studies of specificity and sensitivity parameters.

## 3.3 Development of a chemiluminescent assay (CLIA)

Various molecular-recognition probes and affinity binders are often forced to utilize special materials and enhancement systems to meet the requirement of high-sensitivity detection. Aptamer labelling with graphene oxide quantum dots,[25] substrate changing for enzyme-binder DHPS and its labelling with fluorophores,[26] and application of modifiers for biosensor electrodes[27] are just some ways of improving assay performance for SA determination.

Here, to improve the sensitivity of detection, we used a chemiluminescent assay known for its high intensity of signal. The magnitude of a signal is measured in relative light units (RLU), and the signal can be donated to decrease reagent concentrations. The latter action usually improves the sensitivity of competitive interactions. The maximal intensity of light emission was registered 2–4 min after substrate addition. During the following 20 min, about 5–15% of RLU loss was

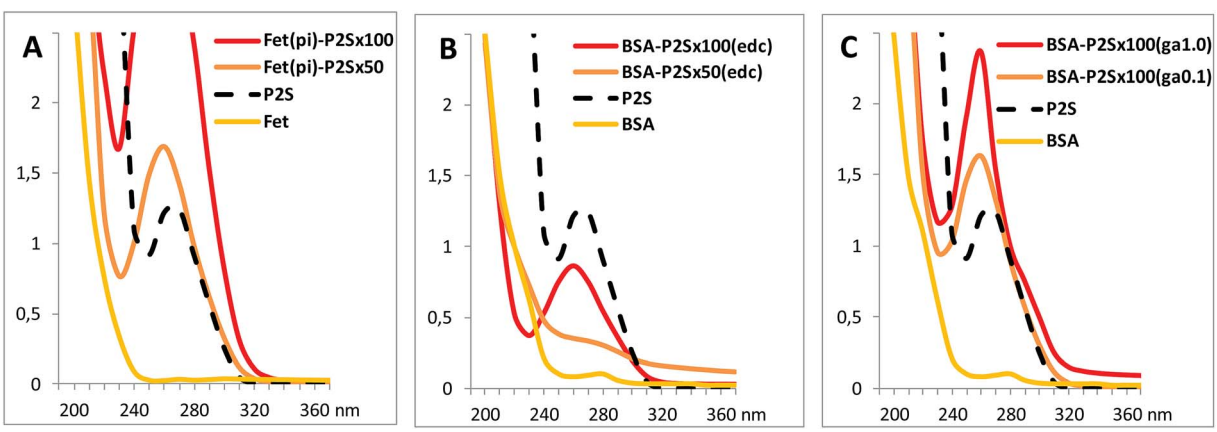



Fig. 2 UV-spectrograms of conjugates based on proteins (Fet and BSA) and P2S prepared at molar ratios of 1 : 50 and 1 : 100 using sodium periodate for glycoprotein oxidation (pi), glutaraldehyde (ga), and carbodiimide condensation (edc) methods.

observed. The higher the initial signal, the bigger the losses that occurred, whereas the lowest background signal remained stable (Fig. 3).

Thus, a high-intensity reaction appeared to be more unstable than a moderate-intensity signal. Detailed examination of the desired point of reading revealed that the signal value was also not stable at the first minutes of the reaction (Fig. 4). Although, the inhibitory activity ($IC_{50}$) of SMX was greatest at the zero time-point and was significantly better in milk solutions, the high variation (13–23%) in repeated readings suggested the poor stability of the signal. However, the chemiluminescent signal stabilized up to 3 min for all the fluids we tested, and the variation was ≤5% and maintained at this level longer. These data indicated that the most reliable CL signal would be registered 3 min after substrate addition.

### 3.4 Comparison of ELISA formats

The effect of heterologous hapten and enhanced chemiluminescent signal on assay sensitivity was demonstrated using a panel of 24 SAs, and could be compared with the $IC_{50}$ values obtained in the corresponding optical ELISAs. Table 1 demonstrates gradual improvement of assay sensitivity ($IC_{50}$) from indirect to direct homologous format,[19,24] from a homologous format to a heterologous format using a novel bis-hapten, and the effect of chemiluminescent detection. Owing to these changes in immunochemical interaction and detection signal, the overall effect of increasing sensitivity was about tenfold.

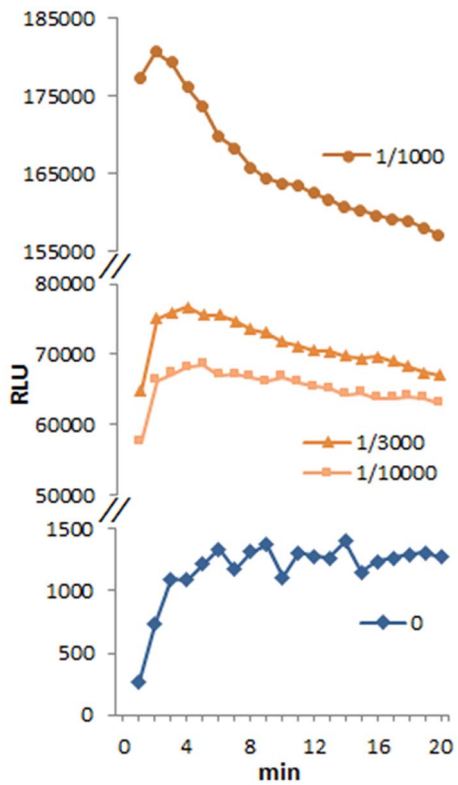



Fig. 3 Kinetic measurement of light signals from interactions between coating antigen Gel–P2S (1.0 μg $mL^{-1}$) and mAb–HRP conjugate 1/1000–1/10 000 and 0 as a background.

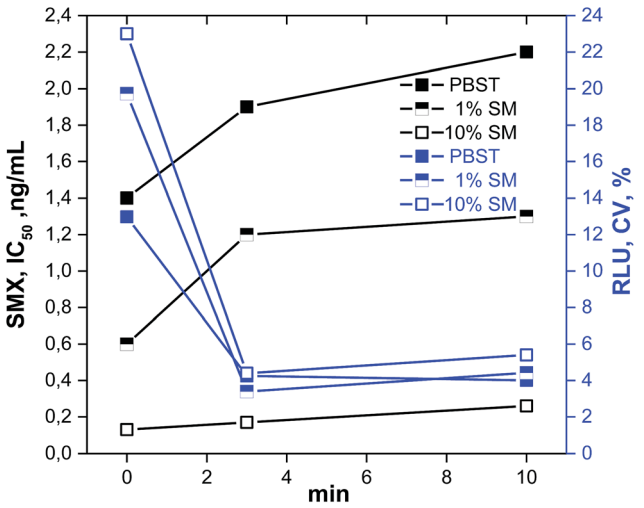



Fig. 4 Time-dependent changes in assay sensitivity and stability of chemiluminescent light signal. CLIA determination of SMX in PBST and the same buffer containing 1% or 10% skimmed milk powder (SM). Coefficient of variation (CV) was calculated from repeated $B_0$ – well readings ($n$ = 5, 0.2 s per well).

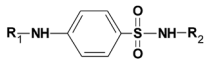
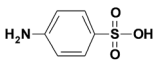
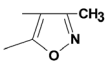
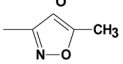
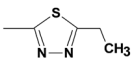
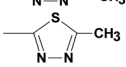
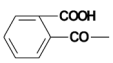
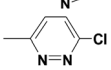
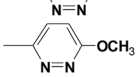
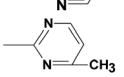
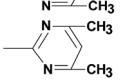
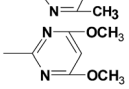
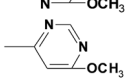
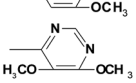
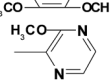
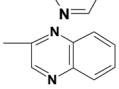
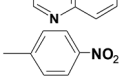
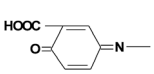
Some anti-SA immunochemical and related methods involving chemiluminescent detection system were also reported, they demonstrated different sensitivity ranges. A chemiluminescent multianalyte chip immunoassay could detect SDZ and SMZ residues in honey with $IC_{50}$ values of 420.5 and 213.5 (limit of detection (LOD) of 192.6 and 81.5 ng $mL^{-1}$).[28] An aptamer-based chemiluminescent assay of SMZ has been described[29] to have a linear range of 1.85–21.57 and LOD of 0.92 ng $mL^{-1}$.

The group-specific SA assay developed here demonstrated more sensitive values for SMZ with an $IC_{50}$ of 1.8 ng $mL^{-1}$ and a linear range of 0.8–10.5 ng $mL^{-1}$. Nevertheless, greater sensitivity for this analyte was realized using additional enhancement systems. Chemiluminescent resonance energy transfer (CRET) by employing hapten-functionalized quantum dots in a competitive immunoassay allowed detection of SMZ up to 9 pg $mL^{-1}$ ($IC_{50}$ = 0.2 ng $mL^{-1}$).[30] Another improvement was associated with use of an alternative enzyme (anionic soybean peroxidase) and a biotin-streptavidin detection system. Specific determination of SMP in this CL-ELISA[31] was established with a sensitivity ($IC_{50}$) of 0.17 ng $mL^{-1}$ *vs.* 0.27 ng $mL^{-1}$ for SMP in the present study. Scholars have reported that sulfamethoxydiazine (unavailable at present work) could be analyzed with high sensitivity using CLIA at a working range of 0.01–2 ng $mL^{-1}$.[32] However, most of those studies were devoted to determination of individual SA analytes, unlike the group determination discussed here. The spectrum of detectable analytes enlarged due to sensitivity upgrade from 11 to 15 SAs for 100 ng $mL^{-1}$ level, and from 6 to 11 SAs for <10 ng $mL^{-1}$ level. Such benefit was comparable with the effect achieved in hybrid two antibody-based immunoassay of SAs.[24]

### 3.5 Estimation of the matrix effect

Milk is a popular beverage a frequent object of safety control and analytical-system testing. Despite its liquid state (which is

Table 1 Comparative sensitivity ($IC_{50}$, ng $mL^{-1}$) of homologous and heterologous coating hapten-based ELISAs and CLIA of 24 SAs in PBST

| Sulfonamides | | | Type of immunoassay [reference] | | | |
|---|---|---|---|---|---|---|
|  | | | IC-ELISA [19] | DC-ELISA [19 and 24] | DC-ELISA [present work] | CLIA [present work] |
| | | | Coating antigen | | | |
| Name | $R_1$ | $R_2$ | Gel–PB(ae) | Gel–PB(ae) | Gel–P2S(ga) | Gel–P2S(ga) |
| SAM | H |  | 4950 | 3111 | 1111 | 623 |
| SGN | H |  | 3525 | 1570 | 376 | 233 |
| SAC | H |  | >10 000 | 7009 | 1815 | 790 |
| ASU | H |  | 105.7 | 125 | 37 | 52.9 |
| SAA |  | | >10 000 | >10 000 | >10 000 | >10 000 |
| SIZ | H |  | 2800 | 1130 | 328 | 153 |
| SMX | H |  | 11.8 | 4.2 | 2.6 | 1.7 |
| SET | H |  | 1280 | 454 | 213 | 115 |
| SMT | H |  | >10 000 | >10 000 | 2214 | 1076 |
| STZ | H |  | 1280 | 454 | 162 | 61 |
| PST |  |  | >10 000 | >10 000 | 3305 | 1980 |
| SCP | H |  | 42.6 | 16.4 | 8.5 | 6.2 |
| SMP | H |  | 4.4 | 1.2 | 0.47 | 0.27 |
| SDZ | H |  | 70.2 | 25.3 | 11.4 | 7.7 |
| SMR | H |  | 24.1 | 17.8 | 4.5 | 6.2 |
| SMZ | H |  | 15.2 | 6.9 | 2.9 | 1.8 |
| SDM | H |  | 1.0 | 0.4 | 0.2 | 0.2 |
| SMM | H |  | 6.8 | 3.4 | 1.4 | 0.9 |
| SDX | H |  | 1090 | 390 | 138 | 77 |
| SLE | H |  | 944 | 454 | 138 | 66 |
| SQX | H |  | 6.5 | 2.1 | 1.3 | 0.73 |
| SNT |  |  | 0.6 | 0.3 | 0.13 | 0.14 |
| SPY | H |  | 5.5 | 2.0 | 0.59 | 0.33 |
| SSZ |  |  | 8016 | 3536 | 1046 | 640 |
| **ng $mL^{-1}$** | | | **Number of detectable sulfonamides with $IC_{50}$ level** | | | |
| **<100** | | | **11** | **11** | **12** | **15** |
| **<10** | | | **6** | **8** | **10** | **11** |

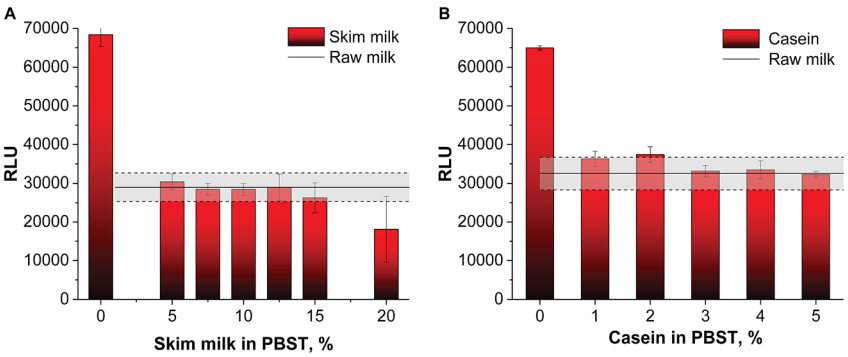


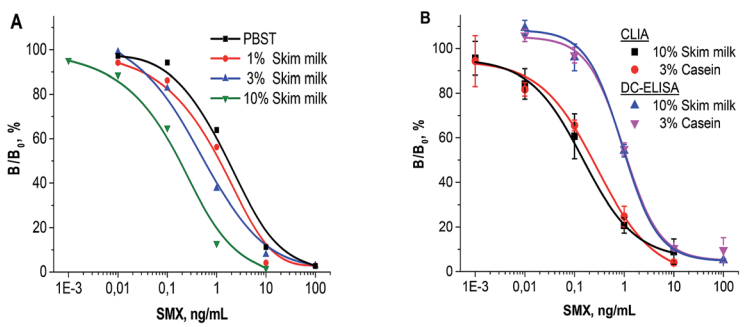

Fig. 5 Correspondence between undiluted raw milk and milk imitators, skimmed milk (A) and casein (B). Each column and line indicate average values ($n = 4$), error bars and dashed line represent the standard deviation (SD), and grey zone is the range ± SD.

convenient due to the target analytes being in a soluble form), the complex composition of milk influences immunobinding and can elicit misleading results. To avoid the matrix effect of milk, a laborious multistep pretreatment procedure is often needed.[33]

The effect of the matrix was retained by milk samples after deproteinization using citric acid and pH neutralization of whey as well as after dialysis (data not shown). A high extent of dilution is effective sometimes but, because of the resultant reduction in sensitivity, it is applicable only for high-sensitivity analytical systems.[34,35]

An approximate and effective approach to neutralize the matrix effect of milk is to imitate it using SM. Previously, such imitation was suitable for group detection of SAs and selective determination of sulfadimidine in ten- and three-fold diluted milk, respectively.[24,36] Here, we first examined SM and casein as matrix-effect imitators of undiluted "crude" milk. The 10% SM solution corresponded to raw milk as declared by the reagent manufacturer, and the influence of 7.5–12.5% SM on immunobinding was shown to be equal to the matrix effect of undiluted milk (Fig. 5A).

Casein is a major milk protein (~3%). Hence, assay buffer supplemented with casein about this level was compared for the extent of the matrix effect with a raw-milk sample. As shown in Fig. 5B, 3% content of casein in the assay buffer resulted in an almost two-fold decrease in the output signal in comparison with PBST, and this influence was most similar to that of undiluted milk. Thus, 10% SM and 3% casein in the assay buffer were selected for matrix imitation in the following study. We generated standard curves of SMX as representative of SAs in these solutions for comparison. The gradual increase in SM content in the assay buffer from 0% to 10% resulted a fall in the signal value and left-shifting of the standard curves (Fig. 6A). The standard curves generated in matrix imitators were almost identical to those of optical and chemiluminescent ELISA systems (Fig. 6B). Thus, the 10% SM and 3% casein solutions as imitators of raw milk provided the conditions for higher assay sensitivity and no loss of sensitivity due to the lack of sample dilution.




| Index | CLIA (skimmed milk) | CLIA (casein) | DC-ELISA (skimmed milk) | DC-ELISA (casein) |
|---|---|---|---|---|
| IC20 | 0,027 | 0,047 | 0,291 | 0,307 |
| IC50 | 0,147 | 0,285 | 0,913 | 0,978 |
| IC80 | 0,798 | 1,740 | 2,866 | 3,118 |

Fig. 6 Influence of the matrix and detection system on SMX determination. Standard curves of SMX generated in PBST with 0–10% content of skimmed milk reagent using an assay with chemiluminescent detection (A). Standard curves and $IC_{20}$, $IC_{50}$, and $IC_{80}$ values (ng $mL^{-1}$) in matrix imitators for SMX determination in milk using DC-ELISA and CLIA (B). Each symbol indicates the average ($n = 4$) and the error bars represent the standard deviation.

### 3.6 Experiments on recovery and detectability

To check the adequacy of SA detection in whole milk using matrix imitator-generated standard curves (Fig. 6B), the experiments "spiked and found" were conducted in developed optical and chemiluminescent ELISAs (Table 2).

Taking in account the differences in sensitivities and measurement range for these assays, samples of raw milk were fortified with several concentrations of SMX, complemented with PBST using 20-fold concentrate, and analyzed. The recovery of low concentrations of SMX in whole milk obtained using SM- and casein-based standard curves in DC-ELISA was 86–108% and 102–133%, respectively. These and analogic values from CLIA (65–109% and 89–134%) demonstrated the acceptable recovery rate and suitability of both matrix imitators for SA measurement in whole-milk samples. However, attention is drawn to significant values of the coefficient of variation when a chemiluminescent detection system was employed. Being more sensitive, CLIA was more susceptible to the influence of external factors on signal integrity. In this regard, measurement of the analyte in whole milk or its simulators was less precise than that of ELISA. Nevertheless, the acceptable reproducibility level (CV, %) of the assay calculated by the Horwitz equation according to the performance criteria and requirements for analytical methods established gives values >64% for concentrations <1 μg $kg^{-1}$.[37] Such high variability is not recommended as acceptable, but the required level has not been legislated.

The possibility to test a sample without dilution saved the initial dynamic range of assay from having to be recalculated due to sample pretreatment, and allowed several weak cross-reactive analytes to be revealed. Hence, the analytes taken at their MRL (25 μg $kg^{-1}$) in whole milk could be discovered using the developed DC-ELISA and CLIA if they exposed the binding level below the cut-off ($B_0 - 3 \times SD$). These levels were different for optical and chemiluminescent assays due to the extent of deviation from the average signal. The data from Fig. 7 showed that 19 anti-bacterial agents, SAC, ASU, SIZ, SMX, SET, STZ, SCP, SMP, SDZ, SMR, SMZ, SDM, SMM, SDX, SLE, SQX, SNT, SPY and SSZ, were detectable by CLIA. Twelve SAs could be revealed in whole milk using ELISA. For analytes exposing the competitive activity within the dynamic range of an assay ($IC_{20}$–$IC_{80}$, grey-marked zone) the residue level about MRL could be quantified using the corresponding standard curves if administration of veterinary drugs is known. Eight analytes (SAC, ASU, SIZ, SET, STZ, SDX, SLE and SSZ) satisfied these conditions and could be measured using CLIA, and only four (ASU, SMP, SDX and SLE) using ELISA. The sensitivity of determination for the remaining SAs was high and allowed the residues below the MRL to be revealed. Thus, application of a chemiluminescent substrate provided more sensitive detection of SAs in comparison with optical signal assays, and the spectrum of detectable analytes was broadened to 19 *vs.* 16 for DC-ELISA.

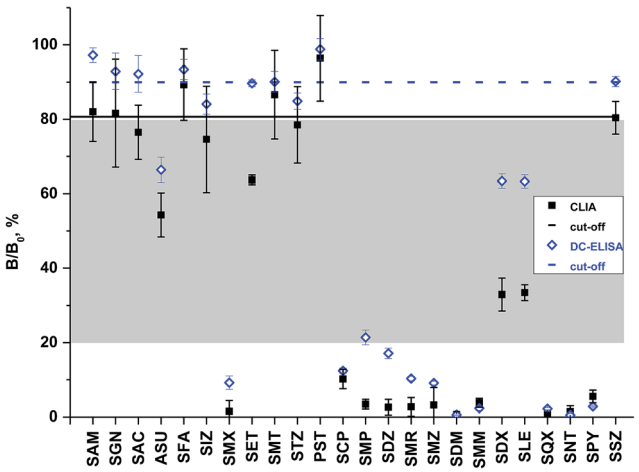



Fig. 7 Detectability of sulfonamides in raw-milk samples in CLIA (black-filled square) and DC-ELISA (blue empty diamond). Solid and dashed lines correspond to the cut-off level for CLIA and ELISA in 10% SM, respectively. Each symbol indicates the average value ($n = 3$), the error bars represent the standard deviation. The grey zone covers the dynamic range of the assay ($IC_{20}$–$IC_{80}$).

Table 2 Recovery of SMX from milk samples in optical and chemiluminescent ELISAs using matrix imitator-based standard curves

| Level of SMX fortification, ng $mL^{-1}$ | Standard curve based on milk matrix imitators | | | |
|---|---|---|---|---|
| | 10% skimmed milk | | 3% casein | |
| | RC[a], % | CV, % | RC[a], % | CV, % |
| **DC-ELISA** | | | | |
| 2 | 98 | 12.5 | 115 | 2.1 |
| 1 | 108 | 0.8 | 102 | 5.5 |
| 0.5 | 86 | 16.4 | 133 | 10.3 |
| **CLIA** | | | | |
| 1 | 109 | 40 | 106 | 25.8 |
| 0.5 | 98 | 34 | 134 | 19.2 |
| 0.2 | 65 | 25 | 110 | 46 |
| 0.1 | 67 | 20 | 89 | 43 |

[a] RC – recovery; CV – coefficient of variation ($n = 3$).

## 4. Conclusions

Three approaches appeared to be useful for improving the sensitivity and detectability of SAs in milk using a previously established immunoassay based on a direct competitive ELISA. A novel bis-SA hapten, P2S, was synthesized, and heterologous coating antigen based on a Gel–P2S conjugate provided 2–4-fold better sensitivity for SA analyses. The additional two-fold improvement in sensitivity was achieved for a broad range of SAs in PBST by a chemiluminescent-enhanced signal. Moreover, a double effect was gained from matrix imitation. This approach allowed avoidance of complex pretreatment of milk and sample dilution. Two simulators, SM and casein, were selected as equivalents of the matrix effect of raw milk. Using simulator-based standard curves, the assay of SAs in whole raw milk, as exemplified by SMX determination, was shown to be 3–6-fold more sensitive than in PBST. Thus, the resultant sensitivity improvement of the developed CLIA permitted detection of 19 SAs in milk at their MRL and below.

## Conflicts of interest

There are no conflicts to declare.

## Abbreviations used

| | |
|---|---|
| Ae | Active ethers method |
| BSA | Bovine serum albumin |
| CBB | Carbonate–bicarbonate buffer |
| DMF | Dimethylformamide |
| CR | Cross-reactivity |
| CV | Coefficient of variation |
| DMSO | Dimethylsulfoxide |
| EDC/edc | 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide/ carbodiimide condensation |
| DC/IC ELISA | Direct/indirect competitive enzyme-linked immunosorbent assay |
| Ga | Glutaraldehyde crosslinking |
| Gel | Gelatin |
| LOD | Limit of detection |
| MRL | Maximum residue limit |
| NHS | *N*-Hydroxysuccinimide |
| NMR | Nuclear magnet resonance |
| PBS | Phosphate-buffered saline |
| TMB | 3,3′,5,5′-Tetramethylbenzydine |

## Acknowledgements

This work was supported by the Ministry of Education and Science of the Russian Federation (RFMEFI60417X0198).

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