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Group-specific detection of 2-deoxystreptamine aminoglycosides in honey based on antibodies against ribostamycin†

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Aminoglycoside antibiotic ribostamycin (RS) was used as the immunizing hapten to evoke antibodies against the 2-deoxystreptamine (2-DOS) fragment for the first time. This moiety of the molecule is a generic structure combining a large number of aminoglycosides. Periodate-oxidized RS was conjugated to the protein carrier with a definite orientation with exposure of the 2-DOS determinant. Two immunogens BSA-RS and BSA-C6-RS were compared. The introduction of a C6 spacer arm between the hapten and the carrier resulted in raising antibodies with a better group specificity. The antibody specificity to the 2-DOS moiety allowed recognition of a wide spectrum of AGs, namely, RS, neomycin, neamin, paromomycin, gentamicin, sisomicin, kanamycin, tobramycin, and apramycin. The developed group-specific indirect competitive ELISA was capable of substituting a number of corresponding selective tests for detection of known analytes (with a LOD of 0.02–0.2 ng mL^{-1}). For analysis of honey, a matrix imitator was developed to avoid honey interferences on immunoassay. The screening procedure including simple dilution of the honey sample and its analysis allowed us to reveal any of the mentioned analytes in honey at a 10 μg kg^{-1} level with a recovery rate of 78–120%.

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1. Introduction

Aminoglycosides (AGs) are a large group of natural antibiotics, produced by *Streptomyces* and some species of bacteria from the genus *Bacillus*, and semi-synthetic ones with a wide spectrum of antimicrobial activity against most Gram-positive and Gram-negative microorganisms. The chemical structures of AGs represent aminocyclitols connected by glycoside bonds. A significant number of AG representatives are derivatives of 2-deoxistreptamine (2-DOS), the B fragment (Fig. 1).

There are several subgroups, including: (1) neomycin B (NM), paromomycin (PM), neamin (NA), and ribostamycin (RS); (2) kanamycin (KM), tobramycin (TM), and amikacin (AM); (3) gentamicin (GM), sisomicin (SSM) and netilmicin (NTM); and (4) a separate representative apramycin (AP) can be distinguished.

Currently, representatives of different aminoglycoside families—GM, NM and PM, KM, AP, and streptomycin (STM)—are approved to treat infectious diseases in animal husbandry. Maximum residue limits (MRLs) for these AGs in products and tissues from edible animals (cows, pigs, rabbits, poultry and aquatic species) are established;[1] however, there are still no common limitations set for antibiotics in honey,[2] except in some EC countries, *i.e.* for AG, STM (10–40 μg kg^{-1}).[3] STM is the most commonly used AG in beekeeping and frequently detected in honey, but several studies reported also about gentamicin[4,5] and kanamycin[6] contamination of honey. Moreover, the simultaneous determination of six to eleven AGs in honey using a special preliminary extraction procedure followed by liquid chromatography-tandem mass spectrometry was reported.[7,8] Thus, the desire to control the residues of most AGs makes a group-specific screening assay with inexpensive and simple sample preparation especially relevant.

Immunochemical methods are widely recognized as an alternative to insufficiently specific and time-consuming microbiological tests and to laborious and expensive chromatographic methods. However, the specificity of previously developed immunoassays was as a rule selective, with cross-reactions only towards the closest analogues belonging to one subgroup, for example, gentamicin–sisomicin–netilmicin;[9–11] amikacin–kanamycin–tobramycin–dibekacin.[12–16] In this regard, the control of AG residues in foodstuffs requires five/six separate immunodetection systems of appropriate specificity or multiplex assay based on five to six antibodies against these analytes.[17,18] A few attempts have been made to develop immunochemical methods capable of group

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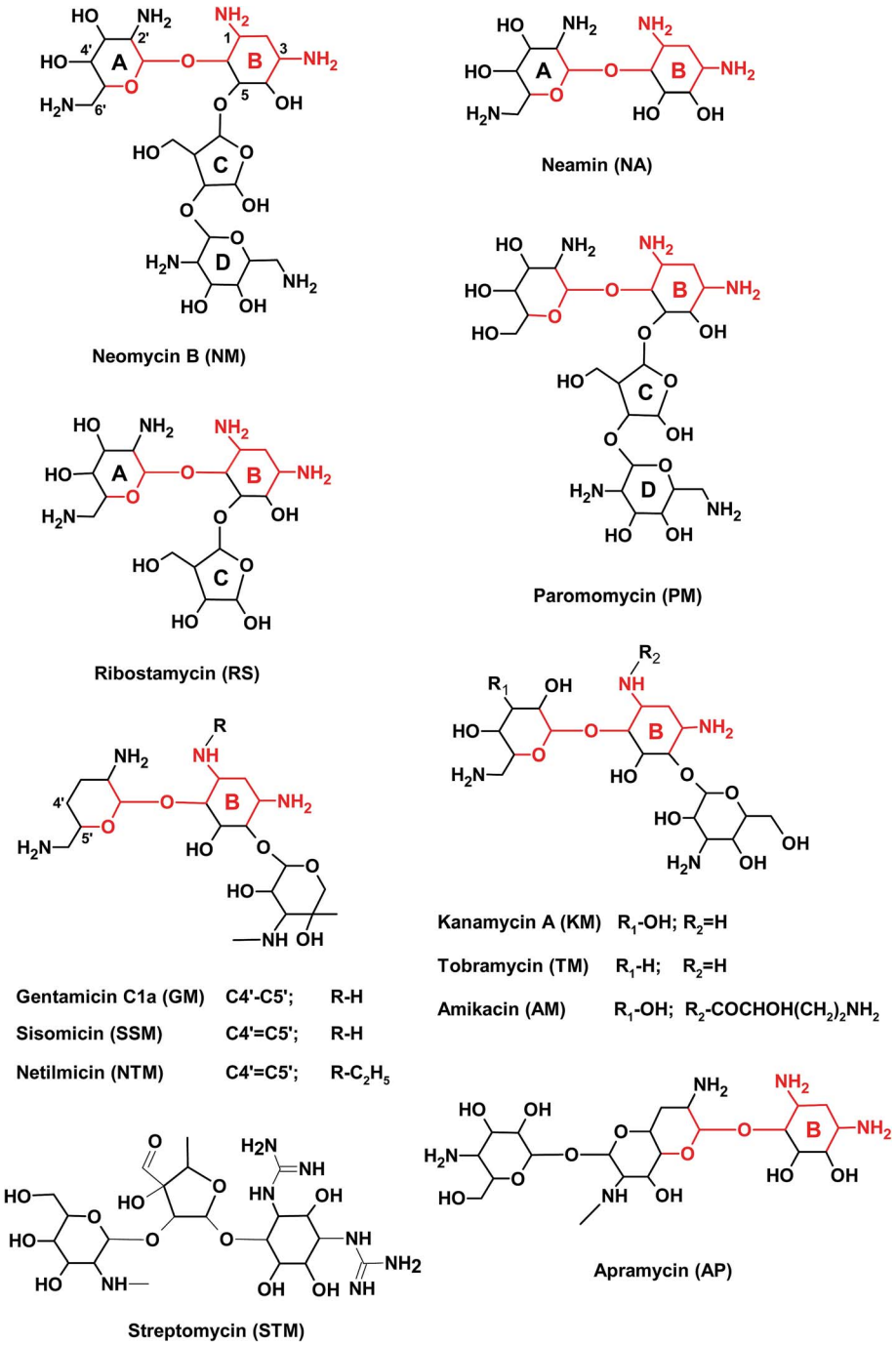


Fig. 1 Structural formulas of 2-DOS (B ring) family aminoglycosides and streptomycin. The constant region of the molecules is highlighted with red.

determination of AGs unlike the other big families of antibiotics.[19–22]

An immunoassay for determination of three AGs in milk was developed by van Amerongen's group, which used NA as a generic hapten for production of antibodies recognizing GM, KM and NM.[23] A carbodiimide coupling method, which was used for preparation of immunogens, provided only random conjugation between protein carboxyls and available amines of NA at C1, C3, C′2 and C′6 positions (Fig. 1). Coupling through C1 and C3 sites resulted in the blockage of the target 2-DOS determinant by the carrier and its immune unreactivity.

Enzyme-linked immunosorbent assay (ELISA) of AG representatives, having OH-substitution in the C′6 position (Fig. 1), was described by Shalev M. *et al.*[24] To create the immunogen, a neamin analogue, NB-82, having –OH and $-CH_3$ at C′6, was chosen. Its conjugation with a protein carrier was carried out site-specifically through the amino group at C3 in the B-cycle while the other amines were protected. As a result, the

antibodies generated against such an immunogen recognized mainly the A-ring (C′6 = OH), while the B-fragment was obscured by the carrier. The developed assay had another practical application, and target AGs differed from those considered in the present work. So, the sensitivity of the reported ELISA for NM, PM, KM and GM (IC_{50} = 100–1000 μg mL^{-1}) was several orders of magnitude higher than the MRLs of these antibiotics in food.[1]

In the present study, a new immunizing hapten ribostamycin was employed to design an immunogen with a definite hapten orientation exposing the target 2-DOS epitope. The antibodies generated against this generic moiety of aminoglycosides were the basis of group-specific screening assay for the detection of residual aminoglycosides in honey.

2. Methods

2.1 Chemicals

Sulphates of neomycin B (NM), ribostamycin (RS), neamin (NA), paromomycin (PM), kanamycin (KM), tobramycin (TM), amikacin (AM), gentamicin (GM), netilmicin (NTM), sisomicin (SSM), geneticin (GC), apramycin (AP), and streptomycin (STM) were purchased from Chimmed (Moscow, Russia). Bovine serum albumin (BSA), human transferrin (TF), complete Freund adjuvant, 1,6-hexanediamine, dimethylformamide (DMF), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (edc), 1,1-carbonyldiimidazole (cdi), sodium periodate (pi), and sodium borohydride were the products of Sigma-Aldrich (Saint Louis, MO, USA). Gelatin (Gel) was from Bio-Rad (Hercules, CA, USA), sucrose from Serva (Germany), two-component tetramethylbenzidine substrate solution was from Bioservice (Moscow, Russia), and goat anti-rabbit IgG antibodies conjugated to horseradish peroxidase were from IMTEK (Moscow, Russia). Honey samples were purchased from local outlets.

2.2 Synthesis of immunogens and coating antigens

2.2.1 BSA-RS(pi), Gel-RS(pi), and Gel-TM(pi). A solution of RS (4.2 mg, 9.25 μmol) in 0.5 mL H_2O was supplemented with sodium periodate (4.1 mg, 19 μmol) and stirred for 15 minutes at room temperature. Gel (4 mg, 25 nmol) in 1 mL 0.05 M carbonate buffer (CB, pH 9.6) was supplemented with oxidized RS (0.75, 2.5 μmol) to compose mixtures with 1/30 and 1/100 ratios. The mixture of BSA (4 mg, 60 nmol) and RS (6 μmol) was prepared at a 1/100 molar ratio. After 1 h-stirring at room temperature, 100 μL of sodium borohydride (2 mg mL^{-1}) was added to each mixture for reducing Schiff bases to amides. One hour later the conjugates were dialyzed against 5 L PBS using dialysis membrane tubes (MWCO 14 kDa) to remove the excess of low molecular weight reagents. An analogical coupling procedure was conducted for the preparation of Gel-TM(pi) conjugates. The molar ratio between Gel and TM was taken as 1/200.

2.2.2 BSA-C6-RS(pi). BSA (10 mg, 150 nmol) and 30 mg EDC in 2 mL of water was stirred for 30 min. Then, 1,6-hexanediamine (1 mg mL^{-1}) was added and stirred for 2 h. The modified protein was dialyzed from the excess of 1,6-hexanediamine against 5 L of CB. The resultant dialysate divided into three equal portions was dropwise supplemented with 10-, 50- and 250-fold excess of periodate-oxidized RS. RS was oxidized with an equimolar quantity of sodium periodate for 20 min. The reaction mixtures were stirred for 2 h at room temperature and additionally for 2 h after addition of sodium borohydride. The prepared conjugates were dialyzed exhaustively against 2 changes of 5 L PBS for two days.

2.2.3 TF(pi)-NM. A water solution of TF containing 20 mg (2 × 130 nmol) in 3 mL was supplemented with sodium periodate crystals (17 mg) and vigorously stirred using a magnetic stirrer for 15 minutes. Oxidized glycoprotein was dialyzed against 5 L of 10 mM acetic buffer (pH 5.0) overnight at 4 °C. The volume of dialysate was divided into two equal portions which were combined with 1 mL solutions of NM in CB and stirred for 2 h. The quantities of NM, 3.6 and 11.8 mg, were taken as 30- and 100-fold molar excess over TF. Then, 100 μL of sodium borohydride (2 mg mL^{-1}) was added to each mixture and stirred for another 2 h. The resultant conjugates were dialyzed against PBS.

2.2.4 Gel-GM(cdi). GM powder (1.12 mg, 2.5 μmol) was added to CDI (405 μg, 2.5 μmol) in 0.5 mL DMF and stirred using a magnetic stirrer for 5 h at room temperature. Activated GM (2.5 μmol) was dropwise added to Gel (4 mg, 25 nmol) in 1.0 mL of CB. The mixture was incubated with stirring overnight at room temperature and then dialyzed against water.

All dialysates were supplemented with glycerol and stored as 1 mg mL^{-1} solutions at −15 °C. BSA-based conjugates were used for immunization, and Gel-based conjugates served as coating antigens.

2.3 Immunization and preparation of polyclonal antibodies

Chinchilla rabbits (2.0–2.5 kg) were obtained from the Scientific and Production Centre for Biomedical Technologies (Elektrogorsk, Russia). All experiments with the rabbits were performed in accordance with the guidelines for the care and use of laboratory animals in biomedical research[25] and approved by the Ethics Committee of the I. Mechnikov Research Institute for Vaccines and Sera.

Animals were subcutaneously injected at 10–15 points on the back with 100 μg of BSA-RS(pi) or BSA-C6-RS(pi) emulsified in the complete Freund adjuvant. The booster immunizations were conducted monthly with the same doses of immunogens in saline. A week after each booster immunization a blood sample from ear veins was taken for the control of immune response. The serum was supplemented with glycerol 1 : 1 (v/v) and stored at −15 °C.

2.4 The study of the immune response and optimization of ELISA

The maturing of the immune response in animals was assessed by indirect ELISA by the interaction of antisera with antigens coated on 96-well polystyrene Costar plates. The wells of the plate were filled with 0.1 mL of the coating conjugate in the concentration range of 0.01 to 1 μg mL^{-1} in CB and incubated for 16 h at 4 °C. The plates were washed 4–5 times with phosphate-buffered saline containing 0.05% Tween 20 (PBS-T). 0.1 mL

of antiserum serially diluted in PBS-T with 1% BSA and 0.1 mL PBS-T (without a competitor, B_0) was added to the plates and incubated for 1 h at 25 °C. After washing, the wells were filled with 0.1 mL of goat anti-rabbit IgG antibody conjugated to horseradish peroxidase and incubated for 1 h at 37 °C. After washing, 0.1 mL of substrate solution containing TMB was added and 30 minutes later the enzymatic reaction was stopped by adding 0.1 mL of 0.5 M sulfuric acid. The absorbance was read at 450 nm using a Stat Fax 2100 plate reader (Awareness Technologies, USA).

The ratios of immunoreagents obtained as a result of such a checkerboard titration which provided an optical density of the reaction in the range of 0.8–1.2 were assessed in a competitive analysis.

2.5 Competitive ELISA and examination of the recognition spectrum

The conduction of competitive assay was the same as above. The competitive step included the addition of 0.1 mL standard AG solutions (1 pg mL^{-1} to 1μg mL^{-1}, B) in PBS-T instead of 0.1 mL PBS-T (B_0). The dependence of relative antibody binding ($B/B_0 \times 100$) on the analyte concentration was plotted as standard curves fitted to a four-parameter logistic equation using Origin 8.0 software (OriginLab, Northampton, MA, USA).

$$Y = \{(A - D)/[1 + (X/C)^B]\} + D$$

where, A is the maximum absorbance at zero analyte concentration; B is the curve slope at the inflection point; C is the concentration of the analyte resulting in 50% inhibition (IC_{50}) and D is the minimum (background) absorbance.

The combinations of coating antigen and antiserum samples with an absorbance level of about 1.0 which provide the most sensitive determination (the least value of IC_{50NM}) were selected for examination of specificity. The cross-reactivity (CR) for every AG representative was calculated as the percentage IC_{50NM}/IC_{50AG}. To estimate the recognition spectrum, a coefficient of group recognition (GRC) was introduced. It was calculated as a ratio between the most active and the poorest analyte–competitor – IC_{50MAX}/IC_{50MIN}.[26]

The dynamic range of assay was accepted as IC_{20}–IC_{80} and the limit of detection (LOD) was calculated as $B_0 - 3 \times SD$.

2.6 Sample pretreatment and analysis of honey

Honey was purchased from a retail network in Moscow. To study the matrix effect of honey and develop a matrix imitator, blank honey samples were selected according to selective ELISA tests[16,27–29] and confirmed by HPLC-MS/MS. The relationship between the effect of the honey matrix on the immunochemical interaction and the similar effect of sucrose solutions used as a honey imitator was evaluated during their parallel titration in accordance with the procedure described previously for the kanamycin and lincomycin ELISA.[16,30]

Briefly, 1 g-honey sample was diluted 5 times with PBST and vortexed thoroughly, and then serial dilutions were prepared. Blank honey samples were spiked with AGs to make concentrations of 100, 10 and 1 ng mL^{-1}. Then, the samples were diluted 50 times with PBS-T and analyzed using ELISA. The recovery rate was calculated as a ratio between the measured and the fortified concentrations.

3. Results and discussion

3.1 Immunogen synthesis

In the majority of publications devoted to immunoassay of AGs, the immunogens, coating antigens, enzyme conjugates or tracers were prepared usually by carbodiimide or glutaraldehyde methods involving AGs' amino groups.[14–18,23,31] Due to several amino groups in AG molecules, the aforementioned conjugation methods contribute to the formation of heterogeneous conjugates with a variable orientation of the hapten.

In the present work, we investigated the immunochemical properties of AG conjugates formed by alternative sites (hydroxyl groups) using the periodate oxidation method (pi) or carbodiimidazole (cdi). For the induction of antibodies against 2-DOS, a fragment common to a great number of aminoglycosides, RS was chosen as an immunizing hapten. Unlike NA and NB-82 used previously,[23,24] RS had the following advantageous features. Its structure is closer to that of NM, since RS corresponds to three carbohydrate residues (A–B–C) of NM, in contrast to NA or NB-82 (A–B). Being a trisaccharide, RS has the size of a molecule comparable to the compounds of the GM and KM families. Finally, the presence of vicinal hydroxyls in the ribosyl cycle (C) of RS made it possible to use a periodate oxidation method for site-specific conjugation with a carrier. Such an approach could provide a certain orientation of the RS molecule on a carrier with a favorable presentation of the 2-DOS determinant. In addition, a C6-spacer (1,6-hexanediamine) was introduced between the carrier and the hapten for better steric availability of the target epitope. Thus, in the resultant immunogens, BSA-RS(pi) and BSA-C6-RS(pi), the hapten was distant from the carrier in different degrees.

The absence of specific UV-Vis spectra of RS and other AGs did not allow us to estimate changes in spectrophotometric characteristics of the prepared conjugates; therefore, the formation of conjugates was confirmed immunochemically using antibodies to NM.[27] This antibody was able to bind with examined conjugates immobilized on the plates. This proves that a determinant (RS) structurally related to NM is present on the carrier and retained its immunochemical activity after coupling (Fig. S1†). The BSA-C6-RS(pi) and BSA-RS(pi) conjugates, synthesized with the ratios between the carrier and hapten of 1/250 and 1/100, respectively, demonstrated the similar interaction intensity. Besides, these two conjugates demonstrated near inhibitory activity when they were used as competitors in anti-NM–Gel-RS(pi) interaction (Fig. S2†). These pieces of evidence confirmed the formation of the conjugates and may indicate a similar hapten load. This became the basis of their comparative application as immunogens.

3.2 Antibody preparation

The study of the immune response in dynamics allowed us to choose antibodies that provide better assay sensitivity and

group specificity. Antibodies to the BSA-RS(pi) demonstrated selectivity towards NM and relatively low cross-reactivity (≤5%) for GM, KM and AP (Fig. S3A†). The application of the C6 spacer in the immunogen apparently contributed to a prominent presentation of the 2-DOS determinant and the induction of antibodies with recognition of different AGs. In addition, anti-BSA-C6-RS(pi) exhibited significantly better sensitivity (IC_{50}), which tended to improve up to the fifth booster immunization (Fig. S3B†). Thus, all subsequent studies were conducted using anti-BSA-C6-RS(pi) antibodies obtained from antiserum # 5.

3.3 Examination of assay specificity and selection of immunoreagents

As can be seen from the structural formulas of AGs, the 2-DOS fragment (B-cycle) is a common structural element for a number of compounds (Fig. 1). Its intermediate position and structural differences between the other glycosidic fragments in different aminoglycosides complicate the task of presenting a generic epitope on the antigen. In addition, the arrangement of glycosidic bonds at 2-DOS is not identical in different families of AGs – 4,6-substitution in KM and GM families, 5,6-substitution in NM, RS and PM. NA and AP have one 2-DOS substitution at the C6 position. Thus, the only C6–C1–C2–C3 region with the existing NH_2 substituents at C1 and C3 remains unchanged in AGs. In this regard, an optimal presentation of the 2-DOS determinant and surrounding structures on the coating antigen is a key factor for group-specific interaction. For this, the study of conjugates of various designs was conducted. A homologous hapten, RS and AG from different families – NM, TM, GM and AP were used as haptens bearing the 2-DOS determinant. A number of AP-based conjugates described in ref. 27 were not able to interact with anti-BSA-C6-RS(pi). The remaining coating haptens were examined, and specificities of corresponding assay variants are presented in Table 1.

For evaluation of assay specificity, a panel of 2-DOS AGs from several subfamilies: (1) NM, NA, RS and PM; (2) GM, SSM, NTM, GC; (3) KM, TM, AM; (4) an individual representative of AP was studied. STM was also included as veterinary AG but non-2-DOS AG. The most of these analytes are used in medical and veterinary areas, but the regulation in animal husbandry applies only to NM, PM, GM, KM, AP and STM.[1] The other representatives have no practical significance in the mentioned spheres, *i.e.* GC (G418) is used mainly in cell biology as a selective agent for eukaryotic cells,[32] and NA is a product of NM degradation. In this regard, the closest analogue of the immunizing hapten, a veterinary antibiotic NM, was taken as the main analyte. Therefore, the inhibitory activity of NM on antibody binding was taken as 100%.

As can be seen from the table, NTM and AM were out of recognition (CR < 0.1%). The presence of extra substituents in 2-DOS at the N1 position was an obstacle to the interaction of these AGs with antibodies. This fact served as additional evidence of the 2-DOS-specificity of the antibodies. The other analytes showed different cross-reactivities, which could vary depending on the design of the coating antigen.

The presentation of the target determinant using a TF(pi)-NM conjugate resulted in the best NM detectability. When the TM hapten was immobilized on polystyrene as Gel-TM(pi), the recognition of AGs from its family was significantly improved. The cross-reactivity of TM and KM was increased up to 46% and 162%, respectively. Replacing the coating hapten by GM (Gel-GM(cdi)-ELISA) contributed to the growth of the inhibitory

Table 1 Influence of coating antigen design on the cross-reactivity profile of aminoglycosides in group-specific ELISA[a]

AGs	ELISA variants based on coating antigens							
	Gel-RS(pi)		TF(pi)-NM		Gel-TM(pi)		Gel-GM(cdi)	
	IC_{50}, ng mL^{-1}	CR, %	IC_{50}, ng mL^{-1}	CR, %	IC_{50}, ng mL^{-1}	CR, %	IC_{50}, ng mL^{-1}	CR, %
NM	**0.19**	**100**	**0.04**	**100**	**0.21**	**100**	**0.53**	**100**
RS	0.08	250	0.01	367	0.05	463	0.77	69
NA	0.03	625	0.005	786	0.05	463	0.73	72.5
PM	**1.1**	**17.3**	**0.4**	**10**	**1.4**	**15**	**3.4**	**15.6**
GM	**2.1**	**9.0**	**0.48**	**8.3**	**0.88**	**24**	**0.99**	**53.5**
SSM	15.8	1.2	2.1	1.9	1.4	15.4	2.6	20.7
NTM	>1000	<0.1	>1000	<0.01	>1000	<0.1	>1000	<0.1
GC	>1000	<0.1	>1000	<0.01	>1000	<0.1	54	1
KM	**0.4**	**47.5**	**0.07**	**57.1**	**0.13**	**162**	**0.29**	**183**
TM	2.4	7.8	0.7	5.8	0.45	46.3	1.3	41.4
AM	>1000	<0.1	>1000	<0.01	>1000	<0.1	>1000	<0.1
AP	**11.5**	**1.7**	**5.8**	**0.69**	**24.3**	**0.86**	**73.7**	**0.72**
STM	>1000	<0.1	>1000	<0.01	>1000	<0.1	>1000	<0.1
	GRC, IC_{50MAX}/IC_{50MIN}*							
All AG	521		1139		538		254	
Vet AG	**59**		**145**		**116**		**254**	

[a] GRC* – group recognition coefficient was determined for detectable AGs. The veterinary 2-DOS antibiotics with established MRLs are highlighted with bold type.

activity for the corresponding family of AGs. The highest CR of GM (54%) and SSM (21%) was registered in this assay variant; and even GC, which was not detected in other ELISAs, showed a CR of 1%. Thus, by changing the type of immobilized hapten/coating antigen, it was possible to change the cross-reactivity profile of antibodies to some extent and create conditions for better recognition of the group as a whole.[33]

To estimate group recognition, a coefficient GRC was defined for 9–10 detectable AGs as a ratio IC_{50MAX}/IC_{50MIN}. Because this work is devoted to the analysis of an agricultural product, honey, this coefficient was determined for all AGs and separately for the veterinary drugs. Table 1 shows that Gel-RS(pi)-ELISA was the best satisfied definition of a group assay. The GRC coefficient for five veterinary 2-DOS AGs from different subfamilies was only 59. At the same time, it should be noted that advantage of this assay variant was due to better AP recognition. If AP was excluded, GRC was similar for all variants of the assay. Differences between IC_{50} for NM, PM, GM, and KM did not exceed 11–12 times. The comparable 12-fold differences were reached by Loomans *et al.*[23] for three AGs (GM, KM and NM; $IC_{50} = 9$, 21 and 113 ng mL^{-1}, respectively). The homologous Gel-RS(pi)-ELISA format was capable of revealing five legislated AGs – NM, PM, GM, KM, AP and additionally RS, TM, and SSM. This assay variant demonstrated better sensitivity ($IC_{50} = 0.2$–16 ng mL^{-1}) and was selected for further experiments.

3.4 Evaluation of the honey matrix effect and selection of the matrix imitator

Honey is a complex product consisting of carbohydrates (75–80%), vitamins, proteins, enzymes, organic acids, trace elements, inclusions and other components. The isolation of analytes such as AGs from the honey matrix is complicated by the fact that they have a similar carbohydrate nature, so simple physicochemical methods are ineffective. And the very honey matrix can interfere with the immunochemical reaction. The intensity of the matrix effect of honey (flower, lime, and buckwheat origin) on antibody binding was evaluated by a method of sample dilution. These experiments showed that the effect of the honey matrix caused a decrease in the absorbance of the immunochemical reaction and persisted even at high sample dilutions (Fig. 2A). Comparison of the effect of sucrose solutions (Fig. 2B) was conducted to simulate the interference of honey and simplify the laborious sample pretreatment. Sucrose (disaccharide) is a minor sugar component in honey (~1%) in comparison with major monosaccharide components, fructose and glucose (30–40%). Nevertheless, sucrose was chosen as the honey imitator since it exposed a stronger honey-matrix-like effect on antibody binding than fructose or glucose. We observed an optical signal decrease due to honey and carbohydrate interference in ELISAs of KM, lincomycin, and tylosin.[16,30,34] The mentioned interference for immunochemical reactions associated with the presence of sugars was also found by other researchers.[35,36] Thus, the nonspecific inhibitory effect of sugar solutions on antibody binding is not associated with the peculiarity of immunoreagents applied, but it is natural. As can be seen from Fig. 2A and B, the concentration-dependent degree of absorbance inhibition was not equal for honey and sucrose. Nevertheless, adequacy was found between solutions of honey and sucrose expressing an almost equal matrix effect (Fig. 2C). This relationship was observed regardless of the

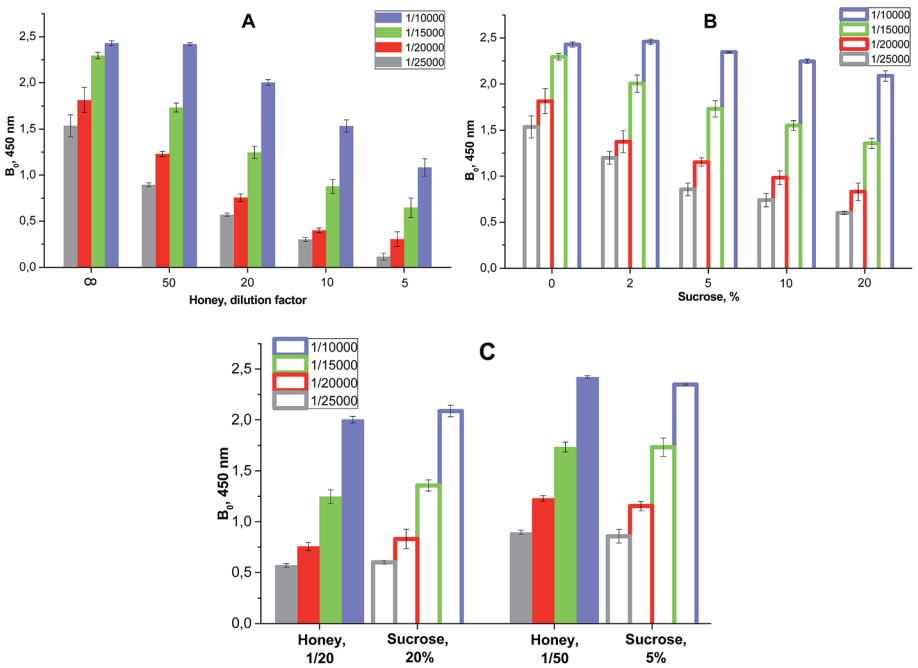


Fig. 2 Influence of honey dilution (A) and sucrose concentration (B) on the antibody binding level and relationship between them (C). The average values ($n = 3$) of absorbance were obtained from antibody (1/10 000–1/25 000) binding to Gel-RS(pi) in honey diluted with PBS-T (filled bars) and sucrose (empty bars) solutions in PBS-T. Symbols ∞ (A) and 0 (B) in the abscissa axis mean PBS-T.

concentration of antibodies (1/10 000–1/25 000). This allowed selection of the desired absorbance level of the reaction depending on honey dilution. Thus, the imitator of the honey matrix effect was chosen. Honey diluted 20-fold with PBS-T expressed the influence on antibody binding similar to that of 20% sucrose solution. 50-fold diluted honey and 5% sucrose were another pair with a similar matrix effect. However, the following experiments showed inconvenience when working with 20% sucrose due to the high viscosity of this syrup solution. Therefore, for honey analysis, the samples were diluted 50 times with PBS-T, and calibration solutions were prepared in 5% sucrose in PBS-T.

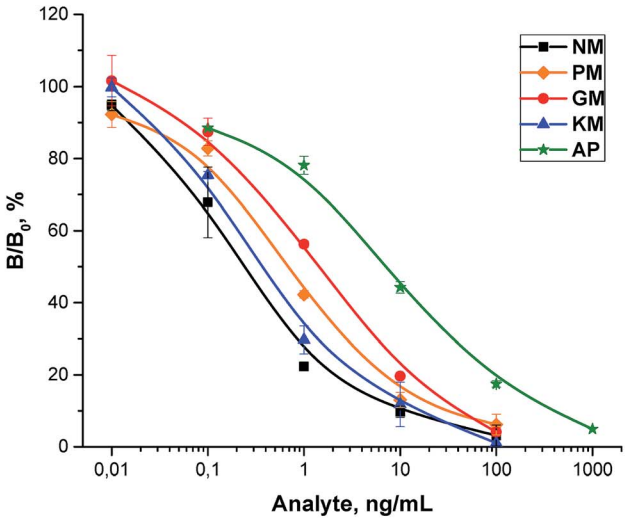
3.5 Determination of AGs in honey and recovery experiments

The determination of AGs in honey could be carried out quantitatively if the analyte to be detected is known. For this, standard solutions of the target AG should be prepared in PBS-T containing 5% sucrose. This medium allowed matrix effects to be eliminated and the matrix imitator-matched standard curves served for measurement of the analyte concentration in honey diluted 50-fold. The typical standard curves of AGs generated in the matrix simulator medium are shown in Fig. 3.

The characteristics of the assay are presented in the accompanying table. The value of the LOD in honey, achieved in this work, satisfies the level of MRL (10–40 μg kg^{-1}) established for STM in some countries.[3] The developed group-specific ELISA was capable of measuring a number of 2-DOS AGs and could substitute a panel of selective tests. Rough elimination of the honey matrix effect was achieved here by application of standard sucrose solution as the matrix imitator instead of a composed blank honey blend. This rendered the complex procedure of sample extraction and isolation of the analyte unnecessary. The recovery examination confirmed the detectability of AGs (78–120%) exemplified by NM, PM, GM, KM and AP which were spiked in honey samples at around the "limitation" level (10–100 μg kg^{-1}). The fortified samples with AP at a 10 μg kg^{-1} level, which was below the LOD of this AG, gave an acceptable value of recovery (Table 2).

The matrix effect of honey of different origins can vary greatly because of its very complex composition influenced by plant sources, climate and treatment.[37] Not only simple dilution of honey samples, but also extraction with acetonitrile, centrifugation and evaporation, followed purification with a C18 column involved in the pretreatment protocol of several commercial ELISA kits had hardly overcome the influence of honey diversity, and the drawbacks in accurate recovery were noted.[38]

To reveal the contamination of honey with 2-DOS AGs the developed group-specific ELISA was used as a screening



Analyte	IC_{50}, ng/mL	Dynamic range IC_{20}-IC_{80}, ng/mL	LOD, ng/mL	LOD in honey, μg/kg
NM	0.2	0.03-2.1	0,02	1.0
PM	0.7	0.08-7.1	0,05	2.5
GM	1.5	0.15-13.3	0,11	5.5
KM	0.35	0.05-3.9	0,04	2.0
AP	6.8	0.5-96.8	0,23	11.5

Fig. 3 Standard curves and analytical parameters of the ELISA-system for group determination of aminoglycosides in honey. Interaction of anti-BSA-RS(pi) with coating antigen Gel-RS(pi) in 5% sucrose solution as the honey imitator. The detection limit in 5% sucrose solution was determined according to LOD = $B_0 - 3 \times$ SD. The detection limit of AGs in crude honey was calculated as the LOD value in buffer multiplied by the factor of sample dilution (50).

Table 2 Recoveries of AGs spiked in blank honey samples in group-specific ELISA using corresponding standard curves

	Fortification level			
	100 μg kg^{-1}		10 μg kg^{-1}	
Analyte	RC, %	RSD, %	RC, %	RSD, %
NM	120	7.2	110	11.3
PM	98	11.7	78	7.4
GM	95	6.7	110	7.9
KM	117	7.3	103	7.4
AP	92	9.5	110	5.9

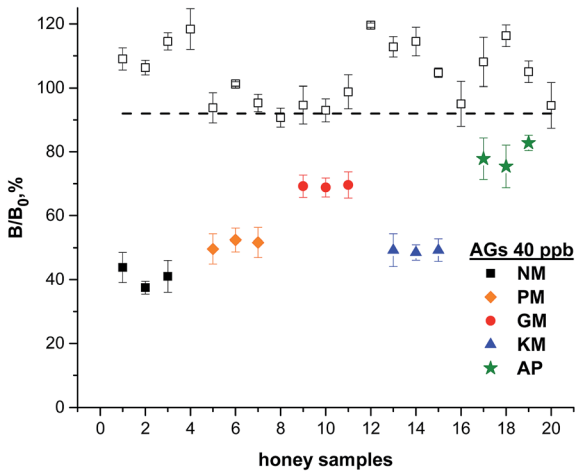


Fig. 4 Detection of AGs spiked in honey samples at a 40 ppb level using group-specific ELISA. Each symbol corresponds to the average relative binding and the error is SD obtained for an individual honey sample analyzed in triplicate. Empty characters represent individual blank honey samples (lime, buckwheat and flower), and filled symbols represent the same samples fortified with AGs at a 40 μg kg^{-1} level. The dashed line corresponds to the limit of assay detection obtained by the matrix imitator (5% sucrose-PBS-T).

qualitative test. In this case, the analyzed sample was considered non-compliant (contaminated) if it caused a relative antibody binding below the detection threshold (Fig. 4). The representatives of this family of antibiotics were spiked in blank honey samples of different botanical origins to maintain a content of 40 μg kg^{-1}. All the samples fortified with NM, PM, GM, KM or AP gave a signal that was below the threshold level unlike 20 blank samples (except #8). Thus, the threshold established as the LOD value for assay in the honey matrix imitator allowed us to distinguish contaminated and non-contaminated honey samples of different floral origins, despite their inequivalent effect on antibody binding.

4. Conclusion

Aminoglycoside antibiotic RS was used as a new immunizing hapten to evoke immune response against 2-DOS, a common moiety of a large number of AG antibiotics. The coupling of periodate-oxidized RS with the carrier provided a definite orientation with exposure of the 2-DOS determinant unlike a usual practice of multi-site conjugation of AG through its amines. The introduction of a C6 spacer arm between the hapten and the carrier in the immunogen allowed antibodies to be raised with better group specificity. The recognition of the 2-DOS moiety of AGs allowed us to detect a wide spectrum of representatives NM, RS, NA, PM, GM, SSM, KM, TM, and AP. The developed group-specific indirect competitive ELISA was capable of substituting a number of selective tests for detection of known analytes (with a LOD up to 0.02–0.2 ng mL^{-1}). For analysis of honey, a matrix imitator was developed to avoid honey interferences on immunoassay. The screening procedure including 50-fold dilution of the honey sample and its analysis allowed us to reveal any of the AGs from the mentioned list in honey at a 10 μg kg^{-1} level.

Conflicts of interest

There are no conflicts to declare.

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