METHOD VALIDATION FOR SIMULTANEOUS DETERMINATION OF 12 SULFONAMIDES IN HONEY USING BIOCHIP ARRAY TECHNOLOGY

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Abstract

A new method for simultaneous multi-analyte determination of drug residues in honey samples was validated according to the criteria set by the *Commission Decision 2002/657/EC*. "*Antimicrobial Array I*", An immunoassay based on multi-analyte array for drug residues testing has been evaluated for simultaneous screening of twelve sulphonamides (sulphamethazine, sulphadiazine, sulphadimethoxine, sulphaquinoxaline, sulphathiazole, sulphisoxazole, sulphapyridine, sulphamerazine, sulphamethoxypyridazine, sulphachlorpyridazine, sulphamethizole, sulphadoxine). The analytes determination was carried out using Biochip Array Technology, an immunoassay testing platform for the simultaneous multi-analyte assessment of a panel of tests, in a single test procedure, using a single undivided sample. The method showed good linearity, sensitivity, specificity, precision (repeatability and intra-laboratory reproductibility), ruggedness and recovery at three concentration levels representing 0.5, 1.0 respectively 1.5% from the maximum accepted residue limits (50, 100, and 150 μg/kg). The decision limits (CCα) were always below 0.5 % from the maximum residue limits (MRL). The results of the validation protocol showed that the method complies with a performant European Union monitoring of drug residues in food. The developed procedure was applied to 16 honey samples, and for two of them there were detected residues of sulphonamides (sulphfadiazine, sulphametazine, sulphatiazol, sulphametizol).

Rezumat

O nouă metodă pentru determinarea simultană, multianalit, a reziduurilor medicamentoase în probele de miere a fost validată conform criteriilor stabilite prin decizia 2002/657/EC a Comisiei Europene (*Commission Decision 2002/657/EC)*. A fost evaluată metoda imunologică de testare multianalit a reziduurilor medicamentoase "Antimicrobial Array I" pentru screeningul simultan a 12 sulfonamide (sulfametazina, sulfadiazina, sulfadimetoxina, sulfaquinoxalina, sulfatiazol, sulfisoxazol, sulfapiridina, sulfamerazina, sulfametoxipiridazina, sulfaclorpiridazina, sulfametizol, sulfadoxina). Determinarea analiţilor s-a efectuat utilizând Tehnologia Biocip, o platformă de testare imunologică pentru determinarea simultană multianalit a unui profil de teste printr-o singură procedură, utilizând o probă unică, neeşantionată. Metoda a dovedit o bună linearitate, senzitivitate, specificitate, precizie (repetabilitate şi reproductibilitate), robusteţe şi recuperare la trei nivele de concentraţie ce reprezintă 0,5, 1,0 respectiv 1,5% din limita maxim admisă pentru reziduuri medicamentoase (50, 100 şi 150 μg/kg). Limitele de decizie (CCα) s-au situat sub

0.5% din limita maximă admisă pentru reziduuri. Rezultatele obţinute în urma protocolului de validare au demonstrat că metoda corespunde standardelor Uniunii Europene de supraveghere a reziduurilor medicamentoase în alimente. Procedura de testare validată s-a aplicat pe 16 probe de miere, iar pentru două dintre acestea s-au detectat reziduuri de sulfonamide (sulfadiazina, sulfametazina, sulfatiazol şi sulfametizol).

Keywords: honey, sulphonamides, drug residues

Introduction

Honey is generally accepted as being a natural food product, free of contaminants and giving health benefits to the consumer. However, over the last few years traces of sulfonamides have been found in a growing number of honey samples worldwide [1]. Contamination of honey may also occur through antimicrobial treatment of plants. The presence of drug residues in honey poses a potential risk to human health. Hypersensitivity reactions to sulphonamide drug residues in food include skin rash, hives and pruritus [9]. The presence of chemotherapeutics in honey can also lead to bacterial resistance. According to the European legislation, the use of sulfonamides for treating the honeybees is illegal in many countries. Sulfonamides play an important role as effective chemotherapeutics for bacterial and protozoal diseases in veterinary medicine. In bee colonies sulfonamides are used for the treatment of foulbrood and this may lead to residues being present in honey. The drugs are administered to honeybees as a sodium salt dissolved in sucrose solution in combination with dihydrofolate reductase inhibitors of the diaminopyrimidine group [6]. The European Union (EU) legislation has set maximum residue limits (MRLs) for sulphonamides in food from animal origin, but no MRLs have been established for honey, thus the use of sulphonamides in this matrix is not accepted. Some countries within the European Union have set efficient limits or tolerated levels. Belgium and the United Kingdom have set efficient limits of 20 and 50 μg/kg and Switzerland has established a fixed limit of 50 μg/kg for the total sulphonamides in honey [15,16]. The availability of rapid and reliable analytical methods for the specific detection of this compound in test samples with low limits of detection (LODs) in order to detect traces of chemotherapeutics is of interest for monitoring and regulatory purposes. Recently, the EU has issued a specific regulation decision (2002/657/EC) concerning the performance of methods and the interpretation of the results in the official control of residues in products of animal origin [11]. The methods currently applied for the analysis of official samples of the substances in group B of *Annex I of Council Directive 96/23/EC* will have to

comply with the decision 2002/657/EC [12]. Some new parameters must be calculated as limit of decision (CCα) and detection capability (CCβ).

Several analytical procedures are currently available for the determination of sulphonamide residues in honey and at present liquid chromatography is the most adopted instrumental technique [5–10]. Because sulfonamides are polar compounds severe matrix influences have occurred; therefore the analytical procedures must use either a very selective but expensive detector (i.e. Mass Spectrometry) or a good matrix clean-up. EU Commission Decision 93/256/EEC stated that "*Methods based only on chromatographic analysis without the use of molecular spectrometric detection are not suitable for use as confirmatory methods*". Liquid chromatography - mass spectrometry **(**LC–MS) is the ideal technique to detect nonvolatile, polar compounds such as sulphonamides (SAs). Several approaches involving LC–MS or LC–MS/MS have been reported for determination of SAs in animal products [10]. However, these methods have the following problems: the extraction and clean-up involves varying analytical steps that are laborious and time consuming, recoveries are sometimes low and variable, the sample preparation or determination is not cost-efficient, a large quantity of toxic solvents such as acetonitrile, methanol, and methylene chloride are used as extracting solvents and LC mobile phases, which can be harmful to the environment [1–10]. The present paper describes the simultaneous determination of 12 sulfonamides (sulphamethazine, sulphadiazine, sulphadimethoxine, sulphaquinoxaline, sulphathiazole, sulphisoxazole, sulphapyridine, sulphamerazine, sulphamethoxypyridazine, sulphachlorpyridazine, sulphamethizole, sulphadoxine) in honey samples using Biochip Array Technology (BAT). The method proposed represents a possibility to comply at the same time with the requirements of decision 2002/657/EC and the resources of an official laboratory with high samples throughput.

Materials and Methods

Reagents

Anti-Microbial Array I kit, Sulphonamide Multianalyte Controls (EV3602, EV3523, AMC5004, Randox Laboratories, Crumlin, Northern Ireland) were used according to manufacturer's instructions. The kit contained bar-coded reagents: ready to use biochips, ready to use liquid multianalyte calibrators completed with parameter details, multianalyte conjugate, conjugate diluent, assay diluent, signal reagent and wash buffer.

Apparatus

The semi-automated benchtop analyzer Evidence Investigator, Randox, UK is a system based on the reproducible and stable protein-biochip microarray technology described for the fully automated high throughput. This instrument has been applied to the multiplexed determination of analytes. The core of the system is the biochip onto which a selection of ligands are precisely immobilized and stabilized forming arrays.

We also used a Thermoshaker, provided by Randox, UK.

Sample preparation

9 mL diluted wash buffer (37^{o}C) was added to 1g of honey sample. After 10 minutes rolling, filtration and dilution, the sample was ready for application to the biochip. Results were multiplied by 20 to take into account the dilution effect of preparation.

General procedure

The Biochip Array Technology was used to perform test samples. Evidence Investigator analyzer was used in conjunction with the Antimicrobial Array I panel. The biochip platform used to perform the immunoreactions is a 9 mm^2 chemically functionalised substrate that facilitates the deposition of capture agents onto spatially distinct test regions. The biochips are held in a carrier accommodating 9 biochips in a 3x3 format. The technology for immunoassay is based on traditional enzyme-linked immunosorbent assay (ELISA) principles, namely the deposition of capture antibodies onto the reactive surface followed by competitive immunoassay. Detection is based on peroxidase-induced chemiluminescence using a highly sensitive charge coupled device (CCD) camera. Each biochip contains 23 distinct test regions and, unlike most current conventional immunoassay analysers, allows multiple assays to be performed simultaneously on a single sample.

This multi-plexing ability has large implications for both sample size and throughput. The immunoassay steps were manually performed and the reaction temperature was controlled with the supplied custom thermoshaker. After completion of the manual procedures, the biochip carrier was inserted in the imaging module of the system followed by a set of automatic operations. The instrument's super-cooled CCD camera in the instrument simultaneously detected the light signals emitted by the discrete test regions (DTRs) of the array.

The obtained results were processed, validated and archived using a dedicated software. A competitive chemiluminescent immunoassay was

employed for the anti-microbial array. The light signal generated from each of the test regions on the biochip was detected using digital imaging technology and compared to the calibration curve. The concentration of analyte present in the sample was plotted and calculated from the calibration curve.

Method validation

The Antimicrobial I Assay is based on a competitive reaction where any free analyte contained in the standards/ samples competes for the binding sites of the capture antibody with horseradish peroxidase labelled conjugate. Following the incubation and washing steps, enzyme substrate is added. Chemiluminescent signal intensity is inversely proportional to the concentration of the analyte present in the sample.

Calibration curves, sensitivity, accuracy, intra-, and inter-day precision were performed to validate the whole procedure. Linearity was evaluated using matrix-matched calibration, spiking blank extracts at nine concentration levels. The analyzer software uses a 4-parameter curve fit method for calibration. In theory this is a non-linear regression method, assuming a central turning point and two asymptotes at the extreme ends. After analysis of calibrator samples the software constructs a derived calibration curve using the pre-defined competitive assay parameters A, B, C, D, the observed results and the respective equation:

$$y = A + [(B+A)/1+(x/C)^D]$$

where:
x = sample concentration (µg/kg),
y = intensity of the chemiluminescent signal (RLU – relative light unit).

The observed results are plotted and the obtained calibration curve is compared with the derived curve. The software performs iterations of the derived curve until an optimal fit is obtained with the observed curve and the correlation coefficient between the re-defined curve and the observed curve is displayed. The calibration curve is validated if determination coeficients (R^2) are higher than 0.950 [4].

Precision and accuracy of the method were studied by spiking blank samples. Precision of the overall method was studied by performing repeatability (intraday precision) and inter-day precision experiments. Repeatability (intraday precision) was performed spiking blank honey at three concentration levels (50, 100, and 150 μg/kg), using five replicates for each concentration level in one day. To evaluate inter-day precision (reproducibility), the same concentration levels were studied, spiking blank honey during five consecutive days. Recovery was studied by analyzing blank samples that were fortified at three concentration levels (50, 100, and

150 μg/kg). Accuracy was estimated through recovery studies. The absolute recovery and precision of each SA were determined on spiked blank samples at three concentration levels: low (50 μg/kg), medium (100 μg/kg) and high (150 μg/kg) representing 0.5, 1.0 and 1.5% from the maximum accepted residue limit (MRL).

Decision limit and detection capability

The limit of decision (CCα) represents "*the limit at and above which it can be concluded, with an error probability of α, that a sample is non-compliant*" (greater than the MRL for group B substances) while CCβ represents "*the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of β*". For the group B substances, α and β errors must be $\leq$5% [13 – 17].

The Regulatory Limit for unauthorized analytes is the Minimum Required Performance Limit (MRPL) or the Reference Point for Action (RPA). The Screening Target Concentration must be at or less than the MRPL (or RPA) [14]. Due to the fact that in real samples it cannot be predicted which combination of sulfonamides will be present, for the assessment of non-compliant results in the case of sum-MRPLs it is not possible to validate and calculate the precise detection capability for each combination of analytes that might occur in practice. Theoretically an infinite number of possibilities exists as the number of concentration level combination approximates infinity. For banned and unauthorized compounds where no MRPL has yet been established, CCβ should be as low as reasonably achievable. Methods for screening should be able to detect the analyte at the CCβ in 95% of the cases [11, 14].

For many drug residues the Reference Point of Action was stated at 1 µg/kg [11]. This is the reason why we set the Desired Screening Target Concentration = 0.5 µg/kg. In order to establish the decision limit and detection capability, 20 blank matrix samples were selected. Replicates of these samples were spiked at the Screening Target Concentration = 0.5µg/kg. The matrix blank samples and spiked samples were analysed.

The decision limits (CCα, where α = 0.05) were calculated from the mean concentration of the 20 spiked samples at Desired Screening Target Concentration plus 1.64 times the corresponding standard deviations. The CCβ was obtained adding to CCα 1.64 times the same standard deviation.

Application to Real Samples

To evaluate the applicability of the proposed method in real samples, 16 honey samples were analyzed using biochip method and positive results were confirmed using LC-MS/MS method. The confirmatory method complies with the requirements of Decision 2002/657/EC concerning the performance of

method for the determination and confirmation of sulphonamides residues in honey samples (Table I) [11]. Analysis were performed on an Agilent 1100 LC system (Agilent Technologies, USA) coupled to a 4000 Q TRAP mass spectrometer instrument (Applied Biosystems, USA). For the method's confirmation the following criteria were used: the signal-to-noise ratio (S/N) of the characteristic ions selected were > 3, the differentiation of the retention time of analyte and corresponding standard were within ±2.5%, the deviation of the relative abundance of the characteristic ions of the target matter and that of the characteristic ions of the corresponding standard were within ± 20% to ± 50%.

Table I

Performance parameters of the confirmatory method (LC-MS/MS)

Sulphonamide	**Mean conc. ± SD (µg/kg)**	**CCα (µg/kg)**	**CCβ (µg/kg)**	**Precision (%)**	**Accuracy (%)**
Sulphfadiazine	44.4 ± 1.67	2.00	3.50	3.76	11.70
Sulphametazine	35.9 ± 1.47	1.00	1.70	4.09	29.70
Sulphatiazol	37.1 ± 1.37	1.00	1.70	3.69	26.80
Sulphametizol	35.9 ± 1.76	0.80	1.20	4.90	28.60

There were analysed 12 samples purchased from local supermarkets and 5 samples were obtained from different beekeepers from different Romanian geographic regions. The samples were stored at room temperature in the dark. In order to ensure the quality of the results when the proposed method was applied, an internal quality control was carried out in every batch of samples in order to evaluate the stability of the proposed method. We found two honey samples with positive results. These positive samples were sent to an external laboratory for interlaboratory study and confirmation of results using LC-MS/MS (Table II). These results show that Romanian beekeepers still use Polisulfamid® and other sulphonamide drugs in their beekeeping practices.

Table II

Positive samples confirmed by the confirmatory method (LC-MS/MS)

No.	**Sulphonamide**	Sample 1		Sample 2	
		Biochip	LC-MS/MS	Biochip	LC-MS/MS
		Conc. (µg/kg)		Conc. (µg/kg)	
1	Sulphfadiazine	11.4	7.3	9.2	8.8
2	Sulphadimethoxine	< LOD	< LOD	< LOD	< LOD
3	Sulphakinoxaline	< LOD	< LOD	< LOD	< LOD
4	Sulphametazine	24.8	32.6	17.1	21.3
5	Sulphatiazol	22.6	18.4	16.2	11.7
6	Sulphisoxazol	< LOD	< LOD	< LOD	< LOD
7	Sulphapyridine	< LOD	< LOD	< LOD	< LOD
8	Sulphamerazine	< LOD	< LOD	< LOD	< LOD
9	Sulphametoxipiridazine	< LOD	< LOD	< LOD	< LOD
10	Sulphachlorpyridazine	< LOD	< LOD	< LOD	< LOD
11	Sulphametizol	51.2	58.9	68.2	72.9
12	Sulphadoxin	< LOD	< LOD	< LOD	< LOD

Results and Discussion

Biochip Array Technology allows simultaneous and selective multi-analyte measurement of antimicrobials in honey at levels well below the established MRLs. No matrix interferences produced by endogenous compounds were observed for these analytes. The use of multi-analyte calibrators and multi-analyte conjugate simplifies the assay procedure, avoids the use of different calibrators and conjugates for the analysis of 12 sulphonamides from single sample preparation. The standard curves for the 12 SA drugs was in the range of 0 – 20 μg/kg. A satisfactory linearity was achieved. The correlation coefficient (R^2) obtained where higher than 0.982 (Table III). The simultaneous immunoassays showed that the specificity for the target analytes and the sensitivity was $\leq$ 2.5 μg/kg. The limits of detection (LODs) ranged from 0.3 μg/kg to 7.5 μg/kg for sulphonamides (Table III) and detection capability (CCβ) ranged from 0.79 to 7.99 μg/kg (Table IV).

Table III

Calibration ranges, determination coefficients and limits of detection

No.	Sulphonamide	Calibration range (µg/kg)	Determination coefficient (R2)	LOD (μg/kg) (n = 13)
1	Sulphfadiazine	0 – 20.00	0.9870	2.50
2	Sulphadimethoxine	0 – 20.50	0.9820	3.70
3	Sulphakinoxaline	0 – 20.20	0.9860	1.10
4	Sulphametazine	0 – 11.20	0.9880	7.50
5	Sulphatiazol	0 – 21.10	0.9910	1.00
6	Sulphisoxazol	0 – 11.20	0.9860	2.50
7	Sulphapyridine	0 – 19.40	0.9930	1.00
8	Sulphamerazine	0 – 2.50	0.9850	2.30
9	Sulphametoxipiridazine	0 – 19.60	0.9910	3.20
10	Sulphachlorpyridazine	0 – 19.60	0.9930	4.90
11	Sulphametizol	0 – 11.70	0.9960	1.70
12	Sulphadoxin	0 – 19.40	0.9890	0.30

Table IV

Decision limit (CCα) and detection capability (CCβ)

Sulphonamide	Spiked samples (Mean conc. - µg/kg) n = 20	SD (spiked samples)	1.64 x SD	Decision limit CCα (µg/kg)	Detection capability CCβ (μg/kg)
Sulphfadiazine	1.71	0.39	0.64	2.35	2.99
Sulphadimethoxine	2.41	0.54	0.89	3.30	4.19
Sulphakinoxaline	1.23	0.11	0.18	1.41	1.59
Sulphametazine	2.29	1.74	2.85	5.14	7.99
Sulphatiazol	0.71	0.24	0.39	1.10	1.49
Sulphisoxazol	2.01	0.3	0.49	2.50	2.99
Sulphapyridine	1.19	0.09	0.15	1.34	1.49
Sulphamerazine	2.17	0.19	0.31	2.48	2.79
Sulphametoxipiridazine	1.89	0.55	0.90	2.79	3.69
Sulphachlorpyridazine	3.81	0.48	0.79	4.60	5.39
Sulphametizol	1.41	0.24	0.39	1.80	2.19
Sulphadoxin	0.29	0.15	0.25	0.54	0.79

The method showed excellent intra-assay precision (Table V), typically less than 10% for 50, 100 and 150 μg/kg (admitted is maximum 23% coefficient of variation - CV). Inter-day precision was also within the required limits (Table VI).

Table V
Intra-assay precision and accuracy

No.	Intra-assay Precision n=20	Level 1 (50 μg/kg)		Level 2 (100 μg/kg)		Level 3 (150 μg/kg)	
	Analyte	Mean conc. (μg/kg)	CV (%)	Mean conc. (μg/kg)	CV (%)	Mean conc. (μg/kg)	CV (%)
1	Sulphfadiazine	57	6	115	7	164	6
2	Sulphadimethoxine	51	7	99	6	140	5
3	Sulphakinoxaline	50	10	89	9	134	5
4	Sulphametazine	51	9	112	7	170	7
5	Sulphatiazol	59	5	131	9	206	6
6	Sulphisoxazol	47	8	113	5	167	7
7	Sulphapyridine	58	7	100	6	140	7
8	Sulphamerazine	50	7	112	10	174	5
9	Sulphametoxipiridazine	53	7	129	6	190	6
10	Sulphachlorpyridazine	48	6	102	8	149	5
11	Sulphametizol	68	5	149	5	236	6
12	Sulphadoxin	45	9	101	8	139	7

Table VI
Inter-day precision

No.	Inter-day precision (n=20)	Level 1 (50 μg/kg)		Level 2 (100 μg/kg)		Level 3 (150 μg/kg)	
	Analyte	Mean conc. (μg/kg)	CV (%)	Mean conc. (μg/kg)	CV (%)	Mean conc. (μg/kg)	CV (%)
1	Sulphfadiazine	84	10	171	12	259	15
2	Sulphadimethoxine	51	11	101	8	160	10
3	Sulphakinoxaline	54	10	141	10	212	10
4	Sulphametazine	61	14	101	7	138	7
5	Sulphatiazol	51	14	122	12	207	11
6	Sulphisoxazol	36	11	90	9	136	10
7	Sulphapyridine	94	11	192	13	275	8
8	Sulphamerazine	62	10	80	14	156	17
9	Sulphametoxipiridazine	58	9	112	8	175	9
10	Sulphachlorpyridazine	78	11	124	11	189	11
11	Sulphametizol	98	9	158	8	231	9
12	Sulphadoxin	38	11	64	10	95	10

Satisfactory results were found, with recoveries higher than the minimum admitted which is 70% for drug residues for all the assayed compounds at the three concentration levels. The percentual recovery was higher than 80% in different honey matrices (Table VII).

Table VII
Recoveries for 50, 100 and 150 μg/kg

No.	Analyte	Level 1 (50 μg/kg)		Level 2 (100 μg/kg)		Level 3 (150 μg/kg)	
		Mean conc. (μg/kg)	Recovery (%)	Mean conc. (μg/kg)	Recovery (%)	Mean conc. (μg/kg)	Recovery (%)
1	Sulphfadiazine	54	112	113	113	159	106
2	Sulphadimethoxine	51	102	96	96	138	92
3	Sulphakinoxaline	49	98	86	86	134	89
4	Sulphametazine	50	100	112	112	169	113
5	Sulphatiazol	57	115	129	129	204	136
6	Sulphisoxazol	46	92	112	112	166	111
7	Sulphapyridine	53	106	97	97	139	93
8	Sulphamerazine	49	99	112	112	173	115
9	Sulphametoxipiridazine	52	105	128	128	190	126
10	Sulphachlorpyridazine	47	94	101	101	148	99
11	Sulphametizol	66	132	148	148	234	156
12	Sulphadoxin	44	88	100	100	138	92

The method showed good linearity, sensitivity, specificity, precision (repeatability and intra-laboratory reproducibility), recovery and decision limits (CCα) below 50% of maximum residue limits (MRL).

The results of the validation process demonstrated that the method is suitable for application, as a quantitative screening method, in drug residue surveillance programmes. The miniaturization of the immunoassays using this technology reduces the volume of the sample and reagent per test and increases test results output. The identity of eventual sulfonamides found in routine samples can be confirmed by LC–MS/MS systems.

Conclusions

The Biochip Array Technology method can be applied in routine analysis due to sample throughput as well as in surveillance programs to control the presence of drug residues in honey samples. Biochip method simplifies confirmatory analysis by providing individual sulphonamide identification and semi-quantitative results. It also allows the simultaneous detection of an extensive number of sulphonamides from a single sample, minimal false positives results and no para-aminobenzoic acid (PABA)

interference in honey, resulting in excellent specificity. The next step of this project is to extend the method capability to detect other sulfonamides.

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