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ATTEMPT TO USE STANDARD MICROBIOLOGICAL AND ENZYMATIC TESTS FOR ANTIBIOTICS IN HONEY

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The Delvotest and Penzym are tests for the detection of antibiotics used in Poland in dairy industry. Those tests use minimal equipment (pipettes, incubator or water bath) and can be read visually. The aim of this study was to adapt these tests to detect antibiotics in honey. Investigation included the possibility of application of standard microbiological test Delvotest and enzymatic test Penzym for detection antimicrobial drugs in honey and the optimization the conditions of analysis. The tested material were 20 honey samples. The not-diluted honeys and 20% (m/m) solutions have been tested. The obtained results indicated, that 90% honeys tested with Delvotest were interpreted as positive. Probably the natural antimicrobial substances present in honeys cause, like antibiotics, inactivation the growth of bacteria. Enzymatic test can not be use for not-diluted honeys. The investigation of 20% solutions of honeys indicated that 5 readings were positive. Penzym can be use for detecting antibiotics in honeys.

INTRODUCTION

Consumers expect that honey is a pure, natural product, in which neither additives nor conserving agents are allowed. However, it can be contaminated from beekeeping practices or from the environment. The results of the veterinary control of honey samples in Poland show that 30% of honeys are contaminated with residues of antibiotics, especially sulphonamides [Posyniak *et al.,* 2006]. Antibiotics are used for the treatment of bacterial diseases of honey bees, such American foulbrood, European foulbrood and nosemosis. Low concentration of streptomycin can be also found in fruit honey from nectar collected on pear orchards since the blossoms are sometimes sprayed with streptomycin preparations for the treatment of fire blight [Reybroeck, 2003]. Because of the residues in honeys can have adverse effects on human health, in Poland and other European countries the using of antibiotics is illegal. Following the European legislation (EEC-Regulation 2377/90 and amendments) no MRLs (Maximum residue limits) are fixed for antimicrobial agents in honey and therefore the use of them is not accepted in apiculture [Reybroeck, 2003].

Numerous analytical methods have been applied for the determination antibiotics in honeys, including gas chromatography (GC/MS), liquid chromatography (LC/MS-MS) and high performance liquid chromatography (HPLC) [Kaufmann *et al*., 2002; Posyniak *et al*., 2003; Szczęsna *et al*., 2006]. Recently automated screening by biosensors has been applied [Weigel *et al*., 2005]. But these methods require special equipment and a lot of expensive reagents, so the use of these methods is limited.

The honey producing industry as well as regulatory agencies is interested in rapid, simple and inexpensive tests for detection of antibiotics and other drugs in honey. The diagnostic tests, used worldwide in dairy industry, can be easily adapted for use in detecting antimicrobial drugs in honey [Legg *et al*., 2003].

The aim of this study was to adapt standard microbiological test Delvotest and enzymatic test Penzym to detect antibiotics in honey. Investigation included the possibility of application of these tests for detection antimicrobial drugs in honey and the optimization the conditions of analysis.

MATERIALS AND METHODS

The tested material was 20 honey samples coming from different stages of the Polish market. Five samples originated from local beekeepers association, the other have been purchased in shops. The honey samples have been stored in room temp. and before the analysis have been liquefied in water bath at temp. 40°C.

The not-dissolved honeys (20 samples) and 20% (m/m) solutions of honeys (20 samples) have been tested.

Penzm is enzymatic, colorimetric test for detecting beta-lactams drugs in milk. The main component of this tests is enzyme DD-carbopeptidase which is responsible for hydrolysis of polypeptide D-D-Ala-D-Ala and cause the reaction with beta-lactam antibiotics, making the persistent substrate. For honey, the 100 μl not-diluted or 20% water solution sample is pipetted onto a test tube (Eppendorf's type), mixed with 20 μL of enzyme and incubated in water bath at temp. 47.5°C for 5 min. After 5 min, the tablet of polypeptide is added and

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TABLE 1. Delvoest SP-NT – limits of detection in milk.

Antibiotics	Limit of detection (μg/mL)
Penicillin	0.002-0.003
Streptomycin	0.25-1.0
Tetracycline	0.4-1.0
Sulphonamides	0.25-1.0
Chloramphenicol	8-12

incubated for 15 min. After 7.5 min the first reading can be make. If the color of the tablet is pink-orange or peach, the sample is negative. If the color is yellow or peach-yellow, the sample is probably positive and needs the incubation for next 7.5 min. After second incubation the results are interpreted due to the color of the tablet as: pink-orange – the sample is negative, peach – the sample contain small amounts of antibacterial drugs (under 10 μg/mL of Penicilin G), and yellow – the sample is positive.

Delvotest SP-NT is the microbiological test for detecting antibiotics and other substances (*i.e.* detergents, disinfecting and washing substances), which can inhibit the growth of bacteria. The test consists of 100 ampoules with nutrient agar and test organism *Bacillus stearotermophilus var. calidolactis* C953. For honey, the 100 μL not-dissolved or 20% water solution sample is pipetted onto an ampoule and incubated in special incubator or water bath at temp. 64±0.5°C for 2 h 45 min. After the incubation the results are interpreted due to color of 2/3 lower part of agar. If the color is purple the sample is positive, if yellow – the sample is negative. The limits of detection are presented in Table 1.

To confirm the results the LC-analysis have been done The levels of antibiotics were investigated using LC technique, modified by Posyniak [Posyniak *et al*., 2003]. Sulfonamides were extracted from honey with acetate buffer and cleaned up by SPE procedure. The LC separation was carried out on RP C_{18} column and sulfonamides were monitored with fluorescence detector. The detection limits were 5 ppb.

RESULTS AND DISCUSSION

The obtained results are shown in Table 2. The results indicated, that majority of honeys tested with Delvotest were positive. 60% not-diluted samples and 95% solutions give the positive readings. Probably the natural antimicrobial substances present in honeys inhibit, like antibiotics, the growth of test organisms. The main antibacterial agent in honey is hydrogen peroxide, produced by honey glucose oxidase. When diluted, peroxide is produced in amounts causing an antibacterial action. The test organisms (*Bacillus stearotermophilus*) are sensitive on H_2O_2 with concentration at 600 ppm. Honeys contain a lot of other substances, like lysosyme and phenolic acids well known as antibacterial agents. These compounds have the influence on the result of our study too.

The thesis about possibility of application the microbiological test for detecting antibiotics in honeys has been verified negatively.

The results of presented studies shows, that enzymatic test can not be use for not-diluted honeys. The enzyme DD--carbopeptidase has not been mixed with the honey and the occurrence of further reaction was impossible. The investigation of 20% solutions of honeys indicated that 7 readings (35%) were positive.

TABLE 2. Results of testing honeys with standard tests.

Sample	Readings with Penzym		Readings with Delvotest	
	Not-dissolved honey	20% solution	Not-dissolved honey	20% solution
1	0	-	+	+
2	0	-	+	+
3	0	+	+	+
4	0	+	-	-
5	0	-	+	+
6	0	-	+	+
7	0	-	+	+
8	0	-	+	+
9	0	-	+	+
10	0	-	+	+
11	0	-	-	+
12	0	+	-	+
13	0	-	+	+
14	0	-	+	+
15	0	+	-	+
16	0	-	-	+
17	0	-	+	+
18	0	-	-	+
19	0	+	-	+
20	0	+	-	+

+ Positive reading, – negative reading, 0-reading impossible

The obtained results have been verified by comparison with results of LC-analysis, made by external laboratory. The results of this analysis are shown in Table 3. Due to veterinary recommendations, in Poland the samples contaminated with the sulfonamides (sum of all sulfonamides) in quantities above 50 ppb are classified as positive. Considering it, the 6 samples in which the residues were found, have been positive, however 2 of them contained a great quantities of sulfonamides (above 1 ppm) and in 4 samples quantities of sulfonamides have been lower than 100 ppb.

The samples 8, 9 indicated by Penzym as negative, have been classified as negative due to results of LC-analysis. However, the samples 15 and 19, which have been classified as a negative by LC, gave the positive reading with Penzym. Sample 19 contained traces amounts of sulfamethazin, but sample 15 have been not contaminated with sulfonamides. The investigation of sample 10 and 20 showed that these honeys are contaminated with sulfonamides, especially sulfatiazole, sulfacetamide and aulfamethazin. The sample 10 was positive when tested with Delvotest and sample 20 was positive when tested with Penzym.

Further analysis, including testing the honey diluted in

TABLE 3. The content of antibiotics in positive honey samples.

Sample Code	Tested sulfonamides	Content of sulfonamides	Sample Code	Tested sulfonamides	Content of sulfonamides
4	sulfathiazole	16 μg/kg	12	sulfathiazole	79 μg/kg
	sulfacetamid	Not detected above 10 μg/kg		sulfacetamid	26 μg/kg
	sulfamethazine	15 μg/kg		sulfamethazine	122 μg/kg
	sulfadiazyne	Not detected above 5 μg/kg		sulfadiazyne	Not detected above 5 μg/kg
	sulfamerazine	Not detected above 5 μg/kg		sulfamerazine	Not detected above 5 μg/kg
	sulfamethoksypirydazine	Not detected above 5 μg/kg		sulfamethoksypirydazine	Not detected above 5 μg/kg
	sulfamethoksazol	Not detected above 5 μg/kg		sulfamethoksazol	Not detected above 5 μg/kg
	sulfadimethoksine	Not detected above 5 μg/kg		sulfadimethoksine	Not detected above 5 μg/kg
5	sulfathiazole	20 μg/kg	16	sulfathiazole	22 μg/kg
	sulfacetamid	Not detected above 10 μg/kg		sulfacetamid	Not detected above 10 μg/kg
	sulfamethazine	35 μg/kg		sulfamethazine	35 μg/kg
	sulfadiazyne	Not detected above 5 μg/kg		sulfadiazyne	Not detected above 5 μg/kg
	sulfamerazine	Not detected above 5 μg/kg		sulfamerazine	Not detected above 5 μg/kg
	sulfamethoksypirydazine	Not detected above 5 μg/kg		sulfamethoksypirydazine	Not detected above 5 μg/kg
	sulfamethoksazol	Not detected above 5 μg/kg		sulfamethoksazol	Not detected above 5 μg/kg
	sulfadimethoksine	Not detected above 5 μg/kg		sulfadimethoksine	Not detected above 5 μg/kg
10	sulfathiazole	343 μg/kg	20	sulfathiazole	515 μg/kg
	sulfacetamid	95 μg/kg		sulfacetamid	297 μg/kg
	sulfamethazine	589 μg/kg		sulfamethazine	809 μg/kg
	sulfadiazyne	Not detected above 5 μg/kg		sulfadiazyne	Not detected above 5 μg/kg
	sulfamerazine	Not detected above 5 μg/kg		sulfamerazine	Not detected above 5 μg/kg
	sulfamethoksypirydazine	Not detected above 5 μg/kg		sulfamethoksypirydazine	Not detected above 5 μg/kg
	sulfamethoksazol	Not detected above 5 μg/kg		sulfamethoksazol	Not detected above 5 μg/kg
	sulfadimethoksine	Not detected above 5 μg/kg		sulfadimethoksine	Not detected above 5 μg/kg

buffer M (a reconstituted milk powder) and the investigating of contamination of honeys with other antibiotics, will be done.

CONCLUSIONS

1. The microbiological tests using in dairy industry cannot be adapt for detecting antibiotics in honey.
2. The using of enzymatic tests is possible on condition that diluted honeys will be tested.

REFERENCES

1. Kaufmann A., Roth S., Ryser B., Widmer M., Guggisberg D., Quantitative LC/MS-MS determination of sulfonamides and some other antibiotics in honey. J. AOAC Int., 2002, 85, 853-860.
2. Legg D.R., Baumgartner A., Salter R., Wheeler A., ROSA (Rapid one-step assay) for antibiotics in honey. Apiacta, 2003, 38, 207--217.
3. Posyniak A., Żmudzki J., Mitrowska K., Śniegocki T., Grzebalska A., Sulfonamide residues in honey. Control and development of analitycal procedure, Apiacta, 203, 38, 249-256.
4. Posyniak A., Żmudzki J., Mitrowska K., Śniegocki T., The control of veterinary drug residues in honey. 2006, *in*: Proceedings XLIII International Apicultural Scientific Conference, Puławy, Poland, pp. 224-225.
5. Posyniak A., Żmudzki J., Mitrowska K., Śniegocki T., Grzebalska A., Sulfonamide residues in honey. Control and development of analitycal procedure. Apiacta, 2003, 38, 249-256.
6. Reybroeck W., Residues of antibiotics and sulfonamides in honey oh the Belgian market. Apiacta, 2003, 38, 23-30.
7. Szczęsna T., Rybak-Chmielewska H., Pohorecka K., Preliminary study on sulfonamide residues in honey, 2006, *in:* Proceedings XLIII International Apicultural Scientific Conference, Puławy, Poland, pp. 227-228.
8. Weigel S., Gatermann R., Harder W., Screening of honey for residues of antibiotics by an optical biosensor. Apiacta, 2005, 40, 63--69.

PRÓBA ZASTOSOWANIA STANDARDOWYCH TESTÓW MIKROBIOLOGICZNYCH I ENZYMATYCZNYCH DO WYKRYWANIA ANTYBIOTYKÓW W MIODACH PSZCZELICH

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Delvotest i Penzym to standardowe testy do używane w przemyśle mleczarskim wykrywania antybiotyków. Test te wymagają minimalnego wyposażenia (pipeta, inkubator lun łaźnia wodna) i umożliwiają szybkie, proste i wzrokowe wykrycie pozostałości antybiotyków. Celem niniejszej pracy była próba zaadaptowania tych testów do oznaczania antybiotyków w miodach. Badania objęły ocenę przydatności testu mikrobiologicznego Delvotest do wykrywania pozostałości antybiotyków w miodach oraz ocenę przydatności testu enzymatycznego Penzym, a także określenie optymalnych warunków tych oznaczeń. Materiał badawczy stanowiło 20 próbek miodów krajowych. Działaniu testów poddawano miody nierozcieńczone oraz 20% (m/m) roztwory miodów.

Uzyskane wyniki wskazują, iż 90% przebadanych za pomocą Delvotestu próbek daje wynik pozytywny. Najprawdopodobniej naturalne substancje bakteriobójcze i bakteriostatyczne obecne w miodach powodują, podobnie jak antybiotyki, zahamowanie wzrostu szczepu testowego. W przypadku testu enzymatycznego stwierdzono, że nie nadaje się on do badania miodów nierozcieńczonych. Testowanie roztworów miodów wykazało, iż 5 badanych próbek zawiera pozostałości antybiotyków. Penzym można stosować do wykrywania antybiotyków w miodach rozcieńczonych.