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SOLID PHASE EXTRACTION PROCEDURES FOR VALIDATION OF CHARM II SULFONAMIDE, STREPTOMYCIN AND CHLORAMPHENICOL POSITIVES AND DETECTION OF TYLOSIN IN HONEY

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ABSTRACT

Certain interferences can cause false positive results in Charm II assays. Some floral source honeys may interfere with the Charm II streptomycin assay. Some dark honeys may interfere with the chloramphenicol assay. Therefore, presumptive positive validation methods were developed for these assays. The procedures involve solid phase extraction to remove interferences followed by the Charm II test of the extract. Positive samples from this research were confirmed by the HPLC Receptorgram method.

Hydrolysis and column extraction are honey sample preparation steps to remove PABA and cleave sugar bound sulfa drugs in the Charm II sulfonamide assay. This year a positive Charm II sample was sent for EU method verification and was found negative. Charm HPLC receptorgram analysis of sample found sulfachloropyridazine, not routinely screened for by the EU method.

Column extraction may also be used as a concentration step for antibiotic detection. A concentration step is used to detect 10ppb Tylosin.

Key Words: *Chloramphenicol/Streptomycin/Sulfonamides/Tylosin/Charm II system*

INTRODUCTION

The Charm II system is a screening method for testing antibiotics that was originally developed for milk and then adapted for testing antibiotics in honey (1, 2). Honey is a complex matrix that varies in composition due to its plant source, climate and treatment (3). Detection of some antibiotics, e.g. Tylosin, at concern levels may require removal of honey components and concentration of antibiotic. In addition it is important to ensure that the presumptive antibiotic positive samples are not due to interfering components in honey.

A simple validation method for testing presumptive positive samples was developed for streptomycin and chloramphenicol. The procedures offer screening labs a rapid and low cost validation step before HPLC or LC/MS confirmation. The procedures were modeled after the sulfonamide assay that involves hydrolysis and solid phase extraction to remove PABA interferences followed by the Charm II test of the extract (4). Positive samples after confirmation are then applied to determinative chemical methods; in this research they are confirmed by the HPLC Receptorgram method (5,

6). A simple concentration step using solid phase extraction allows detection of 10 ppb Tylosin in honey.

MATERIALS AND METHODS

Apparatus

Liquid Chromatograph: Waters 996 HPLC Dual pump with gradient programming,

Photodiode array detector¸ 200 µl injector loop and Millennium software

system (Waters, Milford, MA); Foxy 200 fraction collector (Isco, Lincoln, NE); with in-line pre-column filter.

Extraction cartridges: C18 500 mg cartridge, CH 500 mg cartridge and C18 500 mg cartridge (Varian, Harbor City, CA).

Extraction System: Vac elut sps 24 (Varian, Harbor City, CA).

Assay system: Charm II system for assay of chloramphenicol, streptomycin, macrolides and sulfonamides (Charm Sciences Inc. Lawrence, MA).

Reagents

Solvents: LC grade acetonitrile and methanol (Fisher Scientific, Pittsburg, PA), HPLC grade water system (Zenon Environmental, Randolph, MA).

HPLC Mobile Phase for chloramphenicol: Buffer A contains 22% acetonitrile and 78% 10 mM ammonium acetate at pH 4.6. Filter under vacuum through 0.45 µm filter. Buffer B contains 75% acetonitrile and 25% HPLC grade water.

HPLC Mobile phase for streptomycin: Buffer A contains 500ml Streptomycin Extraction Buffer (Charm Sciences, Inc) and 500 ml of methanol, filtered under vacuum through 0.45 µm filter. Buffer B contains 75% acetonitrile and 25% HPLC grade water.

HPLC Mobile phase for sulfonamides: Use buffer A mobile phase for chloramphenicol.

McIlvaine/EDTA Buffer: Mix 0.27 M citric acid, 0.045 M dibasic sodium phosphate, 0.13M ethylenediaminetetraacetic acid (EDTA) and adjusted to pH 4.5.

Chloramphenicol extraction buffer: Reconstitute MSU extraction buffer (Charm Sciences, Inc, Lawrence, MA) by dissolving in 1000 ml water. Mix well and bring to room temperature before use.

Streptomycin Extraction Buffer: Charm Sciences, Inc.

Charm II Test for Chloramphenicol, Charm II Test for Streptomycin, Charm II test for Macrolides or Charm II test for Sulfonamides (Charm Sciences Inc. Lawrence, MA).

Zero Control Standard: Charm Sciences Inc. Lawrence, MA.

Antibiotic Standards: All standards were purchased from U. S Pharmacopoeia Convention, Rockville, MD). Diluted stock standards and spiked samples were prepared as necessary.

Sample Preparation

Sample preparation for streptomycin: Weigh out 20.0 g of honey and add 60 ml of streptomycin extraction buffer. Mix well. Activate one C8 cartridge by pushing through 5 ml of 100% methanol. Wash cartridge with 5 ml of streptomycin extraction buffer. Add 10.0 ml of diluted honey sample (or controls) one drop at a time. Wash with 5 ml of streptomycin extraction buffer. Elute remaining residue by slowly pushing through

2 ml of 100% methanol and collect in a 13x100 mm test tube. Evaporate to dryness. Sample is ready for Charm II or HPLC analysis.

Sample preparation for chloramphenicol: Weigh out 10.0 g of honey in a 50 ml centrifuge tube and add 30 ml of MSU extraction buffer. Cap and mix well. Adjust pH to 7.5. Activate one CH cartridge by pushing through 5.0 ml of 100% methanol. Wash cartridge with 5.0 ml water. Add 5.0 ml of diluted honey sample (or positive or negative control) slowly to the cartridge. Wash with 5 ml of 20% methanol. Elute remaining residue by slowly pushing through 2 ml of 75% methanol into a 13x100 mm test tube. Evaporate to dryness. Sample is ready for Charm II analysis. The HPLC sample preparation has been previously described (5).

Sample preparation for sulfonamide HPLC with hydrolysis step: Weigh 20 g honey sample in a 50 ml centrifuge tube and add 30 ml of 1 M HCl. Mix well and incubate at room temperature for at 1 hour. Using a pH meter adjust the pH to 7.7-8.0 with NaOH. Filter the sample through the glass fiber filter. Activate one C18 cartridge by pushing through 5.0 ml of methanol. Wash cartridge with 5.0 ml water followed by additional 5 ml water. Slowly push filtered honey sample through the C18 cartridge. Wash with 5.0 ml water. Elute sample with 2.0 ml of methanol and collect eluate in a 13x100 mm test tube. Evaporate sample to dryness.

The sample preparation procedure for Tylosin involves combining 20 grams honey sample with 30ml MSU buffer and glass fiber filtering. Activate one C18 cartridge by pushing through 5.0 ml of 100% methanol. Wash cartridge with 5.0 ml water. Apply diluted honey sample (or positive or negative control) slowly to the cartridge. Wash with 5 ml of water. Elute remaining residue by slowly pushing through 3 ml of 100% methanol into a planchette and evaporate to dryness. Rehydrate sample with 1ml ZCS and add 4 ml ZCS for analysis using Charm II Macrolide Assay.

HPLC Receptorgram Analysis

The HPLC receptorgram methods for chloramphenicol and sulfonamides have been described previously (5, 6). For sample analysis dissolve dried sample extract in 75 µl of mobile phase buffer A and filter through a 0.45 µm filter into an Eppendorf vial. Wash sample with another 75 µl of mobile phase buffer A and filter through same filter. Wash filter with 75 µl of mobile buffer A. Prior to loading sample, flush the sample loop with 1 ml of methanol followed by 1 ml of mobile phase buffer A and repeat two additional times. Load sample into the sample loop and then inject. Also, start fraction collector to collect fractions and the streptomycin peak. Elute sample at 1 ml/min with gradient that starts with 100 % A at zero time. At 5 min start increasing the %B so at 16 min the % B is 100. Continue %B at 100 for 2 min and then at 19 min drop the %B to 0. The time window for the streptomycin peak is determined by injecting 200 µl of a 1000 ppb streptomycin standard and monitored the absorbance at 210 nm. Set the time window for fraction collection by taking 1 minute on either side of the calculating retention time. The fractions are then dried in a plachette over a hot plate. Each fraction and the streptomycin peak is dissolved and assayed according to Charm II assay for streptomycin.

RESULTS AND DISCUSSION

Of 29 honey samples supplied by a laboratory 12 honeys were Charm II Streptomycin presumptive positive, 11 of which were from an acacia floral source, Table 1.

Table 1. Charm II and Charm II validation method results for 29 European honey samples tested for Streptomycin.

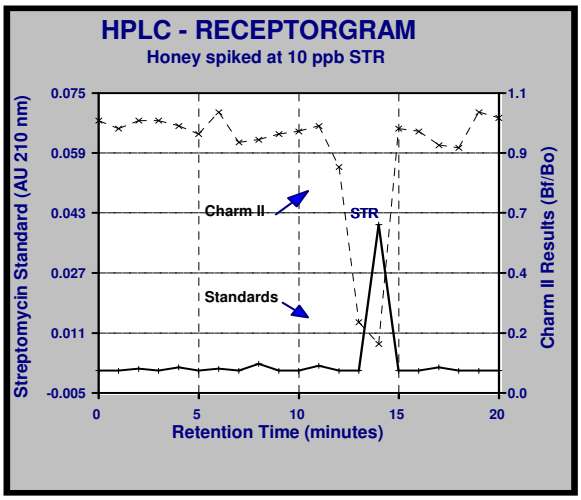
Initial Charm II			**Validation Method**	
sample number	average cpm (N=2)	result*	average cpm (N=2)	result
1	2214	not found		
2	2204	not found		
3	1423	not found		
4	2668	not found		
5	2376	not found		
6	2215	not found		
7	2737	not found		
8	1188	PP	3106	not found
9	1082	PP	3224	not found
10	2246	not found		
11	3108	not found		
12	1381	PP	3300	not found
13	1325	PP	2796	not found
14	1439	not found		
15	1120	PP	2887	not found
16	1052	PP	2952	not found
17	2568	not found		
18	1314	PP	2799	not found
19	2496	not found		
20	1210	PP	2664	not found
21	2258	not found		
22	1882	not found		
23	1273	PP	2501	not found
24	1643	not found		
25	1314	PP	2462	not found
26	1738	not found		

27	852	PP	461	PP
28	1652	not found		
29	2367	not found		
negative	1903	not found	2338	not found
10 ppb spiked	1151	PP	1073	PP

*PP= presumptive positive, cpm is counts per minute

Initial Charm II control point is the cpm average of 10 ppb spike + 20 %(1381). Validation control point is the average 10 ppb spike + 30% (1395). Numbers higher than the control point are considered "not found" while numbers equal or less than the control point are considered presumptive positive at action level 10 ppb streptomycin.

Validation method on the 11 acacia honey samples found all 11 as negative samples. Sample 27 which was not an acacia honey sample remained as a presumptive positive and was analyzed by HPLC receptorgram analysis (Figure 1A, 1B). The sample showed a positive result on the Charm II streptomycin assay in the fraction corresponding to the streptomycin retention time.



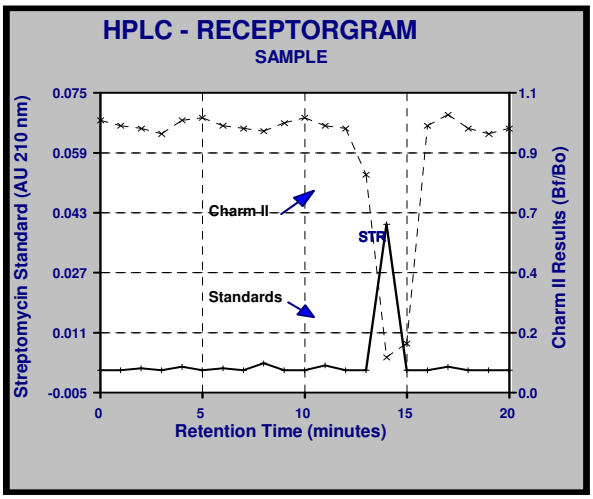


Figure 1A. HPLC Receptorgram for 10 ppb streptomycin (STR) spiked honey

Figure 1B. HPLC Receptorgram for streptomycin (STR) sample 27

Of 123 Charm II Chloramphenicol honey samples supplied to Charm Sciences in 2003, 16 honey samples were analyzed by all three methods: initial Charm II chloramphenicol assay, validation procedure, and HPLC receptorgram. Results are reported in Table 2. In the initial assay 8 samples tested "not found" and 8 samples were presumptive positives. Of the 8 presumptive positive samples 5 were not found after the validation method and 3 remained presumptive positives. The 3 presumptive positives after the validation method were all confirmed as positive for chloramphenicol by the HPLC receptorgram. The 5 presumptive positives that were not found after the validation method were confirmed as negative by the HPLC receptorgram.

Table 2. Initial Charm II screening, Validation Method and Receptorgram results for Chloramphenicol.

Initial Charm II			**Validation Method**		**HPLC Receptorgram**
sample number	average cpm	result	average cpm	result	result
1	1133	PP	1035	not found	negative
2	1344	not found	NR		negative
3	1408	not found	NR		negative
4	1192	PP	973	not found	negative
5	1218	PP	1044	not found	negative
6	1507	not found	NR		negative
7	1140	PP	1150	not found	negative
8	1240	not found	1058	not found	negative
9	1334	not found	1051	not found	negative
10	1763	not found	NR		negative
11	1201	PP	1017	not found	negative
12	1549	not found	NR		negative
13	1010	PP	812	PP	positive
14	1011	PP	767	PP	positive
15	1588	not found	NR		negative
16	1145	PP	846	PP	positive
negative	1548	not found	1172	not found	negative
0.2 ppb spiked	1027	PP	690	PP	positive

NR=not run
PP=Presumptive Positive

Initial Charm II Control point positive is determined from Negative Control Average (N=6) - 20 %= 1238. Validation method control point is determined from Negative average cpm - 20% = 938. Numbers higher than the control point are considered “not found” while numbers equal or less than the control point are considered presumptive positive at action level 0.1 ppb chloramphenicol

In 2003 a test laboratory reported a positive sulfonamide sample by the Charm II Sulfonamide assay that was determined negative by the EU method verification. This sample was subjected to the sulfonamide receptorgram method and was found to contain sulfachloropyridazine which is not identified in the 11 drug EU method. A

later eluting sulfonamide active peak remains unidentified and could be another unidentified sulfonamide drug (Figure 2).

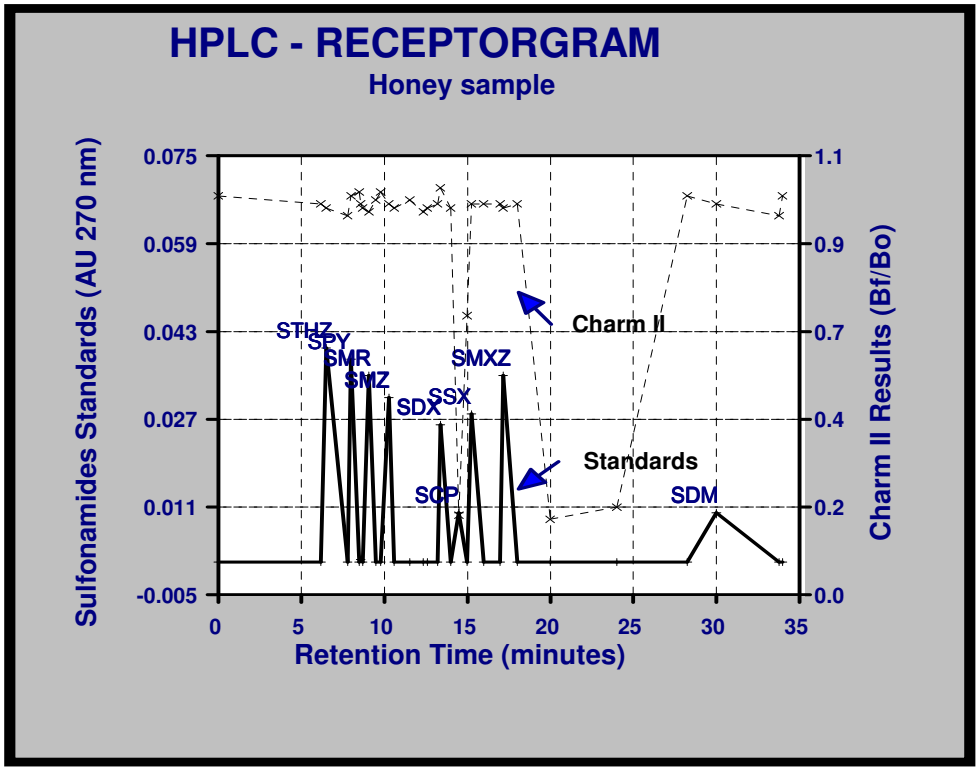


Figure 2. HPLC receptorgram for sulfonamide honey sample STHZ= sulfathiazole SDZ=sulfadiazine, SPY=sulfapyridine, SMR=sulfamerazine, SMZ=sulfamethazine, SSX=sulfisoxazole, SCP=sulfachloropyridazine, SDX= sulfadoxine, SMXZ= sulfamethoxazole, SDM=sulfamethoxine

Fortified honey with 10ppb Tylosin is detected with a 71% B/Bo (Table 3).

Table 3- Results of Charm II Macrolide test with 10 ppb fortified tylosin

sample	CPM	result
Unfortified	2112	not found
fortified	1510	PP

PP=Presumptive
PositiveControl point to determine presumptive positive is determined from Unfortified honey (N=2) - 20 %= 1690. B/Bo (CPM fortified/CPM unfortified) = 0.71

CONCLUSION

The validation method for each antibiotic has been incorporated as an appendix to the Charm II manuals for honey. A presumptive positive sample should be validated using these methods to remove possible interferences and verify that the sample contains antibiotic. Only verified positive samples should be sent for more expensive

HPLC or LC/MS confirmation procedures. Tylosin detection at 10ppb is possible using solid phase extraction as a preparation/concentration step.

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