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Research Note

Modifications and Adaptations of the Charm II Rapid Antibody Assay for Chloramphenicol in Honey

SARAH E. McMULLEN,* JOHN A. LANSDEN, AND FRANK J. SCHENCK

U.S. Food and Drug Administration, Southeast Regional Laboratory, 60 Eighth Street N.E., Atlanta, Georgia 30309, USA

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ABSTRACT

The Charm II screening method for the presence of chloramphenicol in honey has a sensitivity of 0.3 ppb. This screening method is a simple, rapid antibody assay using [^{3}H]chloramphenicol and a binding reagent. Analysis of different types of honey revealed considerable differences in results. Honey can be liquid, crystallized (creamed), or partially crystallized and is classified by the U.S. Department of Agriculture into seven color categories: water white, extra white, white, extra light amber, light amber, amber, and dark amber. Fortified and nonfortified liquid amber honey tested appropriately with the Charm II unit and the negative control provided with the unit after slight modifications were made. However, approximately 70% of creamed honey samples fortified at 0.6 ppb did not test positive for the presence of chloramphenicol using the provided negative control. Matrix quenching effects were evaluated, and these effects were accounted for by establishing different assay conditions for different honey types.

Recently, chloramphenicol, a broad spectrum antibiotic, has been discovered in several food commodities from Asia, including honey. Because of serious reactions, such as aplastic anemia, that can occur in susceptible individuals *(10),* chloramphenicol is allowed for human use only when other antibiotics fail. It is banned in the United States for use in food-producing animals, including bees *(5, 11).* Inspectors in Canada, the United Kingdom, the European Union, and Japan have found chloramphenicol in honey from China *(9).* Recently uncovered transshipping schemes have indicated that China is not the only country of concern *(1).* The current Food and Drug Administration–approved method for detection of chloramphenicol in honey involves liquid chromatography and mass spectrometry (LC-MS) *(12).* This method includes extraction with ethyl acetate, defatting with hexane, and solid phase extraction cleanup before analysis by LC-MS. The instrumentation time is also lengthy; run time for each sample is 28 min (not including standards and blanks). A method for screening samples before proceeding to the confirmation method could save time and money. The screening method evaluated here is the Charm II assay (Charm Sciences, Lawrence, Mass.) for antimicrobial drugs in honey *(4).* Sample preparation involves dilution of the honey in aqueous buffer, and the total time to run the assay (for four samples) is approximately 25 min.

Because honey is a complex matrix, problems with the Charm II assay can occur. The U.S. Department of Agriculture (USDA) classifies honey types as liquid (free from visible crystals), crystallized (solidly granulated or crystallized, often referred to as creamed honey), and partially crystallized (mixture of liquid and crystallized) *(7).* These types are not indicators of quality but rather reflect methods of processing and preparation. Many countries utilize more creamed honey than liquid honey. The demand for creamed honey has increased in many countries, including the United States.

The USDA has seven color designations for honey: water white, extra white, white, extra light amber, light amber, amber, and dark amber. Color is not an indicator of quality and varies with the nectar source *(2, 5),* and there are many different nectar sources in North America *(8).* The two main sugars in honey are glucose and fructose, and the ratio of these two sugars influences the viscosity of the honey *(3).*

Additives to enhance honey nutrition or taste have become increasingly popular. Because of the wide array of additives, including propolis, pollen, royal jelly, eucalyptus, strawberries, pistachios, hazelnuts, black seed oil, mumie, herbs, and black seed oil, all honey cannot be considered the same. Adaptations of the Charm II assay were made to account for various types of honey.

MATERIALS AND METHODS

Charm II assay. The Charm II assay is a rapid antibody assay that uses a binding reagent and [^{3}H]chloramphenicol. The sample extract is incubated with a binding reagent to allow any sample chloramphenicol to bind to the binding reagent. The [^{3}H]chloramphenicol is added and incubated to allow the [^{3}H]chloramphenicol to bind to the binding reagent. The sample is centrifuged, and the supernatant is discarded. The remaining pellet is reconstituted in a small amount of water, and scintillation fluid is added. The amount of [^{3}H]chloramphenicol in the sample is measured using a scintillation counter. A control point (the point above which a sample is considered to be negative for the pres-

* Author for correspondence. Tel: 404-253-1200, Ext 5370; Fax: 404-253-1208; E-mail: smcmulle@ora.fda.gov.

ence of chloramphenicol and below which the sample is considered to be positive for the presence of chloramphenicol) must be established before any unknowns are tested. This point is established by averaging the counts per minute for six negative controls and subtracting 20%. The negative controls used for the liquid honey assay are provided by Charm Sciences, and those for the creamed honey assay are known chloramphenicol-free (by MS; limit of detection [LOD], 0.5 ppb) creamed honey samples.

Control point determination and daily performance monitoring, liquid honey method. The liquid honey negative control (Charm Sciences) is reconstituted in MSU extraction buffer (Charm Sciences) according to the manufacturer's instructions. The positive control is made by taking 6 ml of diluted negative control and adding 300 μl of diluted MSU multi-antimicrobial concentrate standard (MSUMA, Charm Sciences; reconstituted in deionized water) (300 μl of standard in 15 ml of MSU extraction buffer). The pH of the sample is obtained with pH indication strips (for pH 7 to 14) and adjusted to 7.5 with M2 buffer (Charm Sciences; reconstituted with deionized water) if necessary.

Control point determination and daily performance monitoring, creamed honey method. Creamed honey (10 ± 0.05 g) known to be free of chloramphenicol (by MS; LOD, 0.5 ppb) is dissolved in 30 ml of MSU extraction buffer, producing the negative control. The positive control is made by taking 6 ml of diluted negative control and adding 300 μl of diluted MSUMA standard (300 μl of standard in 15 ml MSU extraction buffer). The pH of the sample is adjusted to 7.5 with M2 buffer as necessary.

Preparation of sample to be tested. A sample of honey to be tested (10 ± 0.05 g) is diluted with 30 ml of MSU extraction buffer and vortexed until a homogeneous mixture is obtained. The pH of the sample is adjusted to 7.5 with M2 buffer as necessary.

Charm II method. Different assays for the creamed honey and liquid honey should be set up in the C2Soft Program for Honey (Charm Sciences) according to the manufacturer's instructions.

After the control and test samples have been prepared, they can be used in the Charm II assay. A white binding reagent tablet (ATBL Reagents, Charm Sciences) is added to a borosilicate test tube (13 by 100 mm, with cap) with 300 μl of deionized water and then vortexed (Thermolyne Maxi Mini II or equivalent). Five milliliters of diluted sample is then added to the tube, which is vortexed again causing the contents to rise and fall 10 times. The tubes are then incubated in a heating block (Charm adjustable temperature heating block or equivalent heating block) at 50°C for 3 min. The tubes are vortexed again (rise and fall 10 times) and incubated at 50°C for another 3 min. A green [^{3}H]chloramphenicol reagent tablet (ATBL Reagents, Charm Sciences) is then added, and the tube is vortexed (rise and fall 10 times) and then incubated at 50°C for 3 min. After incubation, the tube is spun in a centrifuge (Heraeus Sepatech Labofuge 200 or equivalent fixed-angle centrifuge) for 10 min at 5,000 rpm. The supernatant is then immediately decanted without disturbing the pellet. The pellet is then redissolved by vortexing with 300 μl of deionized water. Opti-Fluor (PerkinElmer Life Sciences, Inc., Boston, Mass.) scintillation fluid (3 ml) is added, and the tube is capped, vortexed, and then immediately placed in the analyzer (Charm II 7600 with C2Soft software). The results are obtained from the ^{3}H channel. The scintillation fluid must not be added until the sample is ready to read because the counts per minute rises over time after the addition of the scintillation fluid. Optimal reading time is within 3 min of the addition of scintillation fluid.

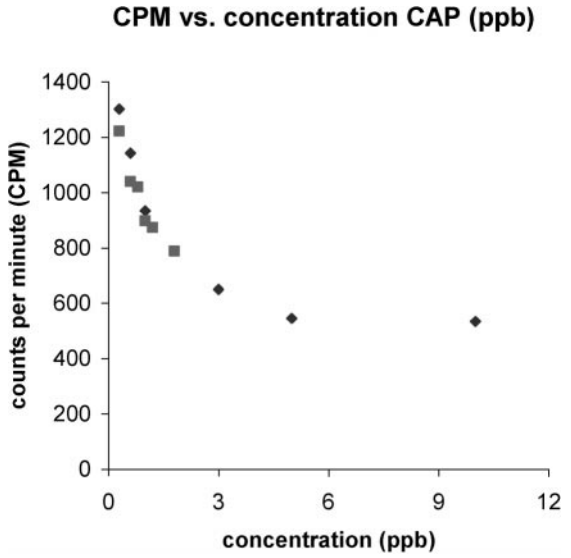

FIGURE 1. *Standard curves (◆ = 0.3 ppb to 10 ppb; ■ = 0.3 ppb to 1.8 ppb) using liquid amber honey.*

The control point is determined for every lot of reagent tablets because the counts per minute differ among lots. An average of three positive controls is also obtained. A positive and negative control should be run every day that analyses are performed for daily performance monitoring. Daily performance monitoring is not necessary on the day the control point is established.

RESULTS AND DISCUSSION

Over 500 Charm II assays were run on many different types of honey that also had been tested using the LC-MS method. The counts per minute for the initial assays of the negative control (mean negative control = 1,388 cpm; control point [CP] = 1,110 cpm) were lower than the mean counts provided by Charm Sciences (mean negative control = 1,617 cpm; CP = 1,294 cpm) for that particular lot (ATBL003), resulting in a lower than expected control point being established. This result was not a particular concern until analysis of commercial brand liquid amber honey (known chloramphenicol free by MS; LOD, 0.5 ppb) produced positive results (1,036 cpm) with the Charm II assay. The centrifuge used for this determination was a swinging arm centrifuge with trunions for test tubes measuring 13 by 100 mm. Replacement of this centrifuge with a fixed-angle centrifuge (Heraeus Sepatech Labofuge 200; 5,000 rpm) caused more of the radioactive pellet to come out of solution on the walls of the test tube, raising the counts per minute (mean negative control = 1,532 cpm; CP = 1,226 cpm) to close to the mean range for the lot provided by Charm Sciences. The commercial brand of liquid amber honey tested negative (mean, 1,693 cpm; $n = 2$) for chloramphenicol using the new control point determined with the new centrifuge. The two standard curves (covering different concentrations) using the same commercial brand honey are shown in Figure 1. At higher fortification levels, the differences in response become negligible. Using the fixed-angle centrifuge, this assay was considered an acceptable screening method for liquid amber honey in our laboratory.

Because scintillation counters measure only net activity (counts per minute), many factors can affect this measurement. Quenching is defined as the reduction of counting efficiency. There are three different types of quenching. Point or physical quenching occurs as the result of incomplete solubilization of the sample in the scintillation fluid. Chemical quenching occurs when various compounds in solution decrease counting efficiency by interaction with excited solvent or fluor molecules. Color quenching occurs when the color components of the sample absorb photons emitted from the fluor before detection by the photomultiplier tubes. Each type of quenching affected the Charm II assay.

Honey with additives presented a new set of problems. Using the control point set with the Charm negative control, many honeys with additives (known chloramphenicol free by MS; LOD, 0.5 ppb) were positive for chloramphenicol by the Charm II assay. However, some of these additives were not soluble (e.g., crushed herbs, strawberries, and eucalyptus) and could be seen suspended in the diluted honey used for the analysis. These particulates precipitated out of solution during centrifugation and when resuspended in scintillation fluid for analysis provided physical quenching, leading to lower counts per minute and creating false positive results. This problem was solved by filtering diluted honey that contained obvious particulates.

Another significant problem was encountered in the analysis of creamed honey. Creamed honey is generally much lighter than liquid honey, ranging from white to yellow in color. The total sugar content is comparable to that of liquid honey, but the ratios of the main sugars, glucose and fructose, vary greatly from those in liquid honey. The counts per minute for the creamed honey (known chloramphenicol free by MS; LOD, 0.5 ppb) were much higher (mean, 2,110 cpm; $n = 7$) than the values for the liquid honey (mean, 1,693 cpm) and the Charm negative control (mean, 1,532 cpm). Although chloramphenicol was not detected in these samples, the following question remained: if chloramphenicol were present, would the counts drop enough for the Charm II assay to detect the presence of chloramphenicol? Unfortunately, the results of further testing indicated that chloramphenicol could be present and yet remain undetected by the assay. Approximately 70% of creamed honeys fortified with chloramphenicol at 0.6 ppb (mean, 1,285 cpm; $n = 7$) escaped detection using the Charm negative control (CP = 1,226 cpm). Apparently, the quenching in the analysis of the amber liquid honey was not present to the same extent in the creamed honey samples, as indicated by the higher counts. The first factor in the additional quenching is color. The amber color of the liquid honey absorbed some of the emitted photons before detection by the photomultiplier tube. This hypothesis was supported by analysis of a control blank (sugar, MSU extraction buffer, binding reagent, and [^{3}H]chloramphenicol reconstituted with water before addition of scintillation fluid; mean, 3,157 cpm; $n = 4$) versus a similar control blank reconstituted in caramel-colored water before addition of scintillation fluid (mean, 2,547 cpm; $n = 2$). The count for a caramel-colored control (no sugar; 3,270 cpm) was much lower than that of a clear control blank (3,880 cpm).

An additional factor in quenching is the sugar ratio. The Charm negative control was 10% honey and 90% sucrose, with a golden tan color. Because sucrose is only a minor and sometimes absent component of honey, the Charm negative control does not adequately represent the creamed honey matrix. The ratio of fructose to glucose, one of the determinants of liquid versus creamed honey, influenced the counts per minute. More work should be done before accepting a trend in the data. A new assay was established, and a known chloramphenicol-free (by MS; LOD, 0.5 ppb) creamed honey was used to determine a new control point (mean negative control = 2,037 cpm; CP = 1,629 cpm). Using this new control point, the fortified (0.6 ppb) creamed honeys that escaped detection by the unmodified assay tested positive for the presence of chloramphenicol.

Work is currently underway using the AOAC International official method 985.25 for color determination of honey *(6)*. A color range to determine proper assay parameters will be established. Determination of the sugar content of various honeys is also important. Total sugar content and moisture percentage (from the refractive index) were easily obtained with a refractometer. Separation and quantification of sugars in honey is being investigated using a high-performance anion exchange LC method with pulsed amperometric detection.

Honey is a difficult matrix to analyze because of intrinsic differences in color, additives, and sugar content. The Charm II assay was an adequate screening method for liquid amber honey using a fixed-arm centrifuge. Physical quenching in honey with additives was eliminated by simple filtration. A new assay was established for creamed honey to account for the color quenching present in the liquid amber honey and the chemical differences in sugar content. The Charm II assay can be used as a screening method for liquid and creamed honey with the modifications outlined here.

REFERENCES

1. Anonymous. 2002. U.S. Customs Service and Food & Drug Administration uncover dumping scheme involving contaminated honey imports from China. U.S. Customs Service and Food and Drug Administration press release 28 August 2002. Available at: http://www.fda.gov/bbs/topics/news/2002/new00831.html.
2. Azeredo, L. da C., M. A. A. Azeredo, S. R. de Souza, and V. M. L. Dutra. 2003. Protein contents and physicochemical properties in honey samples of *Apis mellifera* of different floral origins. *Food Chem.* 80:249–254.
3. Bhandari, B., B. D'Arcy, and S. Chow. 1999. Rheology of selected Australian honeys. *J. Food Eng.* 41:65–68.
4. Charm Sciences, Inc. 2002. Charm II test for antimicrobial drugs in honey. Charm Sciences, Inc., Lawrence, Mass.
5. Code of Federal Regulations. 2000. Drugs prohibited for extralabel use in animals. 21 CFR 530.41. U.S. Government Printing Office, Washington, D.C. Available at: http://frwebgate.access.gpo.gov/cgi-bin/getcfr.cgi?TITLE=21&PART=530&SECTION=41&YEAR=2000&TYPE=TEXT.
6. Cunniff, P. (ed.). 1995. Method 985.25. *In* Official methods of analysis of AOAC International, 16th ed. AOAC International, Gaithersburg, Md.

7. Federal Register. 1985. United States standard for grades of extracted honey, section 52.1391–52.1405. *Fed. Regist.* 50:15861.
8. Gheldof, N., X. Wang, and N. Engeseth. 2002. Indentification and quantification of antioxidant components of honeys from various floral sources. *J. Agric. Food Chem.* 50:5870–5877.
9. Louisiana Register. 2002. Chloramphenicol in honey: testing and sale. *La. Regist.* 28:1684–1686. Available at: http://www.state.la.us/osr/reg/0208/0208EMR.pdf
10. Schenck, F. J., and P. S. Callery. 1998. Chromatographic methods of nanalysis of antibiotics in milk. *J. Chromatogr. A* 812:99–109.
11. Settepani, J. 1984. The hazard of using chloramphenicol in food animals. *J. Am. Vet. Med. Assoc.* 184:930–931.
12. Turnipseed, S., C. Burns, J. Storey, R. Lee, and A. Pfenning. 2002. Confirmation of multiple phenicol residues in honey by electrospray LC/MS Laboratory information bulletin no. 4281. U.S. Food and Drug Administration, Division of Field Science, Rockville, Md.