CHARM II SYSTEM - COMPREHENSIVE RESIDUE ANALYSIS SYSTEM FOR HONEY

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Abstract

Antibiotics and pesticides in agricultural foods is a recurrent problem. Publication of reports of chloramphenicol in honey has recently affected the honey industry. Milk, meats, farmed fish, eggs and honey are all produced in agricultural environments that utilize the benefits of modern medicine (antibiotics) and pest control. Residues in foods create trade disputes, public health consequences and consumer perception problems that have enormous negative economic impact on the food industry. A rapid screening program of raw materials prior to purchase or sale is the solution. The Charm II system is a multi-analyte receptor assay system that has proven rapid, robust and reliable at all levels of farm to table food production.

The Charm II is a scintillation based detection system for chemical families of drug residues utilizing class specific receptors or an antibody in immuno-binding assay formats. Results are numerical counts. The first Charm test for beta-lactams in milk became AOAC-A1 method in 1981. The Charm II assay for beta-lactams, tetracyclines, macrolides, aminoglycosides, sulfa drugs and chloramphenicol in milk became AOAC-A1 method in 1989.

Data are presented that show Charm II sensitivity in fortified raw and heat processed honey to beta-lactam, tetracycline, aminoglycoside, sulfa drugs, macrolide and amphenicol antibiotics. The amphenicol assay modification to detect 0.3ppb chloramphenicol is used as an example to explain how sensitivity is determined from probit analysis using 90% detection with 95% confidence parameters. Results of commodity honey samples screened in a certified lab for chloramphenicol, tetracycline and streptomycin are reported. These samples are predominately from South East Asian region and demonstrate multi-analyte contaminations and positive rates as high as fifty percent.

HACCP analysis and preventative raw material screening have been the solution to antibiotic contamination of food. Implementation of these programs makes the food industry proactive rather than reactive.

Introduction

Antibiotics have been a problem contaminate in foods for many years[1]. The first case of economic loss to the food industry due to antibiotic contamination was loss of fermented product (cheese) to the dairy industry. To prevent loss, industry has utilized screening assays to identify antibiotic contamination. The first assays were microbial inhibition assays that took several hours to a day to develop[2, 3]. The demand for

rapid testing prior to raw material purchase led to development of rapid diagnostic type assays that work in minutes.

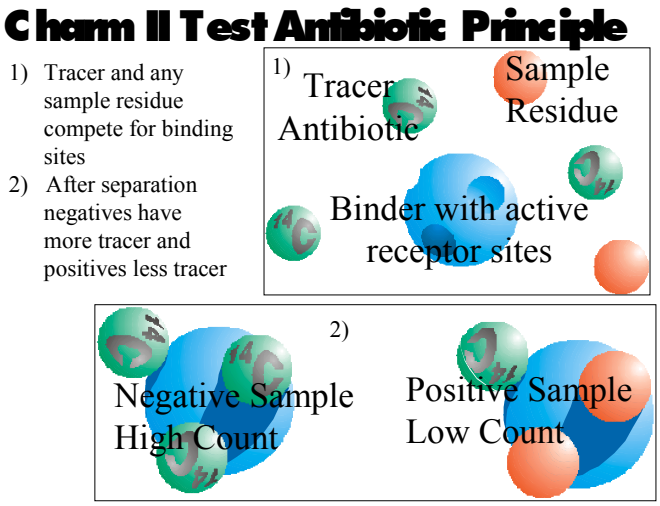
The Charm Test (microbial receptor assay) was the first rapid test developed for the dairy industry[4]. The microbial receptor assay principle was robust in industry environments and broadly specific to entire drug class families of antibiotics. It was expanded to the Charm II Test to include tests for a comprehensive list of antibiotics and organo-phosphate and carbamate pesticides[5].

Antibiotic screening programs for foods have generally followed a pattern of voluntary industry screening in a HACCP (Hazard Analysis Critical Control Point) approach to prevent economic loss (or to protect product image) to subsequent mandatory regulatory screening. The dairy industry is the most advanced in programs that protect product contamination. The current model in the US (Appendix N of the Pasteurized Milk Ordinance) comprises mandatory screening for the most common cow antibiotics in trucks/lorries transporting product to dairies, mandatory reporting of positives with trace back and penalty to the farmer source, and random spot checks of industry samples by public health officials[6]. A similar model of dairy practice is a recommended EU guideline and is becoming mandatory in some EU countries[7].

Other food industries have also been impacted by antibiotic contamination. Meat residues have caused loss of fermented sausage. International commerce departments have screened meat residues and rejected freighters loads of material back to origin countries. Finfish and shrimp from aquaculture have also presented positive in importing countries commerce labs and have resulted in product bans in some EU countries[8]. Honey manufactured by bees treated for bacterial ailments have also contained antibiotic residues. Some EU countries have banned Chinese honey and their products[9]. Honey like milk has a purity image that is critical to maintain consumer confidence. These industries will need to develop control mechanisms that are proactive to prevent antibiotic contamination and maintain product image. Mandatory HACCP regulations are already implemented for the meat and fish industries.

The robustness of the Charm II assay has allowed adaptation of the assay to include other matrices such as tissue, eggs, grains and honey. Economic pressures to screen the test in a raw material HACCP plans favor rapid diagnostics. The Charm test adapted to honey is presented in this report. The test can be done in 12-20 minutes for

a multitude of antibiotics. Levels of sensitivity match current reported sensitivities of LC-MS and HPLC methods being utilized in EU for drug confirmation. Results of field honey samples (predominately from SE Asian origin) sent for analysis are presented.



Methods

Charm II Principle- The Charm II uses H3 and C14 tagged drug tracers with broadly specific binding agents in a receptor assay format. (See figure). Samples with high count (CPM) results are considered negative while samples with low count are considered positive. There are separate reagents for each antibiotic drug class. Honey is diluted 1 part to 3 parts supplied MSU buffer and pH adjusted to 7.5 with M2 buffer. This honey extract has active reagents added in sequential and competitive assay formats at various incubation temperatures (see Table I) optimized for drug detection.

The detection reaction is stopped with a centrifugation step where unbound tracer is separated from bound tracer-binder complex. The pellet (tracer-binder complex) is analyzed in a scintillation counter for 1 minute to give a resulting count. The higher the count, the less drug contamination in the sample. The lower the count, the more drug contamination in the sample. The result is simplified to a present/absent result using a control point. The control point is a number determined from a negative reference (2 SD less than average negative count) or positive spiked sample (2 SD greater than positive count) . Samples with counts greater than the control point are considered negative for drug presence, while samples with counts equal or less than the control point are considered presumptive positive for drug presence. Control points are based on LOD (limit of detection) principles by subtracting 2 or 3 standard deviations (expressed as a percentage of the average count) from a zero count average, or can be based from spiked samples at a specific detection level and adding 2 to 3 standard deviations to the count average to assure a high confidence in detecting that concentration as positive (see Table II).

Table I- Charm II Assay Formats and Detection Levels

Drug Class	Assay Style and Timing Sequence	Incubation Temperature (°C)	Total Time	Target Detection Level* (ppb)
Beta-Lactam	Sequential 2min and 2min	55	12 min	50 Penicillin G
Tetracycline	Competitive 5min	35	12 min	20 Chlortetracycline
Aminoglycoside	Sequential 2min and 2 min	35	12 min	10 Streptomycin
Macrolide	Sequential 2min and 2 min	55	12 min	200 Erythromycin
Chloramphenicol	Sequential 6min and 3min	50	20 min	0.3-0.4 Chloramphenicol
Sulfonamides†	Competitive 3min	85	1 hour†	10 Sulfamethazine
Organo-phosphates and Carbamates	Sequential 10min and 5min	35		20ppb Carbaryl

*Target detection level is to a particular drug considered representative of the class of drug. In most cases the assay is cross-reactive to the entire drug family. Within family drug cross-reactive levels and drug LOD will be different from this reported value.
†Sulfonamide assay uses a more complex acid hydrolysis and reverse phase preparation procedure to eliminate PABA (para-aminobenzoic acid) interference and to release sulfathiazole sugar complexes if present.

Table II- Charm II Assay Control Point Calculation

Drug Family	Reference Sample	% Of Count to add or subtract from Reference Sample Average
Beta-Lactam	Negative Control	-20%
Tetracycline	20 ppb Chlortetracycline	+20%
Aminoglycoside	10 ppb Streptomycin	+20%
Macrolide	200ppb Erythromycin	+30%
Chloramphenicol	Negative Control	-20%
Sulfonamides	10ppb Sulfamethazine	+25%
Organo-phosphates and carbamates	Negative Control	-40%

Determination of Sensitivity- Negative samples are spiked with USPC (United States Pharmacopoeia Convention) [10] standard drugs at concentrations above and below their detection levels. Multiple replicates (15 or 30) at each concentration are randomized and blind coded along with negative samples and run in a single experiment. Results (%positive/number tested) are analyzed via statistical analysis (probit, gombit, logit) to determine the 90% detection level with 95% confidence [11].

Test of Natural Samples- Raw and pasteurized samples of multiple types (clover, alfalfa, early season, late season, crystallized, light, dark) are analyzed versus control points. Positive samples are confirmed by additional HPLC analysis to verify the sample as true positive. After a low false positive rate is verified, negative samples are spiked at the sensitivity level and tested to verify a low false negative rate.

Incurred Samples- When possible samples that are naturally contaminated, and verified positive by alternative methods, are collected and analyzed [12, 13]. Naturally incurred samples should test appropriately on the method.

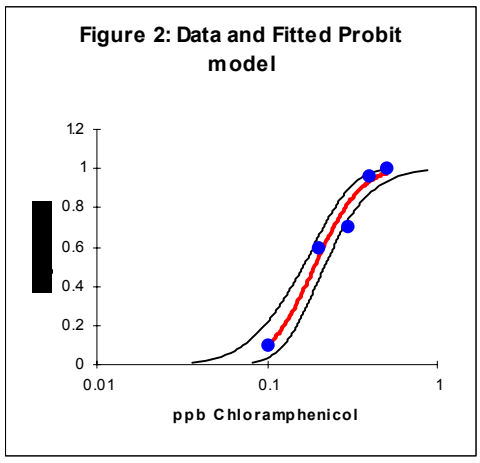
Results and Discussion

Sensitivity determination is exemplified with the Charm II Chloramphenicol Assay in honey. Table III lists the drug concentration, the number of positives encountered at each concentration versus the number tested and the % positive rate.

Table III: Blind Study Results of Fortified Honey Samples Tested by the Charm II Chloramphenicol Assay

Chloramphenicol Concentration (ppb)	Number of Positives	Number of Samples	% Positive
0	0	30	
0.1	3	30	10
0.2	18	30	60
0.3	21	30	70
0.4	29	30	97
0.5	30	30	100

Probit statistical analysis is demonstrated in Figure 2. The 90 percent positive rate with 95% confidence is calculated at 0.43ppb.



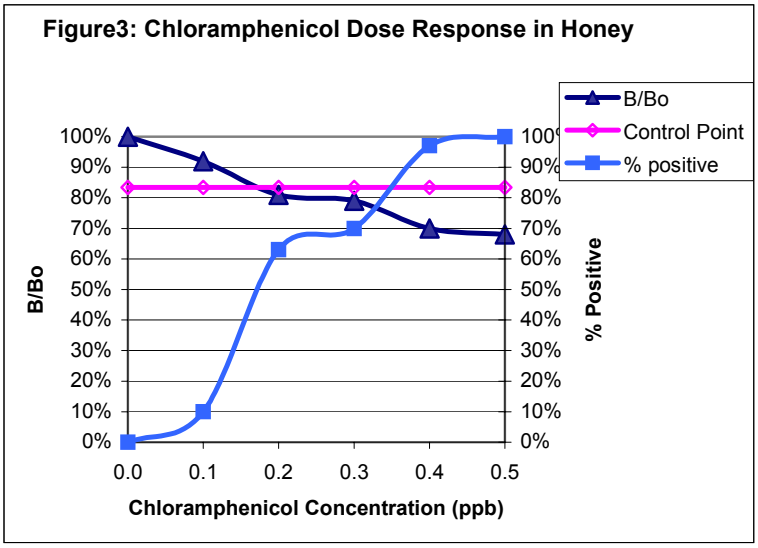


Figure 3 is a standard curve of the raw count averages normalized with the zero value (B/Bo) versus concentration. This is termed a concentration response or dose response curve. The control point represents an interim B/Bo between zero concentration and the target/detected concentration of an assay; and the control point determines positive and negative interpretation.

Information contained in Figure 3 is summarized in Table 4 for all the Charm II tests in honey. Table IV lists the determined B/Bo value each Charm II assay's target sensitivity level (listed in Table I) and the approximate B/Bo value of the assay control point. These sensitivities are similar to the reported sensitivities of LC-MS and HPLC methods currently being utilized to regulate antibiotic contamination in honey[14]. It should be noted that the Charm Test detects cumulative effects of drugs within the same family and the biologically active drug metabolites while HPLC and LC-MS methods detect specific drug compounds. Therefore the Charm sensitivities tend to err on the side of safety when compared to determined levels by the confirmation procedures.

Table IV: Charm II Assay in honey Dose Response Curve Performance

Drug Family	Reference Standard	B/Bo of Standard	B/Bo of Control Point
Beta-Lactam	50 ppb Penicillin G	0.37	0.71
Tetracycline	20 ppb Chlortetracycline	0.64	0.77
Aminoglycoside	10 ppb Streptomycin	0.59	0.70
Macrolide	200ppb Erythromycin	0.40	0.52
Chloramphenicol	0.3ppb Chloramphenicol	0.81	0.75
Sulfonamides	10ppb Sulfamethazine	0.46	0.58
Organo-phosphates and Carbamates	20ppb Carbaryl	0.35	0.6

Assay sensitivity determination is based on drug fortifying a few honey sample known to be negative. It is also important to evaluate assay effectiveness in screening a variety of different samples and those samples fortified with drug. Table V lists a variety of off

shelf honey samples screened on the chloramphenicol assay and the same honey samples spiked with 0.3ppb chloramphenicol.

Table V: Negative Market Honey Samples Tested on Charm II Chloramphenicol Assay and then Fortified with 0.3ppb Chloramphenicol

Honey Type	Charm II Chloramphenicol (CPM)	0.3 ppb Fortified (CPM)	0.3ppb B/Bo
Raw (Crystallized)	1617	1023	0.63
50% Canola, 50% Alfalfa	1398	1050	0.75
Heat Treated (Clear)	1339	895	0.67
Heat Treated (Clear)	1324	910	0.69
Clover (Heat treated)	1337	992	0.74
Raw (slight crystallization)	1450	988	0.68
Raw (clear)	1419	1083	0.76
Late Season	1401	950	0.68

Concern about assay effectiveness can arise with a high incidence of positives in market samples. In the case of recent chloramphenicol assay validation a large number (55/85=65%) of positive market and raw samples were identified, Table VI. The positives samples were verified as true positive by HLPC receptorgram [13] before the assay was released for use. It is possible these samples are positively biased because they are from a specific region (SE Asia) where chloramphenicol was used in bee agriculture. Table VI is results of these same market samples also analyzed for other drugs families. High incidences of other antibiotic families with multiple drug residue contaminations were found.

Table VI: Incidence of Positive in Honey Samples Provided to Lab Feb. 2002-June 2002

Drug Family	Positive Incidence (%)	Number of Samples Tested
Beta-Lactam	6	16
Aminoglycoside	62	24
Tetracyclines	31	16
Sulfonamides	45	33
Chloramphenicol	65	85

These findings are corroborated by other analyses of honey from the S.E. Asian region using various methods of analysis [15]. However S.E. Asian honeys are not the only honeys demonstrating antibiotic contamination. Between 10% and 30% tetracycline and streptomycin positive samples by Charm II and other methods are reported in honey from other production regions including countries in the EU [16, 17,18]. This indicates antibiotic use in honey is not being well controlled or monitored and appears to be a worldwide issue.

Conclusion

The Charm II Assay system effectively analyzes honey samples in little as 10-20 minutes for a complete spectrum on antibiotics and pesticides residues. The detection ranges for various drug families is similar to LC-MS and HPLC methods currently being used to regulate honey in the EU. Charm II is being used to address chloramphenicol residues that have recently appeared in honey from SE Asian origin. There is a 65% incidence of chloramphenicol at levels above 0.3ppb in honey from this geographical region. In addition there are other antibiotic residues found with high incidence in world wide produced honey that indicate a broader residue/management/control problem confronting the honey industry. HACCP programs developed by other food industries should be considered as possible model solutions to residue problems confronting the honey industry. These generally involve use of screening tests as raw material tests when bulk material is being bought and sold. If the industry wishes to protect the image of purity and health in their product they need to be pro-active in developing control programs.

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