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July 19, 2002

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BEFORE FEDERAL COURTS AND AGENCIES

Dockets Management Branch
Food and Drug Administration
Department of Health and Human Services
Room 1-23
12420 Parklawn Drive
Rockville, Maryland 20857

CITIZEN PETITION

The undersigned submits this petition under Section 402 of the Federal Food, Drug, and Cosmetic Act (FDC Act) and 21 C.F.R. § 10.30 to request the Commissioner of Food and Drug grant relief from inaccurate and unwarranted testing for chloramphenicol in imported crabmeat. This petition is submitted on behalf of Miami Crab Corporation, 10585 S.W. 109 Court, Suite 200, Miami, Florida 33176, a family owned company that imports and distributes crabmeat for human consumption.

A. ACTION REQUESTED

Miami Crab Corporation requests that FDA:

1. Immediately cease and desist from using unvalidated testing methodology to evaluate crabmeat for the presence of chloramphenicol.
2. Reinstate previous testing limits of 5 parts per billion (ppb) using existing testing methodology.

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3. Perform a health hazard evaluation relating to the exposure to naturally-occurring chloramphenicol at levels of less than 5 ppb before taking any action against crabmeat containing such levels and revise or clarify Import Alert Nos. 16-124 and 68-01 to specify limits on allowable chloramphenicol in crabmeat.
4. Provide assurances that the presence of naturally-occurring chloramphenicol in crabmeat at levels of less than 5 ppb does not result in such crabmeat being deemed adulterated.

B. STATEMENT OF GROUNDS

1. Background

Recently, the U.S. Food and Drug Administration (“FDA”) began testing samples of crabmeat imported from China for chloramphenicol. To that end, FDA was recently quoted as “upgrading its test procedures” to provide for testing at levels below the 5 ppb previously used (*see* Tab A – report from Houma Courier). We believe that the agency has recently implemented this proposed change to the testing methodology and is currently using a test method—electrospray liquid chromatography/mass spectroscopy—that has not been validated for detection of chloramphenicol in crabmeat. We also believe that, as result of use of this test method, the agency has also lowered the acceptable level for chloramphenicol from approximately 5 ppb to 1 ppb or less.

This agency testing and concerns about use of chloramphenicol in shrimp feed have led to unwarranted regulatory attention being paid to a wholly different shellfish, imported crabmeat. It has resulted in FDA’s request that Miami Crab Corporation conduct a “voluntary” recall, placed a “regulatory cloud” over imported crabmeat, and resulted in substantial marketplace uncertainty.

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These events are causing serious economic injury to Miami Crab Corporation. This harm will continue unless FDA grants the relief sought by this petition.

2. **The Electrospray Liquid Chromatography/Mass Spectroscopy Methodology Is Flawed**

The new testing methodology is described in a paper authored by FDA scientists, "Confirmation of Multiple Phenicol Residues in Shrimp by Electrospray LC/MS" (Tab B). As set forth in that paper, the new testing methodology has not been validated for assessment of chloramphenicol residues in crabmeat.

First, the test methodology assessment was performed using shrimp meat. We are not aware of any validation work performed using crabmeat. Shrimp and crabmeat are substantially different matrices of complex protein. Before use, validation should be performed in the tested matrix. Shrimp and crabmeat differ substantially. To that end, every consumer of shellfish is aware of significant differences in taste between shrimp and crab, due to the presence of differing substances in the meat product. As a result of these differences, the test methodology would have to be validated in crabmeat. Therefore, even if the test methodology had been validated in shrimp, which the aforementioned paper states has not been completed, positive test results using this test methodology on crabmeat do not establish that chloramphenicol is actually present in the crabmeat.

Second, as also set forth in the paper at Tab B, shrimp meat products provide a complex protein "matrix" from which analytes must be isolated using harsh chemical techniques. Once extracted after chemical treatment, the test method is designed around the identification in the resulting fluid, on mass spectroscopy, of signals allegedly unique to the double chlorine atoms

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present in chloramphenicol. Shellfish, and fish in general, are known to contain high levels of iodine and most likely have methods of trapping or concentrating similar isotopes (fluorine, chlorine, bromine, etc.) in their matrices. The test method's development, therefore, assumes without evidence that there are no naturally-occurring chlorine containing compounds within shrimp (or crab) that could be falsely detected by the test method as appearing similar to chloramphenicol. Such naturally-occurring bivalently-bound chlorine atoms might act similarly to chloramphenicol in the test method and be confused for added chloramphenicol. Additionally, the test method assumes without evidence that the harsh chemical extraction techniques do not create a compound that has similar behavior to chloramphenicol in the test method and could also deceive investigators into believing that chloramphenicol is present.

Apparently, during development of the test method using shrimp meat, an attempt was made to control for these possible confounding features by testing unspiked shrimp meat for the presence of similar signals. Tellingly, however, unfortified shrimp meat *was* found to contain a similar signal in 1 out of 6 tests performed. Indeed, the FDA paper (Tab B) states at page 4:

“[C]onfirmation of the drug was complicated by the fact that low-level false positives were observed. Therefore, although the chromatographic program would allow for its detection, confirmation limits for the amine were not evaluated in shrimp at this time.”

Thus, this developmental work on the test method limited to shrimp, which confirmed that false positives take place, demonstrates that the test method is inadequate to determine whether added chloramphenicol is present in shrimp, versus the test merely identifying some natural substance that acts similarly under the test conditions or after the chemical extraction methods.

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3. Chloramphenicol Is A Naturally Occurring Substance

Chloramphenicol is a naturally-occurring compound produced by soil organisms (*Streptomyces sp.*). It was originally discovered in 1947 in soil samples taken in Venezuela. See Goodman and Gilman's *The Pharmacological Basis of Therapeutics*, 8th Edition at page 1125 (Tab C).

Crabs are harvested from coastal waters. Crabs prefer tidal areas where water originates both from the ocean (salt water) and as run-off from land (rain water or rivers). These areas may be adjacent to areas where aquaculture is practiced for other species, such as shrimp. In comparison with shrimp, crabs are not, and cannot be, subject to aquaculture or "farming" techniques. Thus, crabs are not provided with antibiotics in feed or in any other manner to improve production, or to prevent or treat disease. Moreover, chloramphenicol is not added to harvested crabs or crabmeat. See Declaration of Richard Sante, President of Miami Crab Corporation (Tab D).

For these reasons, chloramphenicol would be expected to be found in extremely low concentrations in coastal waters that contain soil run-off as a result of natural events—production from *Streptomyces*. Given that chloramphenicol is a natural substance, the substance would have been present at low levels in the general environment and in crabmeat for eons. Thus, given the known absence of chloramphenicol feeding to crabs and the known environmental presence of chloramphenicol based on its discovery in nature, any chloramphenicol found in crabmeat at levels below 5 ppb most likely represents naturally-occurring chloramphenicol (or another chemical substance that is being falsely detected by methodology designed to identify dual chlorine atoms).

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Since chloramphenicol at such low levels is naturally-occurring, it is not a food additive or drug and its presence is historically safe.

4. Chloramphenicol At Levels Below 5 ppb Does Not Represent A Health Risk

Assuming for purposes of discussion without concession that FDA's test results are scientifically valid, there is no scientific evidence to suggest that naturally-occurring chloramphenicol present at levels below 5 ppb represents a health hazard. As set forth in Harrison's Principles of Internal Medicine, 14th Edition at Volume 1, page 354 (Tab E) and in the aforementioned section of Goodman and Gilman (Tab C), chloramphenicol can result in two forms of bone marrow suppression. The first is a *dose-related* suppression that occurs, generally, in all patients.¹ The typical dosage of chloramphenicol for human use is between is 250 to 500 mg per oral dose. Peak serum or blood concentrations are generally in the range of 10 to 13 µg/mL, which is 1,000 to 1,300 ppm or over 6 orders of magnitude greater than even 5 ppb. This reversible form of bone marrow suppression is reported to occur at whole serum concentrations that exceed 25 µg/mL (25 million ppb) (see USP summary of chloramphenicol at page 2 (Tab F)).

The second form of chloramphenicol toxicity is a unique cytotoxicity resulting in aplastic anemia that occurs rarely and idiosyncratically in between 1 in 30,000 to 1 in 50,000 patients

¹ "A second hematological effect of chloramphenicol is a common and predictable (but reversible) erythroid suppression of the bone marrow that is probably due to its inhibitory action on mitochondrial protein synthesis." Goodman and Gilman at page 1128 (Tab C).

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exposed to *therapeutic* doses.^{2,3} This estimate of incidence was derived on the assumption of an average therapeutic course of 4 g of chloramphenicol (Tab G⁴ and Tab H⁵). Stated differently, the subpopulation with this form of sensitivity to chloramphenicol must be exposed to therapeutic doses and not to incidental environmental concentrations to develop aplastic anemia. This form of reaction is most likely genetically based and operates through a toxic mechanism comparable to other forms of drug or chemical induced aplastic anemia in being associated with a benzene ring (e.g., quinacrine, dipyrone, phenylbutazone, indomethacin, phenytoin, chlorpromazine, tolbutamide). As noted in the review at Tab G, this idiosyncratic reaction has a dose relationship. As described at page 309 of Tab G, benzene and other aromatic compounds are metabolized by the P-450 monooxygenase enzyme system unless there has been induction or presence of an aryl hydrocarbon hydroxylase. Animals unable to induce metabolism through aryl hydrocarbon hydroxylase also develop aplastic anemia in response to benzene exposure. Thus, reactive intermediates from the P-450 system appear to be required for toxicity and generally explains the genetic predisposition and dosage relationship of idiosyncratic aplastic anemia induced by benzene ring containing compounds.

² Wallerstein RO, Condit PK, Kasper CK et al. Statewide survey of chloramphenicol therapy and fatal aplastic anemia. JAMA 1969;208:2045.

³ Smick KM, Condit PK, Proctor RL, Sutchter V. Fatal aplastic anemia: an epidemiological study of its relationship to chloramphenicol. J. Chronic Dis. 1964;17:899.

⁴ Young NS. Pathogenesis and Pathophysiology of Aplastic Anemia in Hoffman R, Benz EJ, Shattil SJ, Furie B, Cohen HJ, Silberstein LE, eds., Hematology: Basic Principles and Practice, 2nd Ed. Churchill Livingstone NY 1995.

⁵ Approved Drug Product Labeling.

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Thus, available scientific evidence does not suggest that low levels of exposure (< 5 ppb) result in any harm. Stated differently, since toxicity reactions such as this idiosyncratic reaction to chloramphenicol are dose-related, and reported cases have been at therapeutic doses 6 orders of magnitude greater than 5 ppb, there is no evidence that exposures at levels below 5 ppb are harmful.

It is important to remember that this cytotoxicity reaction has never implicated an allergic or immunological mechanism. While immunologically-mediated manifestations of disease can occur at very low levels of exposure, and justify a zero tolerance, this is not true for the idiosyncratic rare cytotoxic reaction to chloramphenicol. For a reaction to be deemed immunological, there must be evidence of antigen sensitization, antibody formation, or activation of cell-mediated immunity. To our knowledge, the medical and scientific literature does not disclose the discovery of any immune haptens or other immunological features as features of chloramphenicol's idiosyncratic cases of aplastic anemia. This supports this petition's contention that any harm from chloramphenicol is dose-related. Correspondingly, there are safe limits of exposure. These limits are likely to be well-above any naturally-occurring exposure.

In sum, we are not aware of any evidence establishing – or even suggesting – that there is an established health risk from exposure to naturally-occurring chloramphenicol in soil samples or in coastal water run-off from such soil. There is no evidence that a level of exposure of less than 5 ppb in crabmeat represents any health risk.

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5. The Presence Of Naturally-Occurring Chloramphenicol At Low Levels Does Not Result In Adulteration Of Crabmeat

We surmise that FDA is proceeding on the assumption that the apparent findings of chloramphenicol residues in imported crabmeat are a result of intentional use of chloramphenicol as in “crab aquaculture.” If this were the case, under a literal reading of the FDC Act, the permitted level of chloramphenicol residues in crabmeat would be zero since chloramphenicol is an unapproved new animal drug for this use. *See* Section 402(a)(2)(C)(ii) and Section 512(a)(1) of the FDC Act. However, as discussed above, there is no evidence to support the assumption or its corollary conclusion that added chloramphenicol is used in crabs; in fact, as discussed above, such use is not possible.

Under these circumstances, the relevant statutory provision is Section 402(a)(1) of the FDC Act regarding contaminants:

A food shall be deemed to be adulterated ... If it bears or contains any poisonous or deleterious substance which may render it injurious to health; but in case the substance is not an added substance such food shall not be considered adulterated under this clause if the quantity of such substance in such food does not ordinarily render it injurious to health....

Assuming without concession that FDA’s test method is valid and is in fact detecting chloramphenicol in crabmeat, for the distribution of crabmeat with less than 5 ppb chloramphenicol to be unlawful, FDA would have to show that this naturally occurring chloramphenicol is present in crabmeat at levels that are ordinarily injurious to health. For the reasons discussed in the previous section, FDA cannot make this showing.

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Even if FDA could establish that the chloramphenicol is somehow “added” (*e.g.*, it is present, in whole or in part, in the environment as a contaminant due to the activities of man, *see United States v Anderson Seafoods, Inc.*, 622 F.2d 157 (5th Cir. 1980)⁶), FDA could not meet even this lower standard of Section 402(a)(1). The Supreme Court has stated that the term “may” is to be given its ordinary and usual meaning, and that FDA has the burden of establishing that the contaminant “may” render the food injurious to health. *United States v. Lexington Mill & Elevator Company*, 232 U.S. 399, 410-11 (1914). Here, FDA cannot make that showing.

Regardless of whether chloramphenicol is naturally-occurring or “added” in the environment in which wild crabs are harvested, FDA must perform a health hazard evaluation relating to exposure to low levels (< 5 ppb) of chloramphenicol before taking any regulatory action against crabmeat containing such levels. In the absence of a risk assessment, FDA has no factual or legal basis for regulatory action. The continuation of FDA’s current enforcement activities in the absence of a factual and legal basis constitutes arbitrary and capricious, unlawful agency action.

⁶ We have been told that, prior to June 2002, chloramphenicol may have been used in shrimp aquaculture in coastal pens in China used to raise shrimp. These pens are flushed and cleared of any added substances by tidal action resulting, most likely, in vanishingly small traces of chloramphenicol in the more open, adjacent waters from which wild crabs are harvested. This use of chloramphenicol has ceased as of June 2002 and therefore would not justify continued testing of crabmeat harvested after June 2002. Additionally, chloramphenicol used as a drug in humans is excreted as a glucuronide conjugate in urine and may find its way into coastal water systems through waste disposal. It is plausible that chloramphenicol is present in the waters where crabs are harvested as an “added” environmental contaminant stemming from its use as an animal or human drug. However, as noted in the main text, the agency must perform a health hazard evaluation to assess appropriate limits for such environmental contaminants.

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6. Clarification of Import Alerts

FDA has heretofore issued two relevant Import Alerts. Import Alert #16-124 (Tab I) indicates that FDA may detain “aquaculture seafood products” due to the “use of unapproved new animal drugs.” This Import Alert is not applicable to crabmeat, which is not an aquaculture seafood product. FDA should clarify that this Import Alert is not applicable to crabmeat.

Import Alert #68-01 (Tab J) calls for the automatic detention of chloramphenicol for use in aquarium fish or other animals since it is a “new animal drug without an approved new animal drug application.” While this Import Alert is plainly inapplicable to crabmeat, the text explaining the reason for the alert contains false and misleading information, as follows:

This irreversible aplastic anemia [due to chloramphenicol] does not seem to be related to the frequency or level of exposure to the drug. In fact, there appears to be a significant subgroup of the population with an apparent predisposed sensitivity to chloramphenicol. This type of blood dyscrasia has been associated with extremely low levels of exposure to the drug. An example is a rancher diagnosed as having aplastic anemia four months after he began treating his cattle with chloramphenicol.

First, we note that judicial decisions and FDA policy do not allow the use of anecdotes to support scientific conclusions. For example, in rejecting the use of anecdotal evidence to support drug efficacy, the Supreme Court stated that “impressions or beliefs of physicians, no matter how fervently held, are treacherous.” Weinberger v. Hynson, Westcott and Dunning, Inc., 412 U.S. 609,

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619 (1973) (footnote omitted). To that end, the aforementioned authoritative text provided at Tab G states, at page 303, that:

The literature on idiosyncratic reactions – case reports and collections of cases – should be approached with skepticism. The data are often of poor quality, underlying mechanisms are seldom offered, and detection bias is an especially important problem. Patients with aplastic anemia are undoubtedly more closely questioned about potentially toxic exposures than individuals with other diseases. Interpretation of single cases is often confounded because many drugs are used in combination. The onset of marrow failure [i.e., aplastic anemia associated with chloramphenicol] is notoriously difficult to date accurately and therefore to place temporally in relation to drug use; the delay in marrow disease following benzene use may be years or decades, and conversely there are trivial case reports of drug use virtually coincident with the onset of marrow failure. Association does not establish causality....

Elsewhere this text notes that agricultural workers and farmers are at increased risk of aplastic anemia based on exposure to pesticides and other aromatic hydrocarbons.

Second, medical texts and reports do not support the conclusion stated in the Import Alert that there is no dose or prior exposure relationship in patients with idiosyncratic sensitivity to chloramphenicol. To the contrary, these publications support the existence of a “no-effect” level even in this subpopulation.

7. **Conclusion**

For the reasons stated, FDA’s electrospray liquid chromatography/mass spectroscopy test methodology has not been validated for use in crabmeat. According to FDA’s own paper, the test methodology is unreliable and yielded false positives when tested in shrimp, a different food product. Chloramphenicol is a naturally-occurring soil substance, and may be present through no

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activity of man in coastal waters where wild crabs are harvested. Crabs are not, and cannot be, raised through aquaculture or "farming." Thus, chloramphenicol is not intentionally fed to crabs as a drug and is not added to crabmeat. There is no scientific evidence to support the conclusion that low levels of chloramphenicol (< 5 ppb) present any risk to human health. For these reasons, FDA has no factual or legal basis for taking regulatory action against imported crabmeat with very low levels of chloramphenicol. FDA should provide assurances that the presence of naturally-occurring low levels of chloramphenicol in imported crabmeat does not result in the crabmeat being deemed adulterated.

C. ENVIRONMENTAL IMPACT

This petition is entitled to a categorical exclusion under 21 C.F.R. § 25.30 and § 25.32.

D. ECONOMIC IMPACT

Information regarding economic impact will be submitted on request.

CERTIFICATION

The undersigned certifies that, to the best knowledge and belief of the undersigned, this petition includes all information and views on which the petition relies, and that it includes representative data and information known to the petitioner which are unfavorable to the petition.

Respectfully submitted,



Jur T. Strobos, M.D.
Arthur Y. Tsien
Counsel to Miami Crab Corporation

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Wal-Mart yanks Chinese shrimp, crawfish in Louisiana

By JOHN DeSANTIS

Senior Staff Writer

May 31, 2002

[Email this story.](#)

The nation's largest retailer is pulling Chinese-shrimp and crawfish from the shelves of its Louisiana stores, a spokeswoman said Thursday, in response to the state's crackdown on a U.S.-banned antibiotic found in some shellfish products from that country.

Karen Burk, a spokeswoman for Wal-Mart Stores, said the retail giant is confident its suppliers meet all federal standards for food safety. Wal-Mart's suppliers, Burk said, provide certification that their products are safe and legal.

But the Louisiana Department of Agriculture and Forestry, which began extensive testing of crawfish and shrimp for antibiotic content late last month, has challenged such certifications, after confirming positive tests for the antibiotic chloramphenicol in Chinese shrimp and crawfish purchased within the state.

One of the samples that tested positive, a spokesman for the agency said Thursday, was a package of shrimp purchased from a Wal-Mart store within the state. In an interview, an agency investigator said the Wal-Mart sample tested positive for trace amounts of chloramphenicol in the amount of 5.2 parts per billion.

Burk said she was not aware of the state's finding, and that Chinese crawfish and shrimp will continue to be sold in Wal-Mart stores in other states.

"We are not aware of any situation where any of our shrimp has tested positive," Burk said. "We deal only with reputable suppliers and they provide us with documentation that shows that the shrimp meets all FDA regulations and is of the highest quality."

A new demand by Louisiana that retailers provide inspectors with specific documentation that shows that shrimp and crawfish are chloramphenicol-free is the reason Burk states for the retail chain's actions.

"The new Louisiana regulations are simply telling us we have to have specific documentation and comply with specific testing regulations that they have set up at state-approved laboratories," Burk said.

Louisiana officials say there are no new regulations regarding actual presence of chloramphenicol, only that they are now demanding proof



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to their satisfaction that products have been tested.

The U.S. Food and Drug Administration has a long-standing zero-tolerance policy for presence of the drug in food. But the federal government's own tests of imported seafood products have never detected the substance. European nations barred Chinese shrimp from their borders because of positive chloramphenicol; in interviews earlier this month an FDA spokeswoman said federal testing for chloramphenicol was only accurate to a level of 5 parts per billion, while European test protocols are capable of detecting far smaller amounts. The FDA is in the process of upgrading its testing procedures.

Louisiana has tested shrimp and crawfish for the antibiotic in its own labs, and sent samples to Canadian laboratories for further confirmation. The Canadian tests thus far, have confirmed the state's results.

Chloramphenicol is a powerful antibiotic used to treat anthrax and other serious infections in humans, and in some nations is used for veterinary purposes, including retarding of illness or infection in pond-raised shrimp and crawfish.

U.S. health policies call for detention of products found after testing to contain chloramphenicol, but the United States has no outright bans on imports from any specific country, but would block shipments of seafood on a company-by-company basis if the substance was found.

Federal food investigators have acknowledged that their agency performs minimal tests on such products as a rule. The FDA itself, in an "import alert" it published, states that there is "a causal relationship between the use of chloramphenicol and the development of a usually irreversible aplastic anemia in man."

"The case fatality rate is approximately 70 percent, and those who recover experience a high incidence of acute leukemia," the import alert continues. "This irreversible aplastic anemia does not seem to be related to the frequency or level of exposure to the drug. In fact, there appears to be a significant subgroup of the population with an apparent predisposed sensitivity to chloramphenicol."

Illness, the alert states, "has been associated with extremely low levels of exposure to the drug."

Scientists are not sure of the precise risks associated with ingestion of trace amounts of chloramphenicol by humans, adding to their desire for caution.

Louisiana's interest in chloramphenicol was prompted by concerns voiced by commercial fishermen in the state, whose dockside prices spiraled sharply downward this spring in the wake of large imports of shrimp from South American and Asian nations, including China. Fishermen and their representatives have alleged that Chinese shrimp containing chloramphenicol were dumped on the U.S. market.

Senior staff writer John DeSantis can be reached at 850-1151 or john.desantis@houmatoday.com

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To Miami Crab

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1-305-412-2129

**CONFIRMATION OF MULTIPLE PHENICOL RESIDUES IN SHRIMP BY
ELECTROSPRAY LC/MS**

Al Pfenning, Sherri Turnipseed, Jose Roybal, Cathy Burns, Mark Madson,
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C. Keyes

SCOPE

The analysis of shrimp for chloramphenicol and related compounds is important for several reasons. Residues of chloramphenicol (CAP) are of particular concern because this drug can cause serious acute reactions, including aplastic anemia, in susceptible individuals (1). Recently it has been reported that chloramphenicol has been found in several foodstuffs from Asia, including shrimp (2).

There are limited reports of the analysis of CAP and other phenicols in food from animal origin substances using electrospray LC/MS (3). Several others government (4,5) methods have also been reported, but are not published in the open literature. Our laboratory has been working with these compounds for many years. The traditional approach to the determination and confirmation of these compounds is isolation from tissue or fluids using liquid/liquid extraction, derivatization with silylating agents to form volatile derivatives, and analysis by GC/ECD and/or GC/MS with negative chemical ion detection (6-8).

The scope of this method is to describe a confirmatory (qualitative) method for chloramphenicol (CAP) and several related compounds (florfenicol [FF] and thiamphenicol [TAP]) in shrimp using negative ion electrospray with ion trap LC/MSⁿ analysis. Because the chromatographic and MS conditions were initially developed to look for the metabolite florfenicol amine as well as these other drugs, the method allows for detection of this compound in the first part of the chromatographic run, but at this time a confirmation limit for this drug has not been determined in shrimp.

Both fixed MS² scans and data dependent acquisition were used successfully to confirm these drugs in shrimp tissue. The fixed MS² program outlined in this SOP was chosen for the final method. Certain parameters, such as matrix effects, reproducibility of the instrument and extractions must be evaluated more thoroughly before this method would meet standards for quantitative analysis. Better performance for quantitation at low residue levels (<1 ppb) will most likely be obtained using a triple quadrupole instrument.

- (1) Roybal, J.E. "Chloramphenicol and Related Drugs" in *Analytical Procedures for Drug Residues in Food of Animal Origin* (1998) ed, S.B. Turnipsced and A.R. Long, Science Technology System, W. Sacramento, CA pp. 227-260.
- (2) <http://www.fst.rdg.ac.uk/foodlaw/news/eu-02031.htm>
- (3) Hormazabal, Y J. Liq Chromatogr. & Related Technique

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- (4) Canadian Food Inspection Agency (CFIA), Dartmouth Laboratory Draft method: Analysis of Florfenicol, Florfenicol Amine, Thiamphenicol and Chloramphenicol in Fish, Shellfish and Crustaceans (2002)
- (5) Florida Chemical Residue Laboratories, Florida Department of Agriculture and Consumer Services, Preparation and Analysis of Chloramphenicol in Shrimp. (2002)
- (6) Pfenning, A.P., Roybal, J.E., Rupp, H.S., Turnipseed, S.B., Gonzales, S.A., Hurlbut, J.A. (2000) *JAOAC Int.* 83, 26.
- (7) Pfenning, A.P., Madson, M.R., Roybal, J.E., Turnipseed, S.B., Gonzales, S.A., Hurlbut, J.A., Salmon, G.D (1998) *JAOAC Int.* 81, 714.
- (8) Kijak, P.J (1994) *JAOAC Int.* 77, 34.

PRINCIPLES

I. Extraction.

Ten grams of shrimp composite is extracted with 20 mL basic ethyl acetate/acetonitrile, homogenized and centrifuged. The extraction steps are repeated and the ethyl acetate/acetonitrile layers are evaporated to dryness. Thirty mL water is added to the flask, sonicated and followed by hexane defatting steps. The aqueous phase is passed through a series of SPE columns. The analyte is extract off the final SPE with methanol. The methanol is evaporated to dryness. The extracts are reconstituted into a small volume of 0.1% formic acid and filtered into LC vials. In addition, only the parent phenicols (not the florfenicol amine) were confirmed by this method (only the C18 cartridge was eluted and analyzed).

II. Mass Spectral Analysis

A. Qualitative Confirmation

The qualitative confirmation of phenicols in shrimp is based on unique mass spectral characteristics of these compounds as evaluated by established guidelines (9,10). One unique aspect of these compounds is the fact that they contain two chlorine atoms, thus giving rise to unique isotopic patterns. In order to take advantage of this fact, the MS² spectra is obtained not only from the parent ion ([M-H]⁻), but also from the corresponding M+2 (³⁵Cl³⁷Cl) isotope peak. For example, in the MS² spectra of CAP ([M-H]⁻ pair m/z 321/323) the predominant ion is m/z 194 which corresponds to [M- H-(NH₂COCCl₂H)]⁻. Also present in this spectra are the ions m/z 176 [m/z 194 - (H₂O)]⁻ (15%), 249 [M-H-(2HCl)]⁻ (30%), and 257 [M-H-(HCOCl)]⁻ (25%). These ions are also present in the MS² spectra of m/z 323, although the peak at 257 is split (into peaks of approximately equal abundance) between ions at m/z 257 and 259, indicating the loss of one chlorine atom (either ³⁵Cl or ³⁷Cl) from the ³⁵Cl³⁷Cl parent ion.

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The florfenicol MS² spectra is dominated by the loss of HF from the parent ions. This is observed as m/z 335.8 when the ³⁵Cl³⁵Cl parent ion (m/z 356) is isolated or m/z 337.8 when the ³⁵Cl³⁷Cl ion is fragmented. To obtain additional confirmatory ions, MS³ is performed on ion 335.8 to give a spectra which includes the ions 219 (usually 100%,), as well as m/z 119, 184, 264. Thiamphenicol [M-H]⁻ equal to 354/356, fragments to give the following ions, m/z 227, 240, 270, and 290/292.

The florfenicol amine spectra is not as unique as the parent phenicols because it does not include the lipophilic chlorine containing moiety. This compound responds very well by positive ion electrospray to give [MH]⁺ of m/z 248. The predominant ion in the MS² spectra is m/z 230, representing the loss of water. The dominant ion in the MS³ is m/z 130. Because of the non-specific ions and losses associated with this compound, as well as, the fact that it elutes very early in the chromatographic run, the confirmation of the drug was complicated by the fact that low-level false positives were observed. Therefore, although the chromatographic program would allow for its detection, confirmation limits for the amine were not evaluated in shrimp at this time.

REAGENTS

Solvents: Distilled-in glass, pesticide-grade, hexane, ethyl acetate (EtOAC), acetonitrile (ACN), isopropanol (IPA), methanol (MeOH).

Formic acid used to prepare the mobile phase was purchased from Baker (88%).

Solid-phase extraction columns: C18: Varian Bond Elut 6 cc/500 mg

PRS: Varian Bond-Elut LRC-PRS 500mg

Syringe filters: 4 mm syringe filter 0.45 μm, PTFE. Phenomenex P/N AFO-0422

Ammonium hydroxide (assay ca. 30% as NH₃),

Glacial acetic acid, LC grade.

EQUIPMENT

1. Ion Trap LC/MS: The instrument used was a Finnegan LCQ DECA Ion Trap Mass Spectrometer coupled to a modular Spectrasystem LC system. The components of the LC system include a SCM1000 degasser, P4000 LC pump, AS3000 autosampler, and a UV6000LP UV/VIS detector. The software used was Xcaliber Version 1.2.

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2. LC Column. The LC Column was an Xterra phenyl (2.1 x 100 mm, 3.5 μ , Waters Corp. P/N 186001180). Other phenyl columns would also be acceptable. In this laboratory an Inertsil phenyl (2 x 150 mm, 5 μ , Phenomenex Corp. P/N 0301-150X020) was also tested during method development. If other columns are used, the time segments in acquisition program need to be adjusted to account for shift in retention times.

3. Other.

Tissue disrupter --High speed shearing tool, i.e. tissuemizer, of a diameter < 20 mm.

Rotoevaporator: with ice trap and water bath set at 50 C

Nitrogen evaporator: 12-sample nitrogen evaporator, with 50 C water bath

Plasticware: 50 mL and 15 mL disposable, conical polypropylene with screw cap

Glassware: pear shape flask, Pastuer pipettes

PROCEDURES

1. Standard Preparation

The compounds were purchased or obtained from: Chloramphenicol (USP), Thiamphenicol (Sigma), Florfenicol and Florfenicol Amine (Schering-Plough).

Fortification Standards. For fortification of shrimp, individual stock solutions of drug at 1000 $\mu\text{g}/\text{mL}$ (1000 $\text{ng}/\mu\text{L}$) were made up in acetonitrile. A combined intermediate standard solution (10 $\text{ng}/\mu\text{L}$) was made by pipetting 1 mL of each individual stock solution into 100 mL volumetric flask and diluting to volume with acetonitrile. Prepare fortification standards, as applicable: Pipet 0.5, 0.2, or 0.1 mL combined standard solution into 10 mL volumetric and dilute to volume with acetonitrile for 5, 2, and 1 ppb fortification standards, respectively.

MS Standards For MS analysis, stock solutions of drug at 100 $\mu\text{g}/\text{mL}$ (100 $\text{ng}/\mu\text{L}$) were made up in methanol. A mixed intermediate standard (1 $\text{ng}/\mu\text{L}$ of each drug) was made by diluting 500 μL of each stock solution to 50 mL with 0.1% formic acid.

Working LC/MS Standards. As applicable, LC/MS standards were made as follows:

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| μL of intermediate standard | μL of 0.1% Formic Acid | [ng/μL] | equivalent in shrimp (ppb)* |
|------------------------------------|-------------------------------|-----------------|------------------------------------|
| 1000 | 4000 | 0.2 | 5 |
| 400 | 4600 | 0.08 | 2 |
| 200 | 4800 | 0.04 | 1 |
| 100 | 4900 | 0.02 | 0.5 |

* Assuming 10 g of shrimp is processed and final extract volume is 250 μL.

Stability. Working LC/MS standard are stable for at least one week.

2. Sample Preparation

Control Samples. At least one control (matrix blank) sample should be run with every set of samples.

Fortified Samples. At least two fortified samples should be run with every set of incurred or unknown samples. The concentration of the fortified sample should be in the range of 1-5 ppb.

Incurred Samples. Were not evaluated during method development.

3. Sample Extraction.

Hold frozen shrimp at room temperature until they feel limber. Remove the heads, chitinous shell and body appendages from partially thawed shrimp. Place shrimp meat in blender, and blend with dry ice with pulsed action until contents are uniform. Accurately weigh about 10.0 g of blended shrimp composite into a 50 mL P/P centrifuge tube. (If spiking control shrimp, add 100 μL of the desired concentration of Standard Solution to completely thawed 10 g blank composite and allow to sit at room temperature for at least 20 minutes before proceeding.) Add 20 mL of extraction solution (EtOAc:NH₄OH, 98:2) homogenize with tissue disrupter until the entire mass is broken up (about 30 sec). Centrifuge for 7 min @ 4000 RPM, 5 °C; decant through medium retention filter paper into 100 mL P-S flask. Repeat extraction with another 20 mL of extraction solution, combining the extracts in the 100mL P-S flask. Repeat extraction a third time with 10 mL of extraction solution + 10 mL ACN combining the extracts in the 100 mL P-S flask. Add 5mL IPA, to prevent bumping and foaming and roto-evaporate at 50-55 °C to dryness. Add 30 mL H₂O, vortex, sonicate 2 min, adjust pH (<4.6) with approximately 0.4mL of 0.1% acetic acid and pour into a 50 mL P/P centrifuge tube. Add 5 mL of hexane to the 100 mL P-S flask; vortex, swirl to dissolve contents, and transfer contents to same tube as the acidified aqueous; repeat with another 5

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mL aliquot of hexane. Shake tube well or vortex for about 30 sec, centrifuge @ 4000 RPM at room temperature for 3 min, aspirate upper hexane layer and discard. Repeat hexane defatting steps two more times with an additional 5 mL portion of hexane each time and discard the hexane each time. Condition each PRS and C₁₈ SPE column with 3 mL MeOH followed by 3 mL H₂O. Transfer remaining aqueous from P/P tube to a (conditioned) SPE system consisting of a C₁₈ SPE column on bottom, PRS SPE column on top of the C₁₈, with a 70 mL reservoir atop the PRS; all on a vacuum manifold (allow to flow through at about 1 drop/sec). When level just reaches the top of PRS column, add 2 mL H₂O to columns. Allow the columns to run dry, separate system, discarding reservoir, identify and place PRS column in 50mL centrifuge tube and store in freezer, if needed for florfenicol amine analysis. Elute the C₁₈ SPE with 4 mL MeOH into 15mL disposable P/P centrifuge tube. Evaporate MeOH eluate to dryness in N-Evap with water-bath set at 50°C. The dried extracts are reconstituted into 250 µL of 0.1% formic acid, and filtered for injection into LC-MS system.

4. Instrument Operating Parameters.

Regardless of the instrument used, certain performance verification criteria should be incorporated into the operating parameters. These include mass calibration, tuning, and appropriate fragmentation patterns. Mass axis calibration should be performed according to the instrument manufacturers' specifications or according to internal laboratory MS standard operating procedures. Signal optimization (tuning) should be adjusted to maximize the abundance of ions of interest. Daily system suitability requirements (described in #7 of this section) should also be met. The following describes the specific operating procedures for the instrument used to validate this method in the developer's laboratory.

(i) Instrumental Configuration. LC/MS analysis is performed using a LCQ DECA mass spectrometer coupled to a TSP P4000 LC via an electrospray interface. The instrument is operated using positive and negative ion detection. The instrument was calibrated according to the manufacturer's instructions. The response for CAP was optimized by tuning on ion m/z 321. For tuning, CAP (1 ng/µL in mobile phase) was pumped through a syringe pump at 10 µL/min and then introduced into the LC flow (250 µL/min 80/20 0.1% formic acid/acetonitrile) via a T before entering the MS source. In the tune file the MS parameters were set to a prescan of 2 and a

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maximum inject time of 100 ms. The MS² parameters were also optimized using the tune function of the instrument. For this mode the prescan was set to 1 with a maximum inject time of 500 ms. The collision energy was optimized for both total MS² ion current, as well as for specific ions (m/z 194, 249) with no significant differences (optimal collision energy was 24-26% in all cases).

(ii) **Monitored Response.** Using the ion trap, MS² was performed on the molecular ions for each of the analytes according to the following program:

Program 1: Fixed MS² Acquisition

Isolation width was set to 2 amu for all MS² transitions. Positive ion tune should be used for time segment 1 if used. Tune file developed for CAP (described above) should be used for other time segments.

Time Segment 1: 2-5 minutes FFA (CAN DELETE THIS SEGMENT)

Scan Event 1: (+) MS [m/z 180-350]

Scan Event 2: (+) MS² of m/z 248.1 (24% CE) [m/z 65-250]

Scan Event 3: (+)MS³ of m/z 248.1 (24%CE) → m/z 230.1 (32% CE) [m/z 60-250]

Time Segment 2: 5-11 minutes TAP

Scan Event 1: (-) MS [m/z 320-375]

Scan Event 2: (-) MS² m/z 354.2, (CE 35%) [m/z 65-250]

Scan Event 3: (-)MS² 356.2 (CE 35%)

Time Segment 3: 11-12.5 minutes FF

Scan Event 1: (-) MS m/z 320-375

Scan Event 2: (-) MS² m/z 356.2,(CE 24%)

Scan Event 3: (-)MS² m/z 358.2 (CE 24%)

Scan Event 4: (-) MS³ of m/z 356.2 (24%CE) → m/z 335.8 (20% CE)

Time Segment 4: 12.5-18 minutes CAP

Scan Event 1: (-) MS m/z 300-350

Scan Event 2: (-) MS² m/z 321.2 (CE 24%)

Scan Event 3: (-)MS² m/z 323.2 (CE 24%)

A UV/Vis diode array detector was also utilized with a scan range of 190-800nm and channel A set to 270 nm (bandwidth 9 nm) and channel B set to 236 nm (bandwidth 9 nm).

(iii) **Specific Operating Conditions.** The electrospray interface was operated with a temperature of 275°C. The sheath gas was nitrogen at approximately 35 psi; the auxiliary gas was also nitrogen at approximately 6 psi (optimized for CAP signal). The mobile phase was at flow of 250 µL/min and a column oven was not used. Automated injections of 75 µL were made using

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"push loop" type injection. The LC flow was diverted away from the mass spectrometer for the first minute. The MS was on from 1-18 minutes. The chromatographic gradient is as follows:

| Time (minutes) | % Acetonitrile | % 0.1% Formic Acid |
|----------------|----------------|--------------------|
| 0-5 * | 2 | 98 |
| 6-18 | 20 | 80 |
| 20-22 | 90 | 10 |
| 23-28 | 2 | 98 |

* note- if not interested in florfenicol amine, chromatographic program could begin at 20% acetonitrile. Time windows might need to be adjusted.

5. Procedures for Instrumental Analysis of Samples, Controls, and Standards

Standards are to be run with each set of samples (at the beginning and end of a set of samples, and in the middle of the sequence if many samples are being analyzed). At least two positive controls, i.e. fortified matrix should be run along with any unknown sample extracts. A blank matrix sample (negative control) should also be run along with any unknown sample extracts and must demonstrate the absence of CAP. At least one of the fortified matrix control samples must demonstrate the confirmation criteria in the Validation Section #2v. A solvent blank (mobile phase) should be run before each sample to ensure that there was no carryover from the previous sample or standard. Solvent blanks are not required between duplicates of the same test sample, or when a fortified sample of higher concentration than a previous fortified sample is analyzed.

6. Calculations

For qualitative analysis, the important factor is to obtain information to determine if the data meet the confirmation criteria described in the Validation Section #2v. Ion chromatograms from the full MS (m/z corresponding to $[M-H]^-$) and from MS^2 (m/z 194 corresponding to $[M-H-(NH_2COCCl_2H)]^-$ from both fragmentation of both m/z 321 and 323) can be shown along with the MS^2 spectra averaged across the chromatographic peaks. In addition, extracted ion chromatograms for several ions (m/z 194, 257/259, 249, and 176) in the MS^2 spectra of 321 and 323 can be shown.

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As scan data are obtained, relative abundances of representative ions can be estimated from the appearance of the MS² spectra, or from tabulation data. Integration of ion chromatograms is not necessary.

7. System Suitability

The instrument should meet calibration and tuning criteria as described above. In addition, for each day's analysis, a standard mixture should be analyzed initially to determine the performance qualifications, or system suitability of the instrument. The analytes need to elute at the correct retention time; within $\pm 5\%$ of what was observed for standards previously (unless column or mobile phase have been changed) and within the time-dependent window if used. It may require one or two injections of standard for compounds to elute at correct retention time if instrument has not been used recently. In addition, the response for 75 μL injection of a 1 ppb standard for CAP should be $> 200,000$ counts for the 321- \rightarrow 194 MS² transition.

VALIDATION INFORMATION

1. Validation Data

Validation data for ion trap MS confirmation of multi phenicol residues in shrimp are shown in Table 1. Figure 1 shows chromatograms for a 1 ppb shrimp fortified extract.

2. Parameters Evaluated

(i) **Recovery.** Fortified samples were analyzed at 1 and 2 ppb with recoveries of approximately 55 percent.

(ii) **Reproducibility.** A series of standard injections (75 μL injection size) were analyzed using the following standards: At 1 ppb (3 ng on-column) the reproducibility of standard injections as measured by the CAP 321 to 194 transition was 16% (n=6), at 0.25 ng (750 pg on-column), 19.9% (n=5) and at 0.1 ppb (300 pg on-column) it was 40.0% (n=4).

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(iii) Specificity. This method meets the specificity guidelines for confirmation methods outlined by Sphon⁹ and recently elaborated in CVM's draft guidance¹⁰. During the course of this investigation, several lots of control shrimp were analyzed and there were no significant interfering peaks in any of the control tissue samples analyzed using the mass filters as described.

(iv) Sensitivity. For CAP, the ion trap instrument was able to confirm approximately 300-500 pg of standards on-column and shrimp tissue fortified at 1.0 ppb was confirmed with a 75 μ L injection volume (final extract volume of 250 μ L).

(v) Accuracy, Proof of Recovery from Authentic Samples.

Using an ion trap instrument the following criteria must be met for positive qualitative confirmation:

For chloramphenicol: 1) The ion m/z 194 $[M-H-(NH_2COCCl_2H)]^-$ must be observed in the MS^2 spectra from both parent ions (m/z 321 and 323), and should be a predominant peak in the mass range m/z 100-270. 2) In addition, at least one of the other structurally significant lower abundance ions (m/z 257/259 $[M-H-(HCOCl)]^-$, m/z 249 $[M-H-(2HCl)]^-$, or m/z 176 $[m/z$ 194 - $(H_2O)]^-$) must also be present in at least one of the MS^2 spectra at an approximate relative abundance to the base peak m/z 194 as is observed in the external standards, and 3) the retention time should be $\pm 5\%$ of external standards run on that day.

The qualitative criteria for the other phenicols is similar. The florfenicol MS^2 spectra is dominated by the loss of HF from the parent ions to give only one ion (335.8 from m/z 356 or m/z 337.8 from m/z 358). To obtain additional confirmatory ions MS^3 is performed on m/z 337.8. For thiamphenicol $[M-H]^-$ (354/356) fragments to give several ions m/z 227, 240, 270, and 290/292. At least two of these should be observed in MS^2 spectra from each parent isotope peak. In addition, the retention times for these other residues must also be $\pm 5\%$ of what is observed from external standards analyzed on the same day.

(vi) Practicality, Sample Throughput, Solvents and Time Requirements. Extraction and LC/MS analysis of 6-8 samples can be accomplished in one day/overnight. For example, initial extraction can be performed in 5 hours. Each LC/MS run takes 28

⁹ J.A. Sphon *J. Assoc. Off. Anal. Chem.* 61, 1247 (1978)

¹⁰ Center for Veterinary Medicine (2001) *Guidance for Industry: Mass Spectrometry for Confirmation of the Identity of Animal Drug Residues*

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minutes therefore 6 sample analyses (bracketed by analysis of standards, separated by solvent blanks) can be done in 8-12 hours.

QUALITY CONTROL POINTS

(1) Critical Points

(i) *Extraction.* When filtering, be careful that the syringe filter does not disengage.

(ii) *Chromatography.* A formic acid/acetonitrile mobile phase at 0.25 mL/min on a semi-micro phenyl column resulted in the best chromatographic performance and electrospray sensitivity. The migration of peaks, especially at the beginning of the chromatographic analysis, can be a problem and several injections of standard may be necessary to allow compounds to "settle" into reproducible retention time. Retention times are stable during continuous sequences, even as long as 40-50 samples.

(i) *Mass spectral analysis.* In addition to obtaining good agreement between samples and standards analyzed on the same day, a review of the data shows that the relative abundances of ions obtained different days is also very reproducible.

(2) Performance Specifications.

Performance Specifications are outlined above in Procedures section #4.ii (tuning of mass spectrometer), #7 (system suitability for standards) and the Validation section #2.v (criteria for confirmation).

(3) Stability

Stability of residues in shrimp stored for extended periods of time was not evaluated.

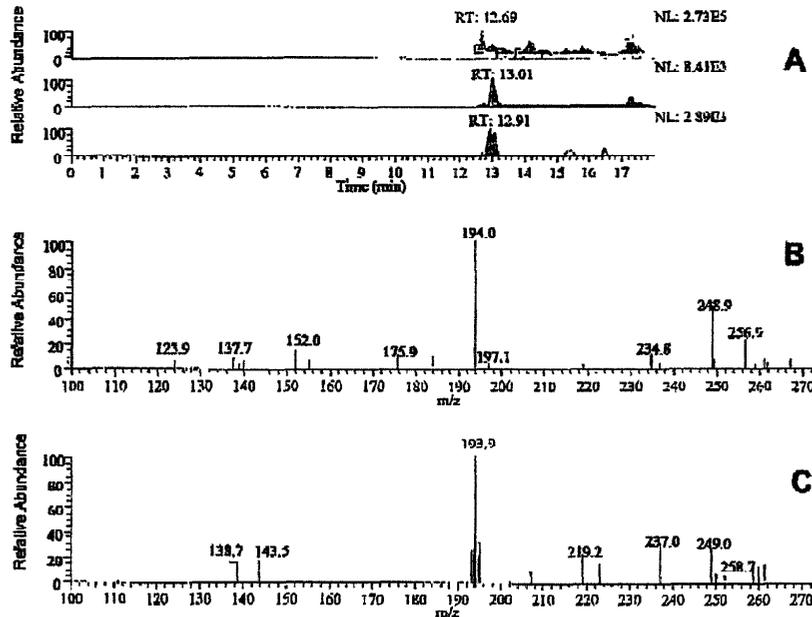
(4) Safety.

Standard laboratory safety practices (lab coats, eye protection) should be followed. In addition any safety precautions listed in the determinative SOP for preparation of reagents should be followed. Also follow instrument manufacturers guidelines for safe operation of electrospray LC/MS (particularly with respect to high voltages, high current, and high temperatures).

Table 1. Summary of Confirmation of Phenicols in Shrimp Using Ion Trap

| Sample | Number Confirmed/Number Analyzed | | |
|---------------------------------------|----------------------------------|-----|-----|
| | CAP | FF | TAP |
| Control Tissue | 0/7 | 1/6 | 0/6 |
| Fortified 0.5 $\mu\text{g}/\text{kg}$ | 3/4 | 1/3 | 0/3 |
| Fortified 1 $\mu\text{g}/\text{kg}$ | 7/7 | 3/4 | 4/4 |
| Fortified 2 $\mu\text{g}/\text{kg}$ | 7/7 | 3/3 | 3/3 |
| Fortified 5 $\mu\text{g}/\text{kg}$ | 7/7 | 6/6 | 6/6 |

Figure 1. Extract from shrimp fortified with 1 ppb CAP.



(A) Extracted ion chromatograms for full MS (m/z 321) and MS² (m/z 194) from m/z 321 and 323.

(B) MS² spectrum for m/z 321 (C) MS² spectrum for m/z 323

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recommended because of the vestibular disturbances that this drug can cause (*see above*).

Urinary Tract Infections. The usefulness of tetracyclines for urinary tract infections has also been reduced appreciably by the increase in the number of drug-resistant microorganisms. As a rule, these drugs are not active against *Proteus* and *Pseud. aeruginosa*. Treatment of urinary tract infections with a tetracycline should be undertaken only if the infecting strain is sensitive. Treatment is usually continued for 7 to 10 days. For severe acute pyelonephritis, tetracyclines should be used only in the unlikely event that no other antimicrobial agent is effective. The acute urethral syndrome in women has been effectively treated with doxycycline (100 mg twice daily for 10 days) (Stamm *et al.*, 1981). While doxycycline may be given to patients with renal dysfunction, the drug concentration in the urine may not be sufficient for treatment of urinary tract infections.

Other Infections. Actinomycosis, although most responsive to penicillin G, may be successfully treated with a tetracycline; in severe infections, intravenous therapy for 1 week, followed by oral administration of drug for a month or more, may be required. Minocycline has been suggested for the treatment of nocardiosis, but a sulfonamide should be used concurrently. Yaws and relapsing fever respond favorably to the tetracyclines and penicillin (Salih and Mustafa, 1977). Although either tetracycline or penicillin G is used to treat leptospirosis, evidence of efficacy is not convincing with these or any other antimicrobial agent. Lyme disease, caused by *Bor. burgdorferi*, is characterized by fever, skin lesions, arthritis, and aseptic meningitis. It responds to either penicillin G or a tetracycline, although tetracycline has been observed to be ineffective in advanced *Bor. burgdorferi* infection (Dattwyler *et al.*, 1987). The tetracyclines have been used to treat atypical mycobacterial diseases, including those caused by *Mycobacterium marinum* (Izumi *et al.*, 1977).

Intestinal Disease. Patients with Whipple's disease may respond to tetracycline, although relapses may occur more frequently than after therapy with penicillin G. The administration of tetracycline to some patients with tropical sprue may be associated with repletion of folate, a favorable hematological response, decrease in diarrhea, improvement in the enzymatic activity and morphology of the superficial epithelium of the jejunal mucosa, gain in weight, and reversal of the abnormal pattern of lipid distribution. Tetracyclines may also be of value in the blind-loop syndrome.

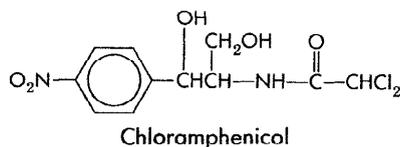
Acne. Tetracyclines have been used for the treatment of acne, and good results have been reported by some investigators. Benefit has been produced by small doses. It has been suggested that these drugs may act by inhibiting propionibacteria, which reside in sebaceous follicles and metabolize lipids into irritating free fatty acids. Although it is generally accepted that the tetracyclines or other

antibiotics have a beneficial effect in acne, some placebo crossover studies raise doubt concerning the value of this kind of therapy. Use of tetracycline seems to be associated with few side effects when given in doses of 250 mg orally twice a day.

CHLORAMPHENICOL

History and Source. Chloramphenicol is an antibiotic produced by *Streptomyces venezuelae*, an organism first isolated in 1947 from a soil sample collected in Venezuela (Bartz, 1948). When the relatively simple structure of the crystalline material was determined, the antibiotic was prepared synthetically. Late in 1947, the small amount of available chloramphenicol was employed in an outbreak of epidemic typhus in Bolivia, with dramatic results. It was then tried with excellent success in cases of scrub typhus on the Malay peninsula. By 1948, chloramphenicol was produced in amounts sufficient for general clinical use. By 1950, however, it became evident that the drug could cause serious and fatal blood dyscrasias. For this reason, use of the drug is reserved for certain patients with serious infections, such as meningitis, typhus, and typhoid fever; it is also a first-line agent for Rocky Mountain spotted fever. An awareness of its activity against anaerobic bacteria, especially *B. fragilis*, has resulted in an increased use of chloramphenicol in recent years (Cuchural *et al.*, 1988).

Chemistry. Chloramphenicol has the following structural formula:



The antibiotic is unique among natural compounds in that it contains a nitrobenzene moiety and is a derivative of dichloroacetic acid. The biologically active form is levorotatory.

Mechanism of Action. Chloramphenicol inhibits protein synthesis in bacteria and, to a lesser extent, in eukaryotic cells. The drug readily penetrates into bacterial cells, probably by a process of facilitated diffusion. Chloramphenicol acts primarily by binding reversibly to the 50 S ribosomal subunit (near the site of action of the macrolide antibiotics and clindamycin, which it inhibits competitively). Although binding of tRNA at the codon recognition site on the 30 S ribosomal subunit is thus undisturbed, the drug appears to prevent the binding of the amino acid-containing end of aminoacyl tRNA to the acceptor site on the 50 S ribosomal subunit. The interaction between peptidyl transferase and its amino acid substrate cannot occur, and peptide bond formation is inhibited (*see Pratt and Fekety, 1986*).

Chloramphenicol can also inhibit mitochondrial protein synthesis in mammalian cells, perhaps because mitochondrial ribosomes resemble bacterial

ribosomes (both are 70 S) more than they do the 80 S cytoplasmic ribosomes of mammalian cells. The peptidyl transferase of bovine mitochondrial ribosomes, but not cytoplasmic ribosomes, is susceptible to the inhibitory action of chloramphenicol. Mammalian erythropoietic cells seem to be particularly sensitive to the drug.

Effects on Microbial Agents. Chloramphenicol possesses a fairly wide spectrum of antimicrobial activity. Strains are considered sensitive if they are inhibited by concentrations of 12.5 $\mu\text{g/ml}$ or less. It is primarily bacteriostatic, although it may be bactericidal to certain species, such as *H. influenzae*. More than 95% of strains of the following gram-negative bacteria are inhibited *in vitro* by 8.0 $\mu\text{g/ml}$ or less of chloramphenicol: *H. influenzae*, *N. meningitidis*, *N. gonorrhoeae*, *Salmonella typhi*, *Bruceella* species, and *Bordetella pertussis*. Likewise, most anaerobic bacteria, including gram-positive cocci and *Clostridium* species and gram-negative rods including *B. fragilis*, are inhibited by this concentration of the drug. Some aerobic gram-positive cocci, including *Strep. pyogenes*, *Strep. agalactiae* (group-B streptococci), and *Strep. pneumoniae*, are sensitive to 8 $\mu\text{g/ml}$, while fourfold higher concentrations are required to inhibit more than 95% of strains of *Staph. aureus* (Standiford, 1990).

The Enterobacteriaceae have a variable sensitivity to chloramphenicol. Although 95% of strains of *E. coli* are inhibited by 12.5 $\mu\text{g/ml}$, only 75% of *Klebsiella pneumoniae*, 50% of *Enterobacter*, and 33% of *Serratia marcescens* are inhibited. Ninety percent of strains of *Proteus mirabilis* are inhibited by 12.5 $\mu\text{g/ml}$. All strains of *Pseud. pseudomallei* are inhibited by this concentration; however, *Pseud. aeruginosa* is resistant to even very high concentrations of chloramphenicol. Eighty-four percent of *V. cholerae* are inhibited by 6.3 $\mu\text{g/ml}$, as are 90% of *Shigella*. Chloramphenicol exerts marked prophylactic and therapeutic effects in experimental infections produced by all rickettsiae. The drug, as a rule, only suppresses rickettsial growth. Chloramphenicol is also effective against *Chlamydia* and *Mycoplasma*.

Resistance to Chloramphenicol. The resistance of gram-positive and gram-negative microorganisms to chloramphenicol *in vivo* is a problem of increasing clinical importance. Resistance of gram-negative bacteria to the drug is usually caused by a plasmid acquired by conjugation and is due to the presence of a specific acetyltransferase that inactivates the drug. At least three types of enzyme have been characterized (Gaffney and Foster, 1978). Acetylated derivatives of chloramphenicol fail to bind to bacterial ribosomes (Piffaretti and Froment, 1978). Strains of *H. influenzae* that are resistant to chloramphenicol contain plasmids that code not only for the production of acetyltransferase, but also invariably for resistance to tetracyclines; they may also code for a beta-lactamase that mediates resistance to ampicillin (Doern *et al.*, 1988). Plasmid-mediated resistance to chloramphenicol in *S. typhi* emerged as a significant problem during the epidemic of 1972–1973 in Mexico and the

United States (Baine *et al.*, 1977). However, the prevalence of resistance of *S. typhi* to chloramphenicol is negligible today, except in some areas of Southeast Asia (Ling *et al.*, 1988). The prevalence of resistance of staphylococci to this antibiotic has also increased; it varies from one hospital to another and is as high as 50% or more in some. Resistant strains of *Staph. aureus* contain one of several related forms of chloramphenicol acetyltransferase that are inducible (Sands and Shaw, 1973). Although loss of sensitivity to chloramphenicol is usually due to acetylation of the drug, both decreased permeability of the microorganisms (which has been found in *E. coli* and *Pseudomonas*) and mutation to ribosomal insensitivity have also been described (Sompolinsky and Samra, 1968; Baughman and Fahnestock, 1979).

Absorption, Distribution, Fate, and Excretion. Chloramphenicol is available for oral administration in two dosage forms: the active drug itself and the inactive prodrug, chloramphenicol palmitate (which is used to prepare an oral suspension). Hydrolysis of the ester bond of chloramphenicol palmitate is accomplished rapidly and almost completely by pancreatic lipases in the duodenum under normal physiological conditions (Kauffman *et al.*, 1981). Chloramphenicol is then absorbed from the gastrointestinal tract, and peak concentrations of 10 to 13 $\mu\text{g/ml}$ occur within 2 to 3 hours after the administration of a 1-g dose. In patients with gastrointestinal disease or in newborns, the bioavailability is greater for chloramphenicol than for chloramphenicol palmitate, probably due to the incomplete hydrolysis of the latter (Smith and Weber, 1983). The preparation of chloramphenicol for parenteral use is the water-soluble, inactive sodium succinate preparation. Absorption after intramuscular injection was previously thought to be highly unpredictable; however, a more recent study demonstrated comparable concentrations of chloramphenicol succinate in plasma after intravenous and intramuscular administration (Shann *et al.*, 1985). It is unclear whether the hydrolysis of chloramphenicol succinate occurs *in vivo*, but esterases of the liver, kidneys, and lungs may all be involved. Chloramphenicol succinate is rapidly cleared from plasma by the kidneys. This renal clearance of the prodrug may affect the overall bioavailability of chloramphenicol, because excretion of up to 30% of the dose may occur prior to

lysis. Poor renal function in the neonate and other states of renal insufficiency result in increased plasma concentrations of chloramphenicol succinate and of chloramphenicol (Slaughter *et al.*, 1980b; Mulhall *et al.*, 1983). Decreased esterase activity has been observed in the plasma of neonates and infants. This results in a prolonged period to reach peak concentrations of active chloramphenicol (up to 4 hours) and a longer period over which renal clearance of chloramphenicol succinate can occur (Kauffman *et al.*, 1981).

Chloramphenicol is well distributed in body fluids and readily reaches therapeutic concentrations in CSF, where values are approximately 60% of those in plasma (range, 45 to 99%) in the presence or absence of meningitis (Friedman *et al.*, 1979). The drug may actually accumulate in brain tissue (Kramer *et al.*, 1969). Chloramphenicol is present in bile, is secreted into milk, and readily traverses the placental barrier. It also penetrates into the aqueous humor after subconjunctival injection.

The major route of elimination of chloramphenicol is hepatic metabolism to the inactive glucuronide. This metabolite, as well as chloramphenicol itself, is excreted in the urine by filtration and secretion. Over a 24-hour period, 75 to 90% of an orally administered dose is so excreted; about 5 to 10% is in the biologically active form. Patients with hepatic cirrhosis have decreased metabolic clearance, and dosage should be adjusted in these individuals. The half-life of chloramphenicol has been correlated with plasma bilirubin concentrations (Camp *et al.*, 1979). About 50% of chloramphenicol is bound to plasma proteins; such binding is reduced in cirrhotic patients and neonates (see Appendix II). The half-life of the active drug (4 hours) is not significantly changed in patients with renal failure compared with those with normal renal function. Full doses of chloramphenicol should be given to achieve therapeutic concentrations of the active drug in uremia. The extent to which hemodialysis removes chloramphenicol from plasma does not appear to be sufficient to warrant adjustment of dosage (Blouin *et al.*, 1980). However, patients undergoing dialysis have complications, such as cirrhosis, the

clearance due to dialysis may become important. In such cases it may be best to administer the maintenance dose at the end of hemodialysis to minimize this effect (Slaughter *et al.*, 1980a). The variability in the metabolism and pharmacokinetic parameters of chloramphenicol in neonates, infants, and children necessitates monitoring of drug concentrations in plasma, especially when phenobarbital, phenytoin, or rifampin are administered concomitantly (McCracken *et al.*, 1987).

Preparations, Routes of Administration, and Dosage. *Chloramphenicol* (CHLOROMYCETIN) is marketed in capsules containing 250 and 500 mg for oral use. *Chloramphenicol palmitate* is a water-insoluble powder; 1.7 g of this preparation is equivalent to 1 g of chloramphenicol base. *Chloramphenicol palmitate oral suspension* contains an amount of chloramphenicol palmitate equivalent to 150 mg of chloramphenicol base, mixed with suitable dispersing and flavoring agents, in each 5 ml. *Chloramphenicol sodium succinate* is marketed as the dry powder; it is intended for solution for intravenous use.

Chloramphenicol may be administered orally or intravenously. Dosage schedules for the therapy of specific infections are presented below. Adjustment in dose must be made when chloramphenicol palmitate is used, as indicated above.

Untoward Effects. Chloramphenicol inhibits the synthesis of proteins of the inner mitochondrial membrane that are synthesized within mitochondria, probably by inhibition of the ribosomal peptidyl transferase. These include subunits of cytochrome *c* oxidase, ubiquinone-cytochrome *c* reductase, and the proton-translocating ATPase. Much of the toxicity observed with this drug can be attributed to these effects (Smith and Weber, 1983).

Hypersensitivity Reactions. Although relatively uncommon, macular or vesicular skin rashes occur as a result of hypersensitivity to chloramphenicol. Fever may appear simultaneously or be the sole manifestation. Angioedema is a rare complication. Herxheimer reactions have been observed shortly after institution of chloramphenicol therapy for syphilis, brucellosis, and typhoid fever.

Hematological Toxicity. The most important adverse effect of chloramphenicol is on the bone marrow; of all the drugs that may be responsible for pancytopenia,

chloramphenicol is the most common cause (Wallerstein *et al.*, 1969). Changes in peripheral blood include leukopenia, thrombocytopenia, and aplasia of the marrow with fatal pancytopenia. These reactions are thought to be idiosyncratic. The incidence is not related to dose; however, it seems to occur more commonly in individuals who undergo prolonged therapy and especially in those who are exposed to the drug on more than one occasion. A genetic predisposition is suggested by the occurrence of pancytopenia in identical twins. Although the incidence of the reaction is low, 1 in approximately 30,000 or more courses of therapy, the fatality rate is high when bone-marrow aplasia is complete, and there is a higher risk of acute leukemia in those who recover (Shu *et al.*, 1987).

A compilation of 576 cases of blood dyscrasia due to chloramphenicol indicates that aplastic anemia was the most common type reported, accounting for about 70% of the cases; hypoplastic anemia, agranulocytosis, thrombocytopenia, and bone-marrow inhibition made up the remainder. Among the patients with pancytopenia the outcome was apparently unrelated to the dose of chloramphenicol taken. However, the longer the interval between the last dose of chloramphenicol and the appearance of the first sign of the blood dyscrasia, the greater was the mortality rate; nearly all patients in whom this interval was longer than 2 months died.

Holt (1967) noted the absence of reported instances of aplastic anemia following parenteral administration of chloramphenicol and suggested that absorption of a toxic breakdown product from the gastrointestinal tract might be responsible. Subsequently, a few cases of aplastic anemia have been described in patients who received parenteral chloramphenicol. However, some of these patients had also received other drugs known to affect the bone marrow (phenylbutazone and glutethimide). The issue thus remains unsettled (Kucers and Bennett, 1987). The structural feature of chloramphenicol that is responsible for aplastic anemia is hypothesized to be the nitro group, which might be metabolized by intestinal bacteria to a toxic intermediate (Jimenez *et al.*, 1987). However, the exact biochemical mechanism has not yet been elucidated.

The risk of aplastic anemia does not contraindicate the use of chloramphenicol in situations in which it is necessary; however, it emphasizes that the drug should never be employed in undefined situations or in diseases readily, safely, and effectively treatable with other antimicrobial agents.

A second hematological effect of chloramphenicol is a common and predictable (but reversible) erythroid suppression of the bone marrow that is probably due to its inhibitory action on mitochondrial protein synthesis. A result is a reduction of uptake of ^{59}Fe by normoblasts and of the incorporation of this isotope into heme (Ward, 1966). The clinical picture is marked initially by reticulocytopenia, which occurs 5 to 7 days after the initiation of therapy, followed by a decrease in hemoglobin, an increase in plasma iron, cytoplasmic vacuolation of early erythroid forms and granulocyte precursors, and normoblastosis with a shift to early erythrocyte forms (Scott *et al.*, 1965). Leukopenia and thrombocytopenia may also occur. The incidence and severity of this syndrome are related to dose. It occurs regularly when plasma concentrations are $25\ \mu\text{g/ml}$ or higher and is observed during the use of large doses of chloramphenicol, prolonged treatment with the antibiotic, or both. Dose-related suppression of the bone marrow has been reported to progress to fatal aplasia, but this does not occur predictably (Daum *et al.*, 1979).

The administration of chloramphenicol in the presence of hepatic disease frequently results in depression of erythropoiesis; this is most intense when ascites and jaundice are present (Suhrland and Weisberger, 1963). About one third of patients with severe renal insufficiency exhibit the same reaction.

Toxic and Irritative Effects. Nausea, vomiting, unpleasant taste, diarrhea, and perineal irritation may follow the oral administration of chloramphenicol. Among the rare toxic effects produced by this antibiotic are blurring of vision and digital paresthesias. Optic neuritis occurs in 3 to 5% of children with mucoviscidosis who are given chloramphenicol; there is symmetrical loss of ganglion cells from the retina and atrophy of the fibers in the optic nerve (Godel *et al.*, 1980).

Fatal chloramphenicol toxicity may develop in neonates, especially premature babies, when they are exposed to excessive doses of the drug. The illness, the "gray syndrome," usually begins 2 to 9 days (average, 4 days) after treatment is started. The manifestations in the first 24 hours

vomiting, refusal to suck, irregular and rapid respiration, abdominal distention, periods of cyanosis, and passage of loose, green stools. All the children are severely ill by the end of the first day and, in the next 24 hours, become flaccid, turn an ashen-gray color, and become hypothermic. Metabolic acidosis has been observed as an early sign of the gray syndrome, especially in patients with liver disease (Evans and Kleiman, 1986). Potentially reversible alterations in myocardial function have also been noted (Fripp *et al.*, 1983). Death occurs in about 40% of patients. Those who recover usually exhibit no sequelae.

Two mechanisms are apparently responsible for this toxic effect in neonates (Craft *et al.*, 1974): (1) failure of the drug to be conjugated with glucuronic acid, due to inadequate activity of glucuronyl transferase in the liver, which is characteristic of the first 3 to 4 weeks of life; and (2) inadequate renal excretion of unconjugated drug in the newborn. At the time of onset of the clinical syndrome, the chloramphenicol concentrations in plasma usually exceed 100 $\mu\text{g/ml}$, although they may be as low as 75 $\mu\text{g/ml}$. Excessive plasma concentrations of the glucuronide conjugate are also present, despite its low rate of formation, because tubular secretion, the pathway of excretion of this compound, is underdeveloped in the neonate. Children 2 weeks of age or younger should receive chloramphenicol in a daily dose no larger than 25 mg/kg of body weight; after this age, full-term infants may be given daily quantities up to 50 mg/kg. Toxic effects have not been observed in the newborn when as much as 1 g of the antibiotic has been given every 2 hours to women in labor.

Chloramphenicol is removed from the blood to only a very small extent by either peritoneal dialysis or hemodialysis. However, both exchange transfusion and charcoal hemoperfusion have been used to treat overdose with chloramphenicol in infants (Freundlich *et al.*, 1983).

Other organ systems that have a high rate of oxygen consumption may also be affected by the action of chloramphenicol on mitochondrial enzyme systems; encephalopathic changes have been observed (Levine *et al.*, 1970), and cardiomyopathy has also been reported (Biancaniello *et al.*, 1981).

Drug Interactions. Chloramphenicol irreversibly inhibits hepatic microsomal enzymes of the cytochrome P₄₅₀ complex (Halpert, 1982), and thus may prolong the half-life of drugs that are metabolized by this system. Such drugs include dicumarol, phenytoin, chlorpropamide, and tolbutamide. Severe toxicity and death have occurred because of failure to recognize such effects. The inhibitory effect of chloramphenicol on hepatic enzymes may protect the liver from the toxic effects of carbon tetrachloride, since metabolism is apparently necessary to convert carbon tetrachloride to toxic products.

Conversely, other drugs may alter the elimination of chloramphenicol. Chronic administration of phenobarbital or acute administration of rifampin shortens the half-life of the antibiotic, presumably because of enzyme induction, and may result in subtherapeutic concentrations of the drug (Powell *et al.*, 1981; Prober, 1985).

Therapeutic Uses. *Therapy with chloramphenicol must be limited to infections for which the benefits of the drug outweigh the risks of the potential toxicities. When other antimicrobial drugs are available that are equally effective but potentially less toxic than chloramphenicol, they should be used (see Kucers and Bennett, 1987; Stan-diford, 1990).*

Typhoid Fever. Although chloramphenicol is still an important drug for the treatment of typhoid fever and other types of systemic salmonella infections, other drugs are also effective. Epidemics in some parts of the world have been due to strains of *S. typhi* highly resistant to chloramphenicol. Ampicillin and amoxicillin are also effective in the management of such infections (DuPont and Pickering, 1980). There appear to be fewer carriers and fewer relapses after ampicillin than after chloramphenicol (Snyder *et al.*, 1976). However, the increasing prevalence of resistance to the drug makes it necessary to determine the sensitivity of the microorganisms recovered from patients with these diseases. Trimethoprim-sulfamethoxazole is also very effective for the treatment of typhoid fever, including disease caused by chloramphenicol-resistant *S. typhi* (Gilman *et al.*, 1975). More recently, cefoperazone and ceftriaxone have emerged as candidates for drugs of choice for the treatment of this disease (see Chapter 46).

Within a few hours after chloramphenicol is administered, *S. typhi* disappears from the blood. Stool cultures frequently become negative in a few days. Clinical improvement is often evident within 48 hours, and fever and other signs of the disease commonly abate within 3 to 5 days. The patient usually becomes afebrile before the intestinal lesions heal; as a result, intestinal hemorrhage and perforation may occur at a time when the clinical condition is rapidly improving. The incidence and the duration of the carrier state are not altered. The dose of chloramphenicol employed in adults with typhoid fever is 1 g every 6 hours for 4 weeks. Al-

US OFFICE PRODUCTS

DECLARATION OF RICHARD G. SANTE

IN SUPPORT OF PETITION FOR RELIEF FROM INACCURATE AND UNWARRANTED TESTING FOR CHORAMPEHNICOL RESIDUES IN CRABMEAT

I, Richard G. Sante, hereby declare that:

1. I am the President of Miami Crab Corporation, located at 10585 SW 109 Court, Suite #200, in Miami, Florida, a family-owned company that imports and distributes foreign crabmeat products hand-picked from swimming (blue) crabs.
2. I have extensive knowledge of and expertise in world-wide production techniques for packing pasteurized crabmeat. My job is to travel throughout the world where crabs are fished and have personally inspected and examined crab trapping and netting methods from a variety of vessels. I own an extensive library of photographs on the subject. I have intimate familiarity with all other phases of production of pasteurized crabmeat over the last 15 years. I am well acquainted with aquaculture or fish farming techniques used throughout the world.
3. Based on my personal knowledge, study, reading, research and hands-on experience in the industry, I declare that crabs cannot be economically produced through "aquaculture" or "farming" techniques. All crabmeat produced and delivered through my company derives from wild caught fisheries that catches uninhibited, free swimming crabs naturally present in coastal waters throughout their entire life cycle. These wild crabs are not and cannot be viably fed fish or other feed for commercial purposes.
4. Crabs are naturally very territorial and quite aggressive. Thus, confining crabs in a restricted space that is fenced or otherwise contained in an area that might be amenable to the addition of feed is simply not possible. When crabs are crowded together in enclosed spaces, the crabs engage in cannibalistic behavior that results in a reduction in total population. Finally, crabs are capable of copious reproduction and rapid, healthy growth to maturity in their natural habitats, which further precludes or forestalls the development of aquaculture or farming techniques for crabs.
5. I am not aware of any practice of providing or need to apply antibiotics to crabs to improve production or to prevent or treat disease by anyone or anywhere in the world. Moreover, there is no need for prophylactic or therapeutic antibiotics since the worst enemy for crab growth in artificially enclosed farms is other crabs themselves and not infections or disease.
6. Crabs do prefer coastal, lower salinity waters and thus are generally found in muddy or tidal areas where water originates both from the ocean (saltwater) and as run-off from land (rain water). These areas may be adjacent to areas where aquaculture is practiced for other species (e.g., shrimp).

7. Chloramphenicol is neither added to harvested crabs nor to crabmeat.

8. In sum, based on my experience and personal knowledge, I declare that crabs and crabmeat are not intentionally exposed to chloramphenicol in any form, whether through feed or in any other way.

I HEREBY DECLARE, under penalty of perjury, that the foregoing is true and correct. Executed on July 15, 2002, in Miami, Florida.

A handwritten signature in black ink, appearing to read "Richard G. Sante". The signature is written in a cursive style with a large initial "R" and a long horizontal stroke at the end.

Richard G. Sante
President
Miami Crab Corporation