Reduction of Animal Use through Validation of a Chemical Method of Detection for Paralytic Shellfish Toxins

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Summary

Paralytic shellfish toxins (PSTs) are produced by algae, accumulate in filter-feeding shellfish, and are potent human toxins. Until recently, the Canadian Food Inspection Agency (CFIA) monitored PST levels in shellfish using the official detection method for PSTs, a mouse bioassay (MBA) procedure that consumed 40,000 mice/year. A collaborative project was initiated with the National Research Council Canada to develop and validate a chemical method to replace the MBA.

Several methods were evaluated, and one was subjected to a pilot study to evaluate real-time challenges. A single-laboratory validation was completed, and a transition period using the method for screening reduced animal use by 75%. An interlaboratory study with participants from 11 countries gained international acceptance for the method. This study confirmed that the method could be implemented in different laboratories, and the method was granted official method status by AOAC International. Animal testing for PSTs has been eliminated in all CFIA laboratories.

Keywords: shellfish toxins, validation, HPLC

The use of animal testing for routine shellfish toxin monitoring has been in place for decades, and in the case of paralytic shellfish toxins (PSTs), the mouse bioassay (MBA) has been widely accepted as the gold standard. Toxins such as PSTs are produced by phytoplankton, accumulate in filter-feeding molluscan shellfish, and have a global distribution. The symptoms of intoxication may range from a slight tingling sensation to loss of feeling in extremities, and even death as a result of respiratory paralysis. The Canadian Food Inspection Agency (CFIA) is responsible for monitoring marine toxin levels in molluscan shellfish as part of the Canadian Shellfish Sanitation Program (CSSP).

The MBA is an efficient method of determining the total PST concentration and has protected human shellfish consumers for decades, but it does have drawbacks: It requires the use of three mice for each sample, it cannot provide information on specific toxin profiles, it is variable, and it is expensive. All these reasons, combined with the desire to reduce and eliminate animal testing, led to a five-year collaboration between the CFIA Dartmouth Laboratory and the National Research Council Canada (NRCC) Institute for Marine Biosciences.

The PST group contains many compounds with different toxicities, and this is a challenge to developing an alternative to the MBA (which produces a single integrated result). The production and availability of analytical standards for the most common and toxic compounds is essential for the success of a chemical method. These challenges were first overcome by the successful validation of a pre-column oxidation high-performance liquid chromatography (HPLC) method (AOAC OMA 2005.06), and the first year of this project was dedicated to comparing the pre-column oxidation HPLC method with a modified version of Oshima's post-column HPLC method (Oshima, 1995). While both chemical methods produced results that were equivalent to the MBA results, the post-column oxidation (PCOX) method was better suited to a regulatory laboratory with a high volume of positive samples (Rourke et al., 2008). The largest drawback to the pre-column method was the amount of time involved in quantifying PST levels. The pre-column method was more efficient for processing samples with no PSTs, but the additional time required to distinguish toxins and interpret data in samples with PSTs did not meet the sample throughput requirements of a regulatory laboratory.

The next phase of the project optimized the post-column method, and this was followed by a real-time pilot study. It is unusual to perform a pilot study before completing the validation studies; however, the method needed to meet a strict turn-around-time in order to be considered for implementation. Every sample received in the laboratory for PST analysis from July-October, 2006 was analyzed by both the MBA and PCOX methods, and the PCOX method was evaluated for turn-around-time, ability to troubleshoot and recover from problems, and ease of use in a real-world setting. The PCOX method met these requirements and was further subjected to a single-laboratory validation (van de Riet et al., 2009) and, subsequently, an international interlaboratory study according to AOAC International protocols (van de Riet et al., 2011).

Method performance was characterized for 12 individual PST compounds and total toxicity in blue mussels (*Mytilus edulis*), soft-shell clams (*Mya arenaria*), sea scallops (*Placopectin magellanicus*), and American oysters (*Crassostrea*

virginicus). Highly-contaminated, naturally incurred samples were used to prepare materials for this study in order to preserve analytical standards and create material most representative of real samples. Validation parameters studied in the single-laboratory validation included ruggedness, linearity, detection limits, recovery, repeatability, and intermediate precision. All results were evaluated and found to be acceptable for the target concentrations.

The successful completion of the single-laboratory validation study indicated that the method was suitable for an inter-laboratory validation study, and this was designed according to the AOAC International protocol for collaborative studies. This study examined method performance in non-expert laboratories, and it was designed to isolate potential sources of error. Laboratories from 17 countries expressed interest in the study, and 15 laboratories from 11 countries submitted data. The study materials included blind duplicate samples, fortified samples, QA samples, and three different toxin levels for each of the four previously mentioned shellfish species. A total of 6336 data points were generated and evaluated as part of this work (van de Riet et al., 2011). A bridging study was included to compare the total PST concentrations obtained from the biological and chemical methods. This comparison showed strong agreement between the two methods. The data for individual toxins was evaluated using standard method performance criteria for chemical methods at the stated concentrations (van de Riet et al., 2011). These validation criteria provided the opportunity to state objectively that the method was fit for purpose. The method received acceptance from different bodies throughout the last two years of the project. The first stage of acceptance was given by Health Canada and CFIA in August, 2009 after the single-laboratory validation study was completed and accepted for publication by AOAC International (van de Riet et al., 2009). The second stage of acceptance was given by the Interstate Shellfish Sanitation Conference (ISSC), which approves methods for use in the USA National Shellfish Sanitation Program (NSSP). These approvals allowed the method to be used for Canadian monitoring of samples and import/export with our largest shellfish trading partner, the USA. In November, 2009 the CFIA Dartmouth and Longueuil laboratories implemented the method as a screening tool to reduce animal use. During this transition all PST samples were screened using the PCOX method, and the MBA method was used to confirm any results that would initiate regulatory action. Ten percent of all samples were analyzed in parallel to continue comparing methods before full acceptance was granted. This translated into a 75% reduction in animal use in the two laboratories using this approach. The final approval was received when AOAC International elevated the method to official method of analysis (OMA) status in the spring of 2011. The method could now be used by all four CFIA labs, completely eliminating animal testing.

This work has introduced another official method to determine PST concentrations in shellfish. At the onset of this project, the pre-column HPLC method (AOAC OMA 2005.06, Official Methods of Analysis, 2009) was the only alternative to the MBA if an official method was to be used. Now, the PCOX method (AOAC OMA 2011.02, Official Methods of Analysis, 2009) and the recently-approved receptor binding assay method (manuscript in press) provide additional alternatives. These methods have the potential to drastically reduce the number of animals used for shellfish testing internationally.

Many physical and conceptual challenges were overcome throughout the project life cycle. The first physical challenge to validation and implementation of any chemical method is the availability of analytical standards. The analytical standards are particularly difficult to manufacture or isolate for shellfish toxins, and there are very few groups in the world carrying out this work. Progress has been made in this field, and the most important PST analytical standards are now available from the NRCC Certified Reference Materials Program. The second physical challenge is the requirement for specialized equipment needed to use chemical methods. Complex instruments are required for the analysis of samples using chemical methods; these instruments are expensive to purchase, but, when viewed over the lifespan of the equipment, the total cost often is lower than maintaining biological methods. Different skill sets are subsequently required to operate equipment, and consideration should be given to whether a single cross-trained group is more efficient than two specialized groups. Two groups were involved at the start of the project, but a single group emerged that was trained in both chemical and biological methods. The third challenge for replacement methods is whether they will be able to detect new toxins. This concern often is cited when integrated biological methods are replaced with specific chemical methods. With this project, a new toxin already has been identified using the PCOX method (Gibbs et al., 2009).

The second group of challenges was more conceptual in nature. Regulatory and individual caution were difficult to deal with, since the MBA method had protected human safety for more than 50 years, and thus many people did not want to consider alternatives. International and country-specific regulations were considered, since the goal of the project was to develop an official method for regulatory testing. Major changes with international impact happen infrequently in any given area, and often an integrated process to facilitate these changes is lacking. Various jurisdictions regulate differently, ranging from explicit legislation to easily-amended policies. Regulatory caution was satisfied by asking a lot of questions and submitting validation data to many groups in appropriate formats. Well-designed validation studies provided an objective demonstration of equivalent method performance to overcome individual reservations. A single method performance characteristic or perception should not be used to assess two methods without consideration of context. One specific example is the turn-around-time of the two methods. The MBA method (with a sufficient number of trained analysts) can produce a result on the same day for a normal volume of samples in a regulatory laboratory, whereas results from the PCOX method are not available until the following day

because samples typically are analyzed overnight by HPLC. This difference is offset by the lower detection capability of the PCOX method, which can detect individual toxins at levels four times lower than the total toxicity detection limit of the MBA. This potential to detect toxins at lower concentrations enables earlier detection of toxic episodes and the ability to adjust sampling frequency and priority.

Implementing a change from a method that has been used for more than 50 years to a new method was challenging, but there were lessons learned that can be applied to other situations to minimize difficulties. The most important factor is planning for the entire process before execution. Large changes, such as introduction of a new methodology, should be implemented during off-season or times of decreased sample volume/priority if possible. All training, documentation, and characterization of required quality assurance materials should be completed before the method is used for routine samples. Communication with stakeholders throughout the transition is also vital, and it is especially important that all such communications are realistic so everyone knows the timeline for changes and what to expect when changes happen. If a new method has drawbacks, it is important to state clearly what is being done to overcome or compensate for these weaknesses, as well as the context that makes the new method a better overall choice. The supporting evidence for this discussion often will be derived from the validation studies.

A five-year project to replace the MBA for PST monitoring was successfully completed, leading to the elimination of animal testing for shellfish toxin monitoring in Canada. This is a reduction in laboratory animal use of 40,000 mice each year. Many physical and conceptual challenges presented themselves during the project, but all were overcome. Most important to the success of this project were the carefully planned validation studies designed to address both method performance questions and comparisons with the existing methodology. Data generated from the validation studies demonstrated that the PCOX method was "fit-for-purpose," and the results were used to overcome resistance to replacing a 50-year old bioassay method.

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