IMPROVED METHODS OF DETECTION FOR THE DIFFICULT TO IDENTIFY MARINE TOXIN, OKADAIC ACID

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A Thesis Submitted to the
University of North Carolina Wilmington in Partial Fulfillment
Of the Requirements for the Degree of
Master of Science

Department of Chemistry and Biochemistry

University of North Carolina Wilmington

2005

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ABSTRACT

The focus of this study was to investigate an alternative, economic approach to the detection of the diarrhetic shellfish poisoning (DSP) toxins in microalgae and shellfish. This group of marine toxins has had a negative impact on human health and economy of the shellfish industry. The method selected for investigation was the evaporative light scattering detector (ELSD).

Chemical detection of any member of the DSP toxins is compromised by the fact that none of them contain a strong chromophore or fluorophore. Preparation of a fluorescent derivative involves a time consuming chemical reaction and specialized equipment that yields questionable toxin concentration accuracy. Alternatively other biochemical methods have been reported, which include an enzyme bioassay using protein phosphatase, or an enzyme linked immunosorbent assays (ELISA). However, these methods are not economical, often requiring specialty reagents, and in the case of the ELISA-based assay, not all the members of the DSP toxin group may be detected with equal sensitivity. The mouse bioassay, used almost universally, can only be used in a broad empirical sense, and there is reluctance among certain countries to continue using this method as a routine monitoring method. More recently, mass spectrometry is often used and may be the best detector available, but the initial capitol cost is high, as is the daily operation of the instrument. Consequently, this detection method is most usually reserved for research laboratories. In selecting an economical detection method for these toxins, the ELS detector offered an attractive, inexpensive approach. The results reported here, where DSP toxins were monitored in phytoplankton and shellfish tissue, support that hypothesis.

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INTRODUCTION

There is a continuing need to develop improved detection methods for marine toxins, not only for the detection of new families of toxins, but also to develop robust and economical methods having utility in regulatory and monitoring laboratories. For the most part, these marine toxins are produced by marine microalgae or phytoplankton an essential part of the food chain in the ocean. These microalgae are grouped into five divisions and include the Chlorophyta, Chrysophyta, Euglenophyta, Cyanophyta, and Pyrrhophyta. Of the approximately 5,000 known phytoplankton only about 40 species produce toxins (Sournia et al., 1991). Of these harmful microalgal species 75% fall into the category of Pyrrhophyta which make up such a large portion of harmful algae due to the diversity of toxins they produce (Hallegraeff 1993). Many of the known toxins produced by dinoflagellates belonging to the class Pyrrhophyta (Daranas et al., 2001) are the main focus of this thesis work. Approximately 2000 species of dinoflagellates described, and around 60 species have been reported to produce blooms that would be considered harmful (Smayda and Reynolds 2003).

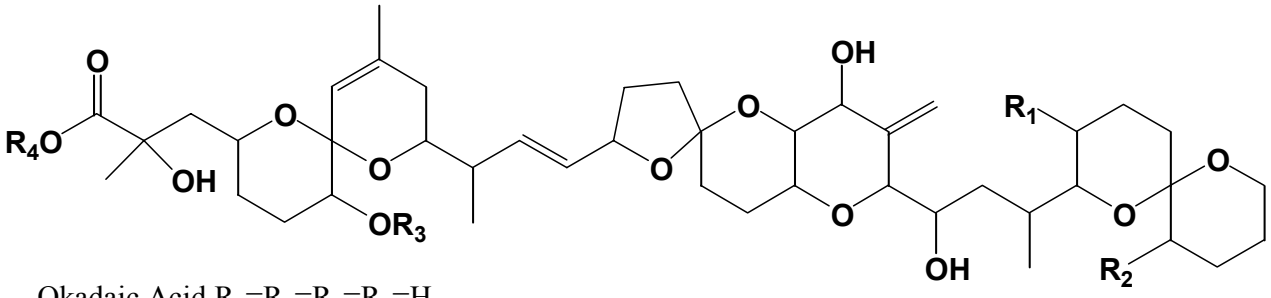
Marine microalgae can increase in numbers to the point of discoloring the water. These "red tides" as they are often referred to, can be produced by almost 300 different algal species and can spread quickly (Sournia et al., 1991). With appropriate temperature, light, salinity, and nutrients a single cell can divide into 7,000 cells in just a week (Anderson et al., 1995). Rapid growth of phytoplankton helps support the marine food chain, but when toxic species are present filter feeding organisms like scallops and mussels ingest and concentrate toxins in their tissues (Lansberg 2002). Such toxic shellfish pose a serious health risk when consumed by humans (Hallegraeff 1993). Finfish that eat plankton can also bioaccumulate the phycotoxins, which then travel up the food chain to birds and marine mammals (Quilliam 2003). For example,

mussels can typically concentrate okadaic acid to levels around 10 μg of toxin/g of digestive gland or hepatopancreas (Morono et al., 2003). To add further complexity, the DSP toxins can be converted to acyl derivatives by esterification with saturated and unsaturated fatty acids at the 7-hydroxy position (Lee et al., 1987; Marr et al., 1992). These acyl derivatives are not found in the producing dinoflagellates, but have been found in Irish and Spanish mussels (Marr et al., 1992; Fernandez et al., 1996). The esters were also found in Portuguese bivalves and were linked to an outbreak of poisonings in humans (Vale and Sampayo, 1999b). Toxic seafood cannot be differentiated from safe seafood by taste or visual inspection, and since their biotoxins are not heat labile cooking does not prevent illness from occurring (Hallegraeff 2001).

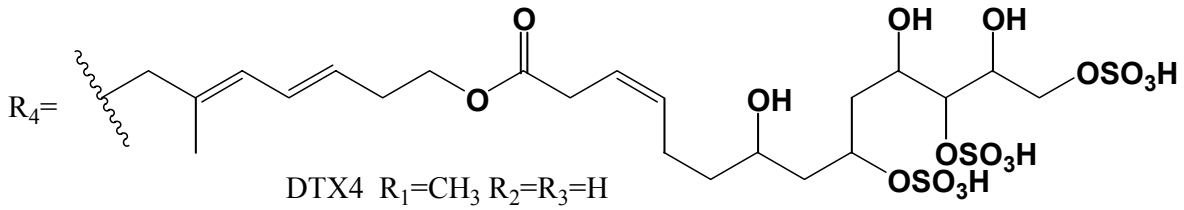
It has been suggested that the frequency of harmful algal blooms (HABs) has increased in the last 20 years (Daranas et al., 2001). One explanation for this trend is that through increased monitoring and improved detection methods, scientists are able to identify toxic blooms more efficiently than in the past. Another reason is the transportation of microalgae in the ballast tanks of ships resulting in blooms where none have occurred before (Hallegraeff and Bolch 1991). In a study of 80 cargo vessels 40% of them were carrying dinoflagellates, and 6% contained dinoflagellates that produce toxins (Hallegraeff and Bolch 1991). Finally, the continual urban development of coastal and estuarine environments has resulted in increased anthropogenic runoff and eutrophication providing microalgae with the nutrients necessary for bloom development and proliferation (Hallegraeff et al., 1995).

Dinoflagellates produce a variety of toxic polyketide compounds of varying structure and size. Among the most common are the polycyclic ethers (Shimizu 2003) sometimes referred to as “ladder-frame” polyethers (Wright et al., 1998). One group of toxins with this structural backbone is the brevetoxins produced by the dinoflagellate *Karenia brevis* formerly known as

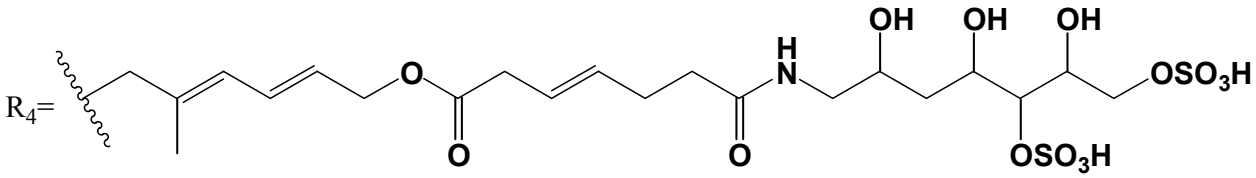
Ptychodiscus brevis (Baden and Tomas 1988; Bourdelais et al., 2005). Other toxins with a similar "ladder frame" structure produced by other dinoflagellates are the yessotoxins from *Protoceratium reticulatum*, the ciguatoxins from *Gambierdiscus toxicus*, maitotoxins from *Coolia monitis* (Daranas et al., 2001). Yet another structural group is described as the linear polyethers and include azaspiracids (Ofuji and Satake et al., 2001) and the diarrhetic shellfish poisoning (DSP) toxins. The latter group includes okadaic acid, DTX-1 (Yasumoto et al., 1984) and DTX-2 (Hu et al., 1992a). The DSP toxin okadaic acid is the subject of this thesis work. Dinoflagellates are among the most troublesome toxic species worldwide, and those belonging to the genera Prorocentrum and Dinophysis produce the diarrhetic shellfish poisoning (DSP) toxins. The first DSP isolated was DTX-1 from the dinoflagellate *Dinophysis fortii* (Murata et al., 1982) the structure was not elucidated until the nearly identical cytotoxic compound okadaic acid was isolated from the black sponge *Halichondria okadai* (Tachibana et al., 1981).



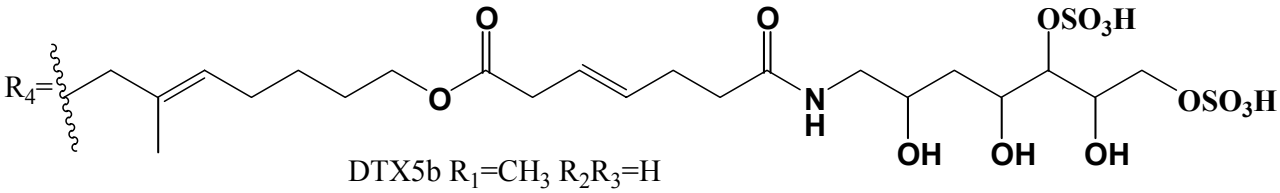
Okadaic Acid $R_1=R_2=R_3=R_4=H$
DTX1 $R_1=R_3=R_4=H$ $R_2=CH_3$
DTX2 $R_1=H$ $R_2=CH_3$ $R_3=R_4=H$
DTX3 $R_1=R_2=CH_3$ R_3=acyl $R_4=H$



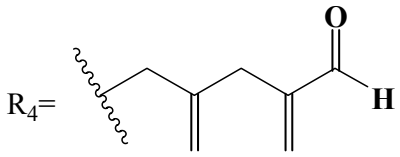
DTX4 $R_1=CH_3$ $R_2=R_3=H$



DTX5a $R_1=CH_3$ $R_2=R_3=H$



DTX5b $R_1=CH_3$ $R_2R_3=H$



DTX6 $R_1=R_2=R_3=H$

Figure 1. Toxins produced by *Prorocentrum sp.* (Daranas et al., 2001; Hu et al., 1992a; Murata et al., 1982).

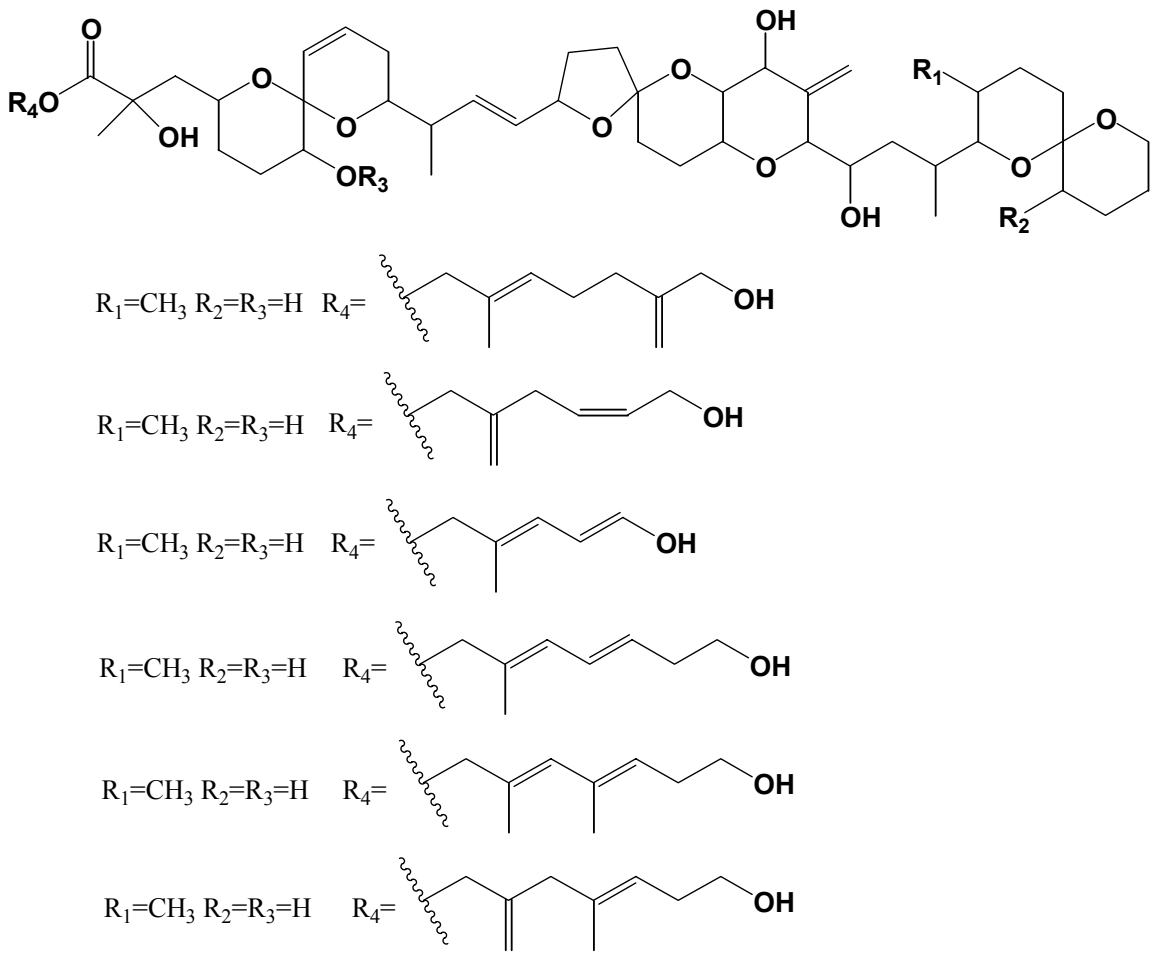


Figure 2. The "diol esters" of okadaic acid (Daranas et al., 2001; Hu et al., 1992b; Norte et al., 1994).

DSP outbreaks occur all over the world: In the Caribbean, *P. maculosum* (Dickey et al., 1990), in Canada, *Prorocentrum sp.* (Yasumoto et al., 1984), southern Europe and Ireland, *D. acuminate* (Puente et al., 2004), in Norway, *D. acuto* and *D. norvegica* (Aune and Yndestad 1993), in Japan, *D. fortii* and *P. lima* (Cembella 1989). Most DSP toxin research has been done using *Prorocentrum sp*., and to a much lesser extent with *Dinophysis sp*. This is because the latter group have not yet been successfully cultured in the laboratory (Subba Rao et al., 1993) and so studies with this organism are confined to naturally occurring blooms when they are observed.

The European Union and the US Food and Drug Administration along with Japan, Canada, and Norway each set the regulatory action limit for shellfish contaminated with DSP toxins at 20 μg of toxin/100 g of shellfish material (Honkanen, et al., 1995; Mountfort et al., 1999, Wright and Quilliam, 1995), other countries have different units like Sweden which limits of 40-60 μg/100g of shellfish, or Korea 5 MU/100g of shellfish (Shumway et al., 1995) A mouse unit (MU) is the amount of toxin required to kill a 20 g mouse in 24 hours and corresponds to approximately 4 μg of okadaic acid (Yasumoto et al., 1978).

DSP toxins are known to inhibit the eukaryotic enzymes PP1 and PP2A (Bourdreau and Hoskin 2005). In humans, ingestion of DSP toxins results in diarrhea, vomiting, nausea, abdominal cramps and headache (Aune and Yndestad 1995). These symptoms usually show up anywhere between 30 minutes and 12 hours and can last up to 4 days (Van Egmond et al., 1993), and there is no effective medial treatment (Honkanen et al., 1995). Furthermore, it is possible that exposure to DSP toxins may also result in intestinal or digestive cancers (Cordier et al., 1999).

Detection Methods

Unlike some algal toxins, the DSPs are more troublesome to detect because they do not possess a strong chromophore that facilitates their detection by various UV detection methods (Wright 1995; Quilliam 2003). As a result a variety of alternative methods have been investigated to detect DSP toxins. The primary detection methods that have been employed are discussed below.

Protein Phosphatase Assay

The protein phosphatase PP assay was developed as a consequence of the ability of DSP toxins to inhibit the eukaryotic serine/threonine protein phosphatases, PP1 and PP2A (Takae and Mieskes, 1991; Takae et al., 1992; Honkanen et al., 1995). In this assay, under normal conditions, phosphate is released from a phosphorylated substrate by the enzymes, PP1 or PP2A, but in the presence of DSP toxins this reaction is inhibited and no phosphate is released (Honkanen et al., 1995). Thus, the level of free phosphates corresponds to the amount of toxin present in the reaction mix. The protein phosphatase assay has been tested using various substrates that provide results in varying formats. The substrates methylumbelliferyl phosphate (MUP) or fluorescein diphosphate (FDP) produces a fluorescence response, whereas using luciferin phosphate (L-P) provides a bioluminescent one, and p-nitrophenylphosphate (p-NPP) results in a colorimetric response (Mountfort et al., 1999). The current problem with using the bioluminescence assay is that it requires the use of luciferase which requires storing at -20ºC and is a harmful chemical that can be inhaled and absorbed through skin (Mountfort et al., 1999). The original assay used p-NPP (Tubaro et al., 1996) as a substrate, but more recently the fluorometric substrates MUP and FDP (Vieytes et al., 1997) have been employed. However, MUP and FDP require a fluorescence detector, costing $25,000 to $40,000, which is more

expensive than the absorbance detector necessary when using the p-NPP substrate, but for accurate measurements the fluorescent substrates are better and may justify the increased cost of equipment (Mountfort et al., 1999). The fluorometric substrate assays have limits of detections of 1 μg/100 g of shellfish (Mountfort et al., 1999), or 150 ng/100 g of hepatopancreas (Nunez and Scoging 1997). The PP assay cannot provide information on the toxin profile, only on the existence of toxin(s) present, which makes it more suitable for use as an intial screening method, but really needs to be followed up with a more specific LC-coupled method such as LC-MS (Mountford et al., 1999). Another drawback to the PP assay is that it lacks sensitivity towards the ester derivates of okadaic acid resulting in a false negative response that could cause unnecessary economic problems to the shellfish industry (Mountfort et al., 2001.) The problem can be resolved by adding an alkaline hydrolysis step to the detection process so that the ester derivatives are converted to okadaic acid and DTX-1 (Mountfort et al., 2001). While the additional hydrolysis reaction might cut down on the number of false negatives, it adds further cost to the process. Another issue has been discovered with this assay. Shellfish have been found to produce enzymes that also inhibit the release of phosphates, so non-toxic shellfish could be labeled as toxic due to the presence of these enzymes (Honkanen et al., 1995). Typical protein phosphatase kits are available for approximately $175.00 as seen in AnaSpec, Inc., permitting 500 assays to be performed.

Mouse Bioassay Method

The mouse bioassay, used for several decades to monitor seafood safety, is presently the only accepted method by the U.S.F.D.A for testing marine biotoxins. It is one of the few internationally accepted assay methods for marine toxins, and has the advantage of highlighting the presence of any toxic substance that may be present in shellfish (Yasumoto 1978). The

mouse bioassay provides a physiological response that can be equated to humans as to whether compounds in a sample are hazardous. Toxin concentrations are generally reported in mouse units (MU). The detection limit for the mouse bioassay is 20 μg/100 g of mussel (Mountfort et al., 1999). While the mouse bioassay can detect the presence of DSP compounds it does not provide specific toxin detection, reproducibility and quantification are variable, and false positive results may result because high levels of polyunsaturated fatty acids in shellfish tissue can adversely affect mice (Aversano et al. 2005; Draisci et al. 1994).

ELISA-Based Method

The enzyme linked immunosuppressant assay (ELISA) requires the production of antibodies specifically made to respond to the DSP toxins. Typically the antibodies that have been raised against okadaic acid are made using mice. Once the complex string of antibodies is made horseradish peroxidase is added and binds to the goat antibody, which was created using a previously obtained okadaic acid specific mouse antibody and injected in a goat, after which a substrate is added, 3,3′,5,5′-tetramethylbenzidine (TMB) that will react with the horseradish peroxidase. The TMB is a dye that will only produce color in its free form. So, unbound TMB will produce a color, typically blue, while TMB that has bound, which will only occur if all of the previous chain is present, is indicative of toxin. Sulfuric acid is added to make the color absorb at 450 nm. The presence of color, unbound TMB, indicates a sample not containing toxin, and samples that are clear are toxic. An absorbance detector is used to provide quantification will allow concentrations of toxins to be obtained

There are several commercial ELISA kits available specifically designed for the detection of DSPs, not all of which use TMB, but from experience TMB has shown to be the most effective dye. The SCETI DSP-check ELISA kit has a detection limit of 10 μg/100 g of mussel

(Mountfort et al., 1999), and other kits available are the "DSP-Check" from UBE Industries, and "Okadaic Acid ELISA Kit" by Rougier Bio-Tech (Morton and Tindall 1996). One drawback to this approach is that the cross reactivity of the kits towards other DSP toxins other than okadaic acid is usually poor. The Rougier Biotech kit is reported to underestimate the amount of toxin present based on known standards, likely attributed to the lower binding affinity to DTX-1, which is approximately 10 times less than the binding affinity for okadaic acid, Rougier Bio-Tek (Lawrence et al., 1998). The UBE ELISA does not detect DTX-1 at all (Morton and Tindall 1996).

Studies have shown that the ELISA kits have accuracy issues with consistently detecting DSP toxins at low concentrations (Nunez and Scoging 1997), and the ELISA kits produced to date seem to have only limited cross reactivity with DTX-1 (Nunez and Scoging 1997) while no data exists for DTX-2 a common toxin in Europe (Hu et al., 1992b, Carmody et al., 1995). The ELISA-based assays cannot detect the ester forms of toxins, thus an additional hydrolysis step is required to convert these esters to a form suitable for detection (Vale et al., 1999a). Thus the high specificity of antibody-based detection methods may be a hindrance when it is important to detect other DSP toxins, and the question of matrix effects (e.g. mussel tissue versus oyster or scallop tissue) poses an additional question mark. However, ELISA-based methods have been effectively used in testing phytoplankton samples for the presence of toxins (Imai et al., 2003).

Fluorescent Derivatives Method

For chemical-based detection methods, the lack of a chromophore in DSP toxins, precludes the use of more traditional detection methods such as LC-UV. One solution that has been employed is to prepare a fluorescent ester derivative of the DSP toxins which contain a free carboxyl group (Lee et al. 1987). The 9-anthryldiazomethane (ADAM) is most commonly used

(Rawn et al., 2005), but several other reagents have been tested such as N-(9acridinyl)-bromoacetamde (Allenmark et al., 1990), 4-bromomethyl-7-methoxycoumarin Br-Mmc (Hummert et al., 1995), and 2,3-(anthracenedicarboximido)ethyltrifluoro-methanesulphonate AE-OTf (Ohrui et al., 1995), and 4-bromomethyl-6,7-dimethoxycoumarin (BrDMC) (Lawrence et al., 1996). Possible successors to ADAM are 9-chloromethylanthracene (CA), which yields the same derivatization product as ADAM (Lawrence et al., 1996), or 1-bromoacetylpyrene (BAP). Both reagents are useful because they are stable, are relatively inexpensive to purchase, and can be kept under normal refrigeration like the ADAM reagent (Gonzalez et al., 2000). However, these methods are still being tested, and currently the ADAM (Lee et al., 1987; Quilliam 1995), BAP (Kelly et al., 1996; Dickey et al., 1993), and 1-pyrenyldiazomethane PDAM (Morton and Bomber 1994) reagents are the ones of choice (James et al., 1997).

The extraction and clean up procedures in this method are reasonably complicated, and at the very least, time consuming. The shellfish material is extracted twice with aqueous methanol and centrifuged, solvent partitioned with dichloromethane-hexane mixtures to remove non-polar compounds, and dried (Lawrence et al., 1996; Rawn et al., 2005). The derivatization process requires the addition of the reagents together with tetramethylammonium hydroxide (Lee et al., 1987; Carmody et al., 1995), boiling for 1 hour in the dark, and then cooling (Lawrence et al., 1996; Rawn et al., 2005). The final clean up is achieved using two column chromatography steps and eluting with chlorinated solvents to purify the derivatized product (Carmody et al., 1995; James et al., 1997; Lawrence et al., 1996; Rawn et al., 2005; Quilliam et al., 1998). Detections limits have been cited at 1 μg/100 g of shellfish (Gonzalez et al., 2000). In addition to the complexity of the process, the ADAM reagent is expensive and must be stored below -70°C otherwise it becomes unstable and degrades (Lawrence et al., 1996). Reagent degradation

is critical and in some cases can result in only a 30% recovery of the derivatized product (Quilliam et al., 1998). The occurrence of artifact peaks in the chromatogram (Quilliam et al., 1998; Vale et al. 1999a), and the time required for each reaction and clean-up are further concerns when using the ADAM method for DSP detection (Wright 1995). Finally, the use of chlorinated solvents has been an increasing concern in the chemical community. Studies have shown that chlorinated solvents, like dichloromethane, a solvent used frequently in this method, have several severe health effects including neurotoxicity (Herr et al., 1997; Rosengren et al., 1986), carcinogenesis (Cassanova et al., 1996), hepatotoxicity (Mizutani et al., 1988), and carboxyhemoglobinemia (Lehnebach et al., 1995). A surprising observation with many of these reagents is that they are not as effective at derivatizing toxins in phytoplankton samples, as they are in shellfish extracts (Nogeiras et al., 2003).

Liquid Chromatography-Mass Spectrometry (LC-MS) Method

In general, liquid chromatography coupled with a mass spectrometer provides a very sensitive chemical method for toxin detection (Puente et al., 2004), and in addition provides some important structural information to further confirm the identification of the analyte. In a typical system, the liquid chromatography system (LC) is coupled with an atmospheric pressure electrospray ionization-mass spectrometry (ESI-MS). This technique has been successfully applied to the detection of many compounds including marine toxins, and is an effective method for the detection of DSP toxins (Draisci et al., 1999; Rawn et al., 2005; Suzuki and Yasumoto 2000; Volmer and Sleno 2004). To accommodate the sensitivity of the mass spectrometry method, and avoid contamination of the mass spectrometer source, samples require more clean-up than with other LC detection methods. A typical work up involves extraction with aqueous methanol, centrifugation, partitioning with hexanes to remove non-polar lipids, and if

appropriate, another washing with chloroform. Further purification using an SPE column is only used when necessary (Puenti et al., 2004; Suzuki and Yasumoto 2000).

Reliable data can be obtained with a single quadrupole mass spectrometer, but a triple quadrupole MS ion trap which generates characteristic fragment ions is considered the optimum for complete confirmation of the presence of a specific toxin (Volmer and Sleno 2004). While even more sophisticated instruments are available (Volmer and Sleno 2004), the expense of purchasing any mass spectrometry system is a major hindrance for many regulatory or monitoring laboratories. Even single quadrupole MS systems still cost around $160,000 and the triple quadrupole ion trap and time of flight instruments are in the range $300,000 - $500,000 each. Most monitoring or regulatory laboratories cannot afford such detectors, let alone the cost of maintaining them, which requires a constant supply of pure nitrogen and trained personnel to operate them. Yet another problem is the absolute size of the detector itself, (some footprints are as much as 20 square feet), and the requirement for dedicated laboratory space. While DSP toxins are amenable to LC-MS detection methods, this type of equipment is not always available to laboratories, and alternative methods are being sought (Rawn et al., 2005).

Evaporative Light Scattering Detector

The evaporative light scattering (ELS) detector provides an alternative and efficient method for detecting various toxins including okdaic acid. The topic of this thesis was to investigate the potential of HPLC-ELS detection for detecting the diarrhetic shellfish poisoning toxins previously requiring derivatization or sophisticated analytical equipment for their detection.

METHODS

Growth Curve

Culture

Four Fernbach flasks (3L) each containing media (1.5L) were inoculated with 500mL of *Prorocentrum lima* culture (CMS TAC PL 010). Cultures were in a growth chamber at 24°C with a 14 hour day 10 hour night light cycle, salinity of 36 ppt, and "f/2-Si" media (Valenzuela-Espinoza et al., 1999). Cell counts were taken on the initial culture used to inoculate the four Fernbach flasks. After approximately 3 weeks of growth, glycine (2 mL of 0.2422 M solution) and sodium bicarbonate (2 mL of 0.9919 M solution) were added to two of the flasks.

Okadaic Acid Analysis (Media)

The concentration of okadaic acid in the media was determined by removing an aliquot of culture media (28.5 mL), which was then centrifuged at 3,000 rpm in a Falcon tube (50 mL). The supernatant was decanted from the cell pellet and loaded onto a conditioned Sep-Pak 6 mL C_{18} SPE column. Under slight vacuum the column was desalted with deionized water and eluted with 60% MeOH:water (12 mL), and again with 80% MeOH:acetone (12 mL). The fractions were dried in a Savant Speedvac Plus.

Okadaic Acid Analysis (Cells)

The okadaic concentration in the cells was determined by extraction of the cell pellet obtained as described above with 75% MeOH:acetone (20 mL), which was vortexed and then sonicated (10 watt) for 15 minutes, and finally left to stand for 5 hours. Following extraction, the mixture was centrifuged at 4500 rpm to remove as much cell debris as possible, and the organic supernatant was decanted and dried. This analyte was suspended with 20% MeOH:water (20 mL) and loaded onto a prepared Sep-Pak C_{18} SPE column (6 mL) under slight vacuum, desalted,

and eluted with 60% MeOH:water (12 mL) and 80% MeOH:acetone (12 mL). The fractions were dried in a Savant Speedvac Plus.

Okadaic Acid Analysis (Combined Cells and Media)

The total concentration of okadaic acid in the culture was determined by removing an aliquot (10 mL) of whole culture, which contained cells suspended in growth media, and freezing it in a solution of acetone and dry ice prior to freeze drying using Virtis freeze drier. The dried sample was then extracted with 75% MeOH:acetone (20 mL), vortexed then sonicated (10 watt) for 15 minutes, and then left for 5 hours. The samples were then centrifuged for 10 minutes are 4,000 rpms, decanted and dried down. The sample was suspended in 20% MeOH:water (20 mL) and loaded onto a prepared Sep-Pak C_{18} SPE column (6 mL), under slight vacuum desalted, and eluted with 60% MeOH:water (12 mL) and 80% MeOH:acetone (12 mL). The fractions were dried in a Savant Speedvac Plus.

Chemical Analysis

The organic fractions produced obtained from the media, cells, or combined media and cells were all treated identically. The dried fractions were suspended in 55% acetonitrile:water (50 μL) containing 0.1% formic acid, which served as the HPLC mobile phase. Samples (5 μL), better for chromatography, were analyzed in triplicate using an HPLC system which consisted of a Waters 515 pump, Waters gradient controller, Waters 2487 Dual Wavelength Absorbance Detector and Sedere, Sedex 75 ELSD detector with low flow nebulizer, and Servogor chart recorder, the flow rate used was 0.2mL/min. The settings on the ELSD were as follows: gain 10, temperature 42° C, pressure of 3.5 bar of zero grade nitrogen gas, and the chart recorder set at 50 mV. The chart recorder for the UV detector was set at 1 mV. The HPLC column was a Phenomenex Luna $C_{18}^{(2)}$ 150 mm x 2 mm. Limit of detection was defined as signal 3 times the

baseline noise. For validation purposes the samples were also analyzed by mass spectrometry with a Waters Micromass ZQ LC-MS using the same injection volume, column, mobile phase, and cone voltages of 25 V using positive and negative electrospray.

Cell Density

The cell density was tracked throughout the growth period by removing an aliquot (2 mL) of the media every 5 days. Each aliquot was treated with a drop of Lugol (Throndsen 1978), and put into a settling chamber overnight (Guillard 1973). The chambers were examined under a Nikon Diaphot B 40x microscope, and by counting the number of cells per microscope field and the number of fields necessary to reach approximately 100 cells, a cell density (cells/mL) could be found. The 100 cell mark was used as a method of keeping consistency for each day of sampling. Using the equation (Stein 1980): M = 338.27

$$\frac{(1/N)*M*C}{V} = \text{Cell/mL}$$

M= Magnification Constant
N= Number of Fields
C= Number of Cells
V= Volume of Culture

NRC Mussel Reference Material

A toxic mussel reference material was purchased from the National Research Council of Canada to examine the practicality of using the ELSD. The reference material (CRM-DSP-Mus-b) consisted of 3.9 g of blended *Mytilis edulis* digestive gland tissue and contained known concentrations of okadaic acid (10.1 μg/g), and DTX-1 (1.3 μg/g). The extraction and clean up steps were essentially the same as those described for the growth curve work with the exception that the volumes used to suspend the fractions for HPLC injections were changed. A flow diagram showing the clean-up process to obtain the fractions for analytical detection is shown below in figure 3.

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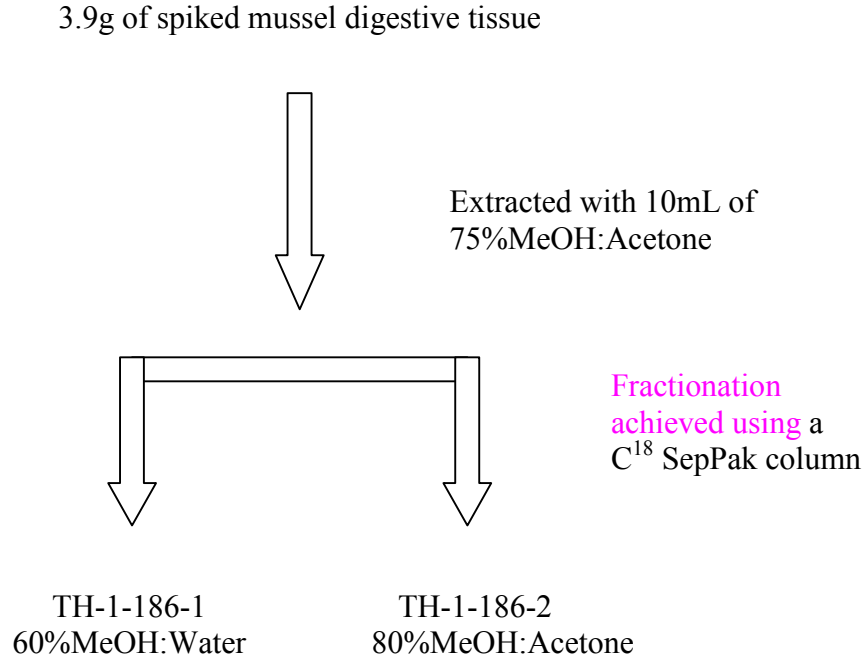

Figure 3. The extraction and purification process for the mussel sample

Each fraction was dried in a Speedvac, and the 60% MeOH:water fraction (TH-1-186-1) was resuspended in 55% acetonitrile:water (1 mL) containing 0.1% formic acid. The 80% MeOH:acetone fraction (TH-1-186-2) was resuspended in of 55% acetonitrile:water (1.75 mL) containing 0.1% formic acid. An aliquot (5 µL) of each and was injected onto the HPLC system. No signal corresponding to okadaic acid could be observed using the UV detector. However, a substantial peak (R.T. 9 mins), which corresponds to the retention time on the Phenomenex Luna $C_{18}^{(2)}$ 150 mm x 2 mm for okadaic acid, was observed using the ELS detector in both TH-1-186-1 and TH-1-186-2 fractions. When the same samples were examined by LC-MS, a molecular ion for okadaic acid could not be observed in the MS-ES+ and MS-ES^-, probably due to matrix effects from the shellfish. To solve this problem each fraction from the SPE column was further purified by using the same conditions as for HPLC ELS detection, the tubing was disconnected after the column and the output flow was collected from the 8^{th} minute to the 10^{th} minute. The timed fraction collection corresponds to fractions denoted with an "A". Following 3 injections, 1.2mL of TH-1-186-1-A and 1.2mL of TH-1-186-2-A were collected. These 2 samples were then dried, resuspended in 55% acetonitrile:water and 0.1% formic acid and an aliquot (5 µL) was injected into the LC-MS.

Due to the complexity of these mussel fractions, confirmation that the peak assigned to okadaic acid by ELS detection was obtained by co-injection with a spike of okadaic acid and also by collection of the peak and analysis by LC-MS (Waters ZQ 2000).

Certified Okadaic Acid Standard

A certified okadaic acid standard (NRC CRM-OA-b) was purchased from the National Research Council of Canada, from which 20 µg of toxin was carefully weighed using a Kahn eletrobalance, and a solution prepared to provide a final concentration of 70 ng/µL. Aliquots of

this solution were examined by HPLC-UV/ELSD, and LC-MS methods, as well as LC-RI (Waters 2414 refractive index detector). The injections were all 5 μL and all done in triplicate. This was done to make a comparison between the ELSD signal produced by the LC Labs okadaic acid standard and the NRC certified okadaic acid standard. The experiments using the RI detector were carried out to compare the performance of this detector with the other LC detectors.

Calibration Curve

A calibration curve, including the standard errors, was constructed from the ELSD and LC-MS data using okadaic acid bought from LC Labs, so the signals produced from these instruments could be converted into ng of toxin/μL. Samples were done in triplicate and run on 3 separate days. The ELSD and MS analytical conditions were the same as described earlier.

Table 1. First Calibration Curve data for okadaic acid, obtained using 4 different detection methods, (UV, ELS, MS-ES+, and MS-ES$^-$) an "*" denotes a concentration not analyzed, "ND" denotes a non-detectable signal. UV and ELSD values are peak heights.

First Calibration Curve				
Concentration ng/µL	**UV (mm)**	**ELSD (mm)**	**ES- Peak Area**	**ES+ Peak Area**
70	1.5	90	6.16E+06	4.79E+07
70	1.5	80	6.12E+06	5.08E+07
70	1.5	82	5.65E+06	5.09E+07
60	1	71	5.09E+06	4.35E+07
60	1	70	5.02E+06	4.29E+07
60	1	69	4.97E+06	4.25E+07
40	0.5	41	3.72E+06	3.17E+07
40	0.5	44	3.69E+06	3.25E+07
40	0.5	41	3.46E+06	3.17E+07
20	ND	13	2.07E+06	1.79E+07
20	ND	13	2.05E+06	1.82E+07
20	ND	13	2.10E+06	1.76E+07
10	ND	4	1.05E+06	1.01E+07
10	ND	4	1.09E+06	9.51E+06
10	ND	6	1.16E+06	9.93E+06
5	ND	2	5.75E+05	4.90E+06
5	ND	2	6.24E+05	5.15E+06
5	ND	2	5.87E+05	4.86E+06
0.15	*	*	1.65E+05	1.19E+06
0.15	*	*	1.27E+05	1.15E+06
0.15	*	*	1.30E+05	1.35E+06
0.08	*	*	ND	7.97E+05
0.08	*	*	ND	6.65E+05
0.08	*	*	ND	7.07E+05
0.02	*	*	ND	4.20E+05
0.02	*	*	ND	ND
0.02	*	*	ND	ND

Table 2. Second Calibration Curve data for okadaic acid, obtained using 4 different detection methods, (UV, ELS, MS-ES+, and MS-ES$^-$) an "*" denotes a concentration not analyzed, "ND" denotes a non-detectable signal (0.02 ng/uL yielded ND). UV and ELSD values are peak heights.

Second Calibration Curve				
Concentration ng/µL	**UV (mm)**	**ELSD (mm)**	**ES- Peak Area**	**ES+ Peak Area**
70	1.5	88	4.12E+06	3.77E+07
70	1.5	96	4.10E+06	3.84E+07
70	1.5	96	4.17E+06	3.98E+07
60	1	71	3.77E+06	3.24E+07
60	1	70	2.75E+06	2.86E+07
60	1	74	1.77E+06	1.99E+07
40	0.5	40	1.38E+06	1.52E+07
40	0.5	41	1.40E+06	1.56E+07
40	0.5	40	1.67E+06	1.58E+07
20	ND	14	8.58E+05	8.88E+06
20	ND	13	1.36E+06	1.28E+07
20	ND	14	1.54E+06	1.44E+07
10	ND	6	8.98E+05	7.48E+06
10	ND	5	9.52E+05	8.25E+06
10	ND	5	9.43E+05	8.34E+06
5	ND	2	4.70E+05	3.53E+06
5	ND	2	4.77E+05	4.30E+06
5	ND	2	4.87E+05	3.75E+06
0.15	*	*	ND	3.34E+05
0.15	*	*	ND	1.10E+05
0.15	*	*	ND	1.88E+05
0.08	*	*	ND	1.81E+05
0.08	*	*	ND	1.47E+05
0.08	*	*	ND	ND

Table 3. Third Calibration Curve data for okadaic acid, obtained using 4 different detection methods, (UV, ELS, MS-ES+, and MS-ES$^-$) an "*" denotes a concentration not analyzed, "ND" denotes a non-detectable signal (0.08 and 0.02 ng/µL yielded ND). UV and ELSD values are peak heights.

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Third Calibration Curve				
Concentration ng/µL	**UV (mm)**	**ELSD (mm)**	**ES- Peak Area**	**ES+ Peak Area**
70	1.5	75	3.06E+06	2.29E+07
70	1.5	84	3.31E+06	2.49E+07
70	1.5	87	3.97E+06	2.83E+07
60	1	66	2.62E+06	1.95E+07
60	1	66	2.62E+06	2.02E+07
60	1	68	3.03E+06	2.37E+07
40	0.5	33	1.79E+06	1.33E+07
40	0.5	33	2.05E+06	1.51E+07
40	0.5	32	2.54E+06	1.84E+07
20	*	9	1.13E+06	8.06E+06
20	*	8	1.13E+06	8.68E+06
20	*	12	1.37E+06	1.11E+07
10	*	4	5.77E+05	5.16E+06
10	*	4	6.52E+05	5.71E+06
10	*	4	8.79E+05	7.49E+06
5	*	1	1.88E+05	1.80E+06
5	*	2	2.80E+05	2.08E+06
5	*	1	2.50E+05	2.10E+06
0.15	ND	ND	ND	3.34E+05
0.15	ND	ND	ND	9.65E+04
0.15	ND	ND	ND	2.39E+05

Table 4. Averaged Calibration Curve data obtained from 3 separate experiments for the detection of okadaic acid at different concentrations obtained using 4 different detection methods, (UV, ELS, MS-ES+, and MS-ES$^-$) an "*" denotes a concentration not analyzed, "ND" denotes a non-detectable signal. UV and ELSD values are peak heights.

.

Calibration Curve Averages				
Concentration ng/µL	**ELSD (mm)**	**UV (mm)**	**ES+ Peak Area**	**ES- Peak Area**
70.00	86.44	1.50	3.80E+07	4.52E+06
60.00	69.44	1.00	3.04E+07	3.52E+06
40.00	38.33	0.50	2.10E+07	2.41E+06
20.00	12.11	ND	1.31E+07	1.51E+06
10.00	4.67	ND	8.00E+06	9.11E+05
5.00	1.78	ND	3.61E+06	4.37E+05
0.15	*	*	5.54E+05	1.41E+05
0.08	*	*	5.00E+05	ND
0.02	*	*	4.20E+05	ND

Each concentration was injected in 5 μL volumes, 3 times, and the entire process was repeated on 3 separate days. All injections were made on an HPLC with a flow rate of 0.2 mL/min and a mobile phase of 55% acetonitrile:water containing 0.1% formic acid with a Phenomenex Luna $C_{18}^{(2)}$ 150 mm x 2 mm column and plumbed into the appropriate detector. Because the concentrations of samples 7 through 10 were below the detection limits of the ELS and UV detectors, they could only be detected using the MS detector. The "ND" indicates a non-detectable signal for okadaic acid and * indicates a non-analyzed sample.

Analysis of Other Microalgae for DSP toxins

To examine the validity of using the ELSD for DSP toxin detection, four other microalgal samples were provided by Dr. C. Tomas (CMS TAC) for chemical analysis. The samples were provided in a blind study format, and labeled PM (24.5 mL), PH (37.5 mL), SC (26 mL), and PL (22.5 mL). Table 5 provides culture identification as well as clone identification number. Cell counts and extraction methods were as described for the Combined Cells and Media section, and HPLC-ELSD/UV/MS analyses were all performed as previously stated.

Table 5. Microalgal cultures used in a blind study to identify the possible presence of okadaic acid.

PM = CMS TAC - PM 009	*Prorocentrum minimum*
PH = CMS TAC - PH 002	*Prorocentrum sp.* - Hawaii
PL = CMS TAC - PL 010	*Prorocentrum lima*
SC = CMS TAC - SC-102	*Scrippsiella trochoidea*

RESULTS

Calibration Curves

Okadaic acid calibration curves were constructed using ELS, UV, MS-ES+ and MS-ES$^-$ detectors using okadaic acid material purchased from LC Labs. The results were then put into figures 4-7 to illustrate the trend line and subsequent equation of the line along with standard error bars.

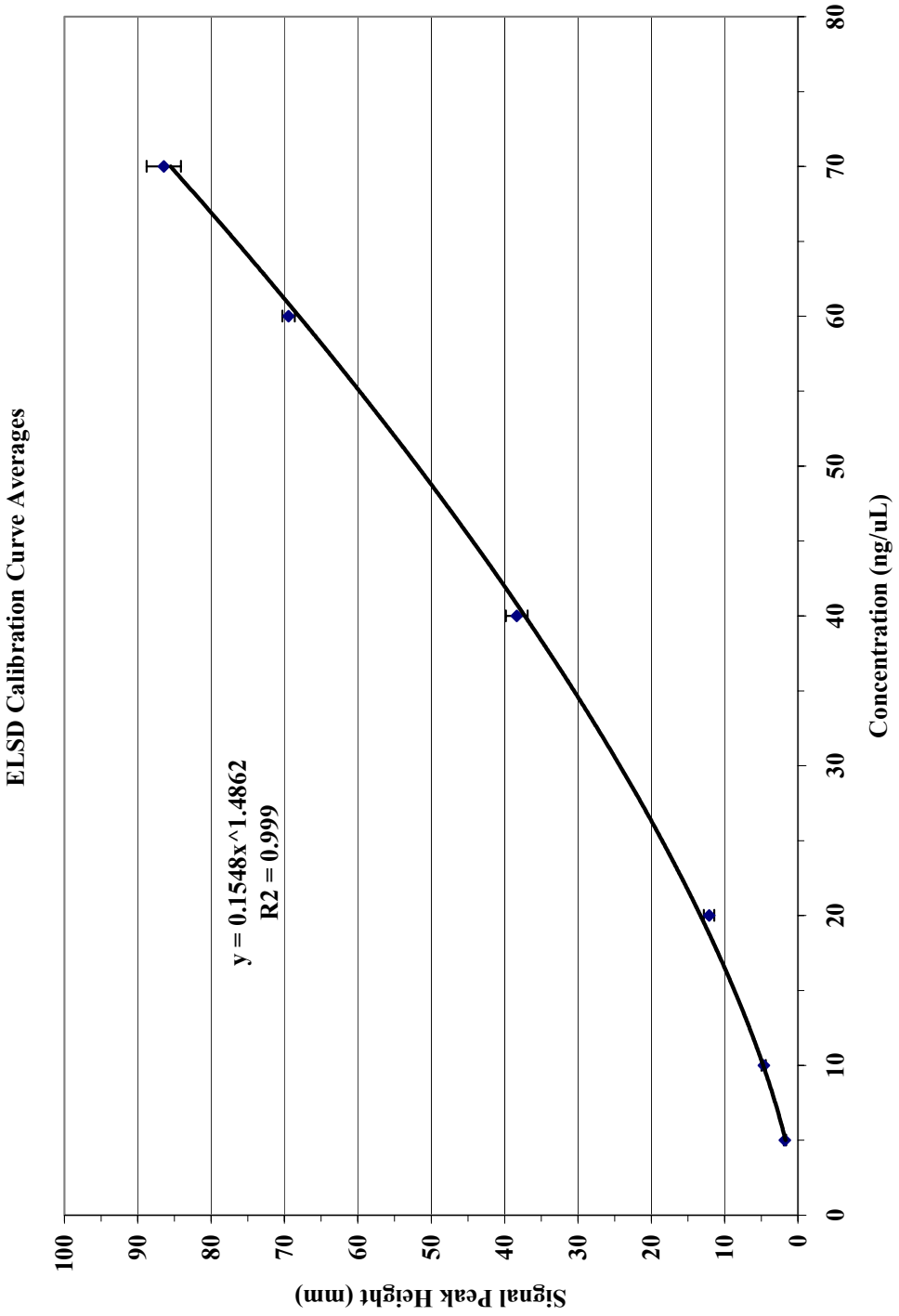

Figure 4. The average ELSD signals graphed at the various concentrations of okadaic acid and trend line applied including standard error bars in order to generate a calibration curve to permit conversion of peak height to ng/µL of toxin.

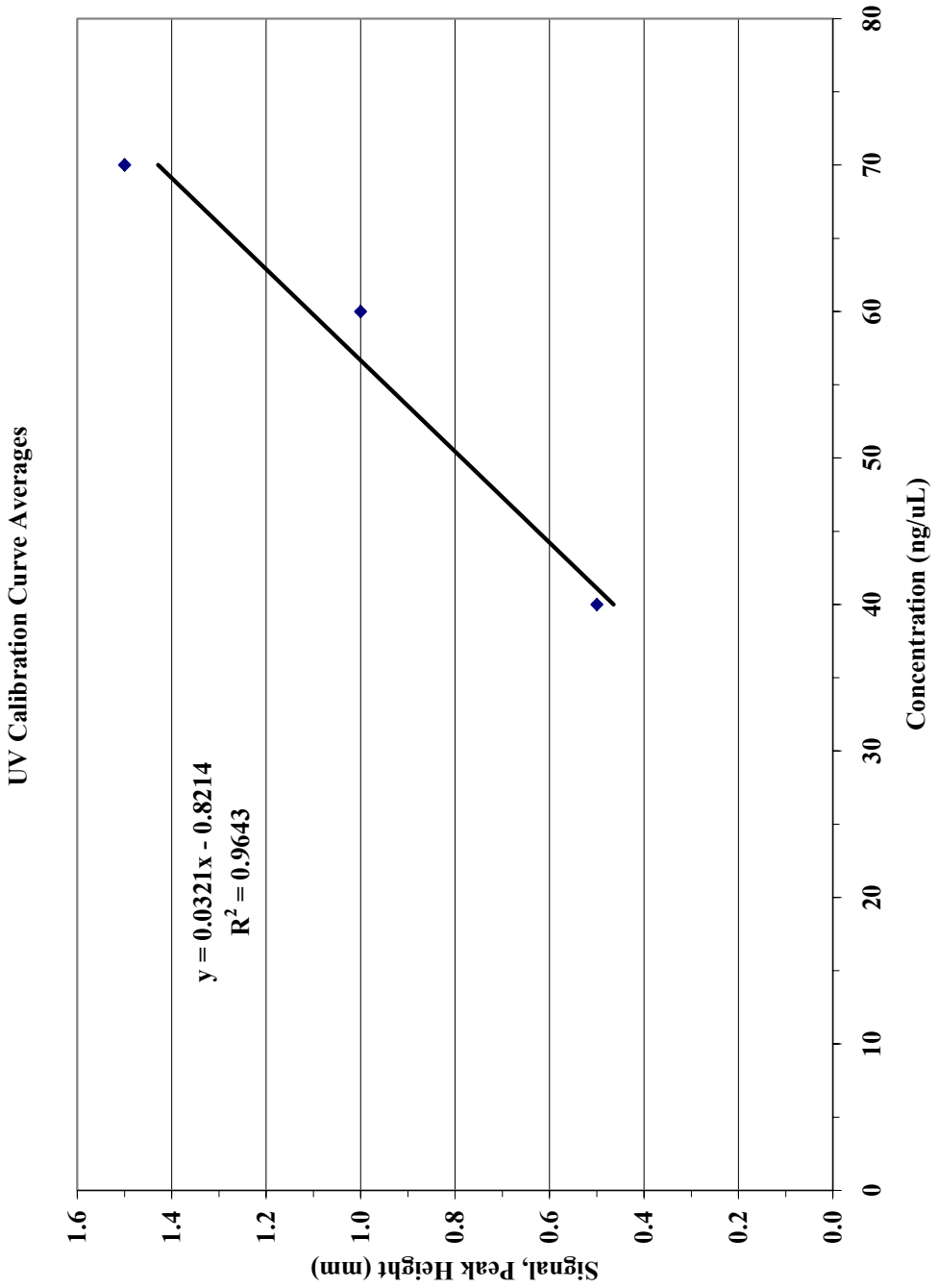

Figure 5. The average UV signals graphed at the various concentrations of okadaic acid and trend line applied including standard error bars in order to generate a calibration curve to permit conversion of peak height to ng/µL of toxin.

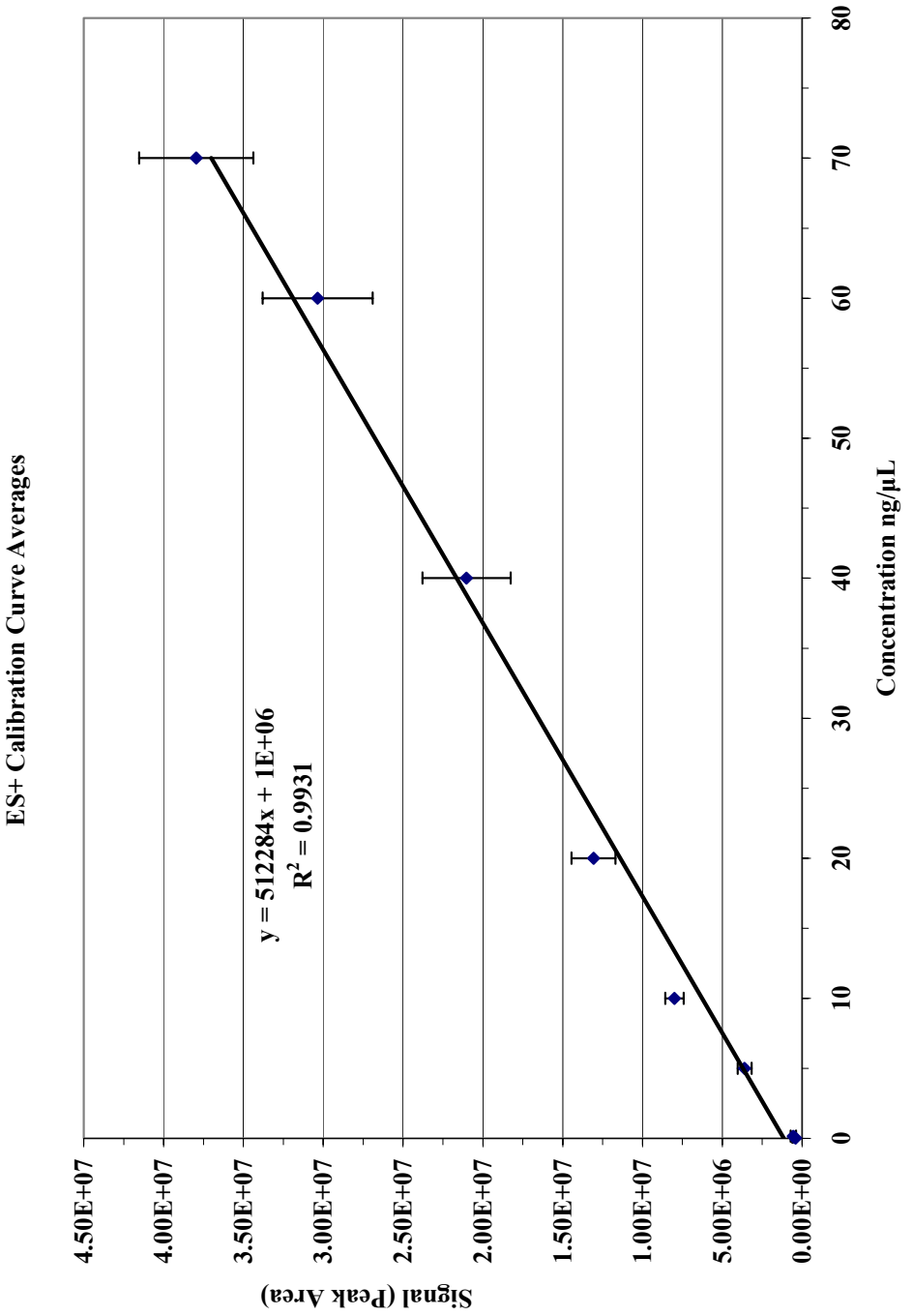

Figure 6. The average ES+ signals graphed at the various concentrations and trend line applied including standard error bars in order to generate a calibration curve to permit conversion of peak height to ng/µL.

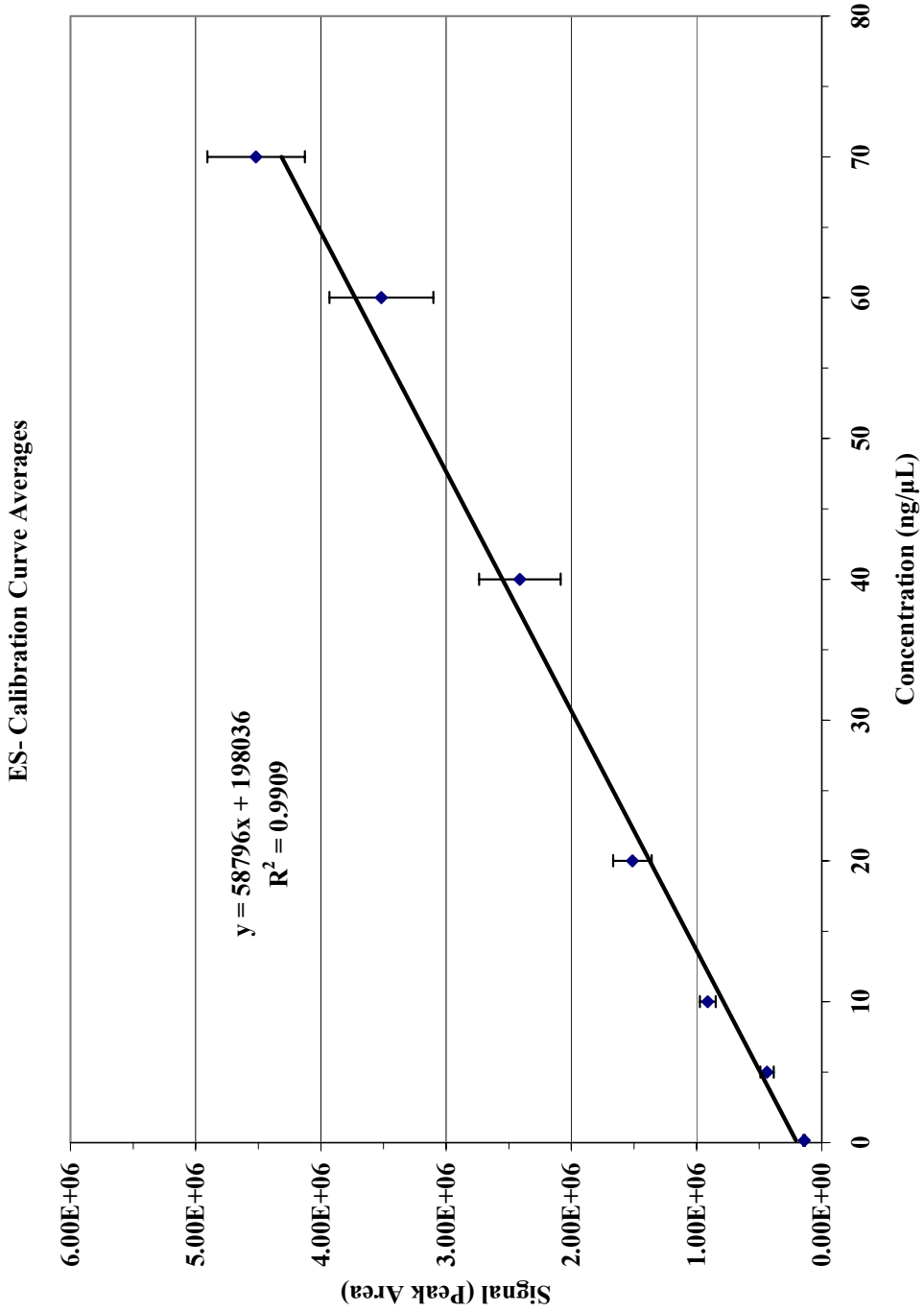

Figure 7. The average ES^- signals graphed at the various concentrations and trend line applied including standard error bars in order to generate a calibration curve to permit conversion of peak height to ng/µL.

Graphing the data for each of the detectors enabled a trend line to be applied, so that okadaic acid concentrations could be calculated in units of ng/µL per 5 µL injection or ng of toxin on column. Based on this data the ELSD has a detection limit of 5 ng/µL with 5 µL injections or 25 ng, UV 40 ng/µL with 5 µL injections or 200 ng, MS-ES+ 0.002 ng/µL with 5 µL injections or 0.1 ng, and MS-ES$^-$ 0.03 ng/µL with 5 µL injections or 0.15 ng. The lower MS-ES- is most likely due to ion suppression due to the 0.1% formic acid in the mobile phase.

Okadaic Acid Certified Standard

A certified okadaic acid standard, CRM-OA-b, was purchased from the National Research Council of Canada. The standard (20 µg) was used to prepare a standard solution with a toxin concentration of 70 ng/µL in 55%acetonitrile:water containing 0.1% formic acid. This solution was used to examine the ELSD, UV, MS, and RI detection methods.

Table 6. Raw detector data produced from injecting the NRC Okadaic Acid Standard at 70ng/µL with 5µL injections using ELS, UV, MS-ES+, or MS-ES$^-$ detectors.

NRC Okadaic Acid Std. 70ng/µL					
	ELSD	**UV**	**ES+**	**ES-**	**RI**
Concentration	**Peak Height (mm)**	**Peak Height (mm)**	**Peak Area**	**Peak Area**	**Peak Area**
70.0 ng/µL	95.0	2.0	2.81E+07	1.41E+06	1.79E+07
70.0 ng/µL	94.0	2.0	2.89E+07	2.08E+06	4.49E+06
70.0 ng/µL	98.0	2.0	3.01E+07	2.04E+06	7.98E+06
Average	95.7	2.00	2.91E+07	1.84E+06	1.01E+07

To make a comparison between the LC Labs and the NRC standards, both were tested at the same concentrations and injected on the ELSD. The ELSD signal between the certified okadaic acid standard solution at 70 ng/µL and the LC Labs standard at 70 ng/µL with 5 µL injections, produced similar peak heights. The certified NRC standard had a signal of 75.49 ng/µL and the LC Labs standard 70.52 ng/µL.

This sample was also analyzed using refractive index (RI) detector. It was found that the RI detector is capable of detecting okadaic acid, but not consistently. The RI detector was not worth forth evaluation as a viable detector.

NRC Mussel Reference Standard

A certified reference material of mussel tissue (3.9 g), containing okadaic acid (10.1 µg/g) and DTX-1 (1.3 µg/g), was purchased from the National Research Council of Canada. The crude extract of the mussel tissue was applied to an SPE column and the 60% MeOH:water (TH-1-186-1) and 80% MeOH:acetone (TH-1-186-2) fractions were examined using the ELSD, UV, MS-ES+, and MS-ES$^-$.

Because of their complexity the SPE fractions were further purified by HPLC to obtain 2 fractions, TH-1-186-1-A and TH-1-186-2-A. These two fractions were then examined by LC-MS. Fraction TH-1-186-1 revealed a peak with R.T. 8.5 mins and displaying ions at (m/z 827.8 ES+, mode; m/z 803.7 ES$^-$ mode), whereas no such signal was observed in fraction TH-1-186-2-A. In the 60% MeOH:water fraction okadaic acid is a major component, whereas the 80% MeOH:acetone fraction is much more complex and contains only a trace of okadaic acid. To conclusively establish the peaks observed in the ELS chromatogram corresponds to okadaic acid, a spike of approximately 150 ng of okadaic acid was added to the samples TH-1-186-1 and TH-1-186-2.

Table 7. LC-ELSD peak heights (mm) before and after the addition of an okadaic acid spike to the sample.

	NRC Mussel Standard		After Spike	
	ELSD (mm)	**ELSD Ave (mm)**	**ELSD (mm)**	**ELSD Ave (mm)**
TH-1-186-1	**76**	**83.3**	**148**	**147**
	91		**145**	
	83		**148**	
TH-1-186-2	**85**	**85.7**	**125**	**124**
	79		**124**	
	93		**123**	

Using the calibration curve constructed for the ELSD the okadaic acid concentration in TH-1-186-1 was 344 ng on column and 500 ng on column after its spike, and TH-1-186-2 had a toxin concentration detected at 351ng on column and 450ng on column after its spike. Additionally prior to the spike, 38 µg, of the possible 40 µg, of okadaic acid was detected total in the mussel extract, providing a recovery of 95%.

Using the 344 ng of okadaic acid and the mass of the extracted mussel digestive tissue, a limit of detection could be found. The limit of detection was calculated to be 7 µg/100g of shellfish digestive material.

Other Microalgal Samples

Unknown microalgal samples were provided by Dr. Carmelo Tomas for rapid analysis of the presence of okadaic acid. The goal was to test the validity of the method developed in this work by quickly determining whether any of the four samples provided contained DSP toxins. The samples were provided as aliquots of culture samples, containing both media and cells. The samples were processed as described in the Methods Section, and analyzed using the standard HPLC-ELSD/UV and LC-MS parameters previously stated.

The PH and PL samples each had high concentrations of okadaic present, PM had trace concentrations at the correct retention time for okadaic acid, but ultimately the presence of okadaic acid in that sample could not be concluded even using the MS detection method. The SC sample, *Scrippsiella trochoidea*, which turned out to be a blind negative control, contained no okadaic acid at all, and provided a validation of the method. The other samples corresponded as the following: PM was *Prorocentrum minimum*, PH was a new species tentatively name *Prorocentrum "hawaii"*, PL was *Prorocentrum lima*. The *Prorocentrum minimum* sample had a

cell count of 3826.2 cells/mL, *Prorocentrum "hawaii"* 2,374.2cells/mL, and *Prorocentrum lima* 3525 cells/mL. Table 8 indicates the concentrations of toxin present the samples.

Table 8. Concentration of okadaic acid detected in 4 blind phytoplankton samples. PM (*P. minimum*), PH (*P. "hawaii"*), PL (*P. lima*), and SC (*Scrippsiella trochoidea*). SC was a blind negative control, and contained no okadaic acid.

Sample	Fraction	ELSD (ng)	(pg/cell)
PM 60%MeOH:water	TH-2-57-1	36.7	0.4
PM 80%MeOH:acetone	TH-2-57-2	0.0	0.0
PH 60%MeOH:water	TH-2-57-3	479.9	5.4
PH 80%MeOH:acetone	TH-2-57-4	0.0	0.0
PL 60%MeOH:water	TH-2-57-7	454.4	5.7
PL 80%MeOH:acetone	TH-2-57-8	105.3	1.3
SC 60%MeOH:water	TH-2-57-5	0.0	0.0
SC 80%MeOH:acetone	TH-2-57-6	0.0	0.0

PH and PL had okadaic acid concentrations at 5.39 pg/cell and 5.73 pg/cell respectively, PM had a much lower level at 0.4 pg/cell (Table 7). In addition, the ELS data revealed several other peaks in the PH and PL extracts that had retention times similar to okadaic acid (R.T. 8.05). These potentially interesting peaks were found in the 80% MeOH:acetone fraction of *Prorocentrum "hawaii"* and *Prorocentrum lima*. The ES^- m/z and R.T. values in *Prorocentrum "hawaii"* fraction B were (674.4, 3.80 min), (720.4, 3.80 min), (745.4, 5.01 min), (815.4, 6.05 min), (816.3, 6.05 min), (789.6, 7.26 min), and (887.3, 7.35 min). *Prorocentrum lima* fraction B contained the peaks with ES^- m/z and R.T. values for (719.4, 3.90 min), (787.4, 3.90 min), (745.4, 5.12 min), and (813.4, 5.12 min). These ion fragments indicate compounds similar to previously identified toxins. While by itself not conclusive, a literature review failed to associate these masses with any previously identified DSP toxins, suggesting that they may represent new derivatives.

Production of Okadaic Acid during a Normal Growth Cycle of *P. lima*

To better understand the production of okadaic acid in *Prorocentrum lima* a study of the growth curve and production of okadaic acid was performed. The media and cells were analyzed separately for okadaic acid and as well as a combined sample containing both media and cells. In a second study, the effect of adding sodium bicarbonate and glycine to the cultures (B+G) was examined to determine if it enhanced toxin production.

Figures 8-13 show the change in cell density and then the change in toxin concentration at each of the 12 sampling times for each group. The concentration graphs illustrate the changes over time in pg/cell. The 80% MeOH:acetone fractions for the Normal and B+G Media did not yield any toxin signals, so those graphs are not included.

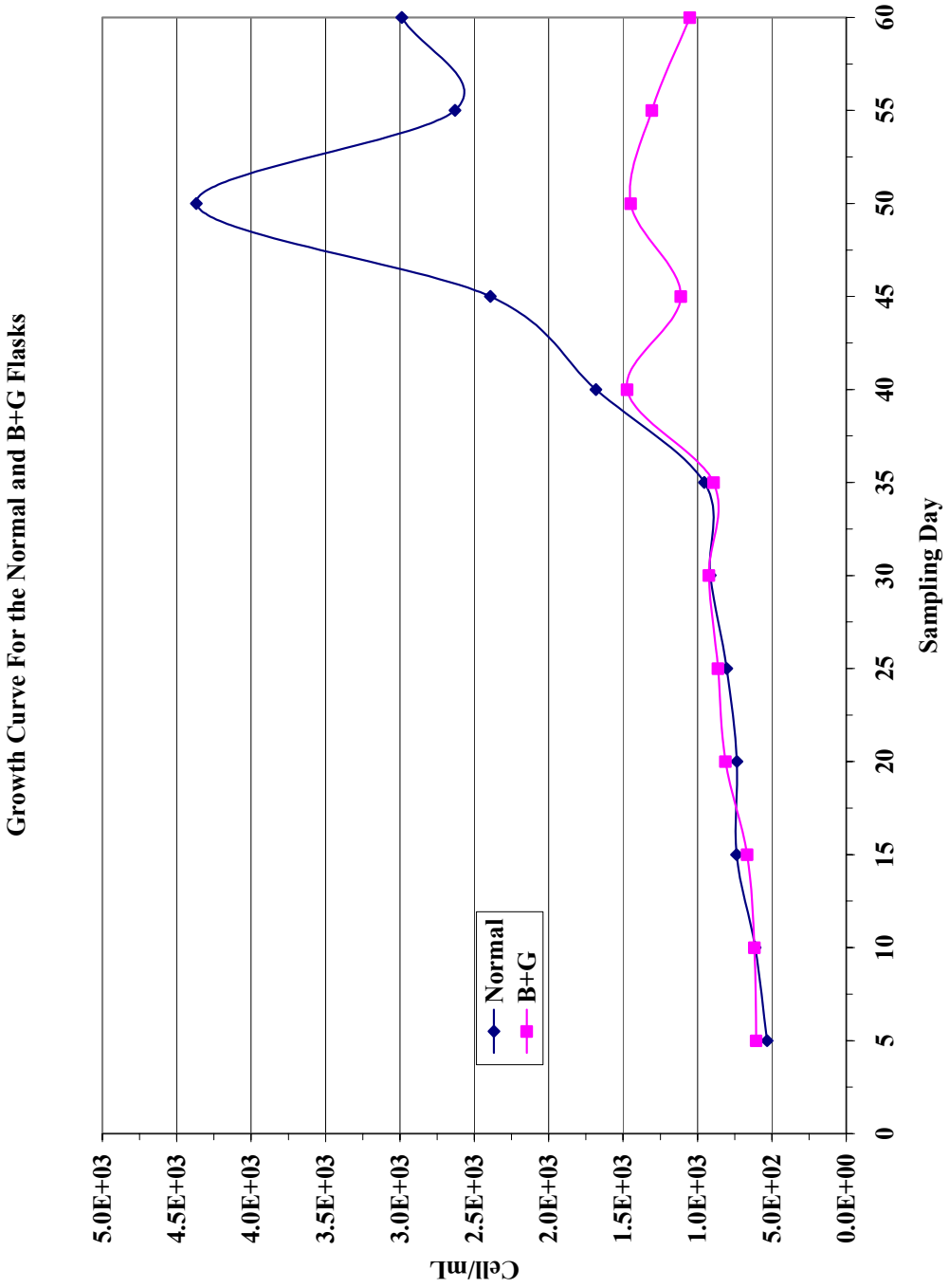

Figure 8. Cell density change in the Normal and B+G (bicarbonate and glycine supplement) culture flasks over the experiment time period of 2 months.

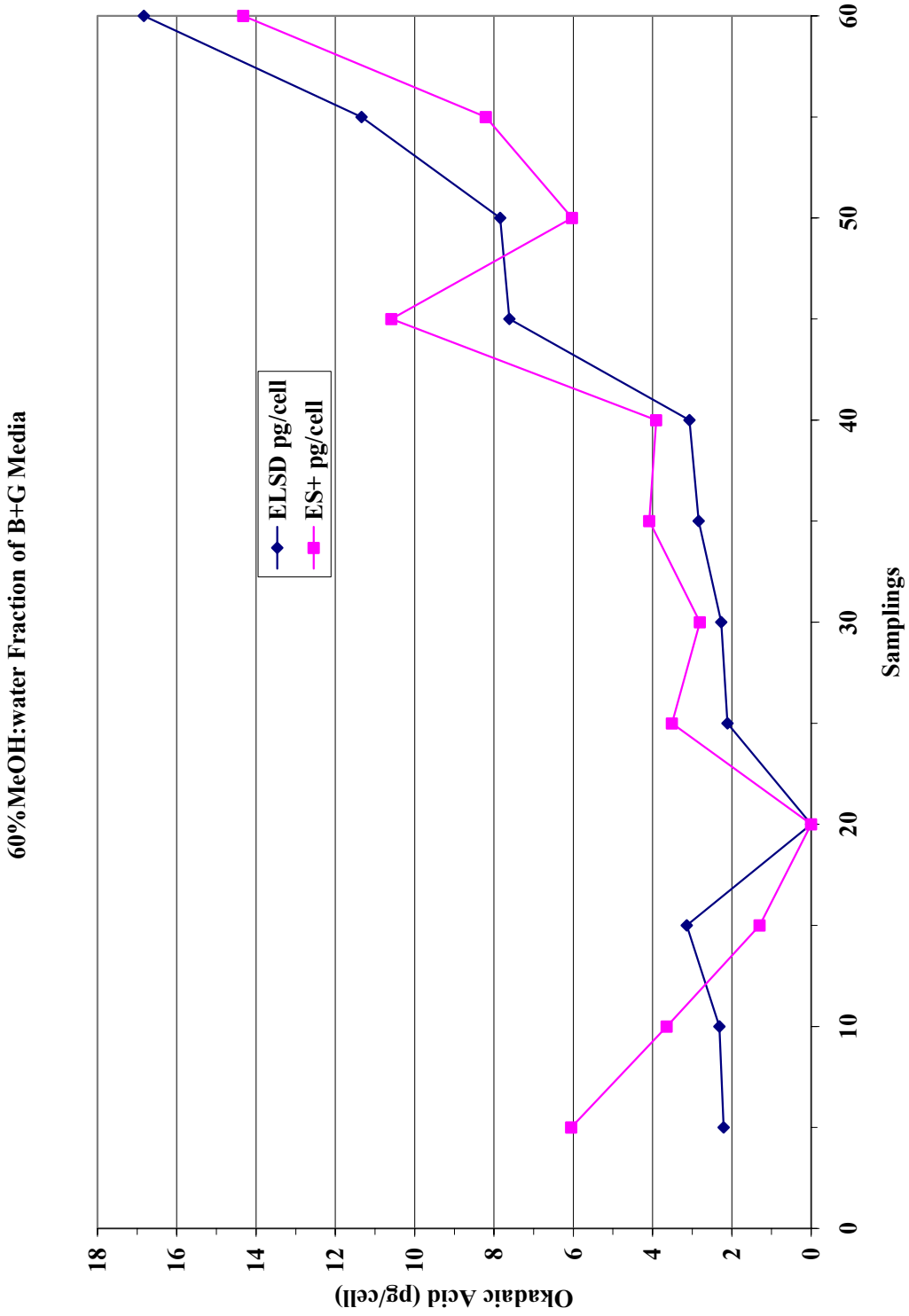

Figure 9.

The 60% MeOH:water of B+G (bicarbonate and glycine supplement) media fraction concentration of okadaic acid (pg/cell) as determined by LC-ELS and MS-ES+ during the growth period of 2 months.

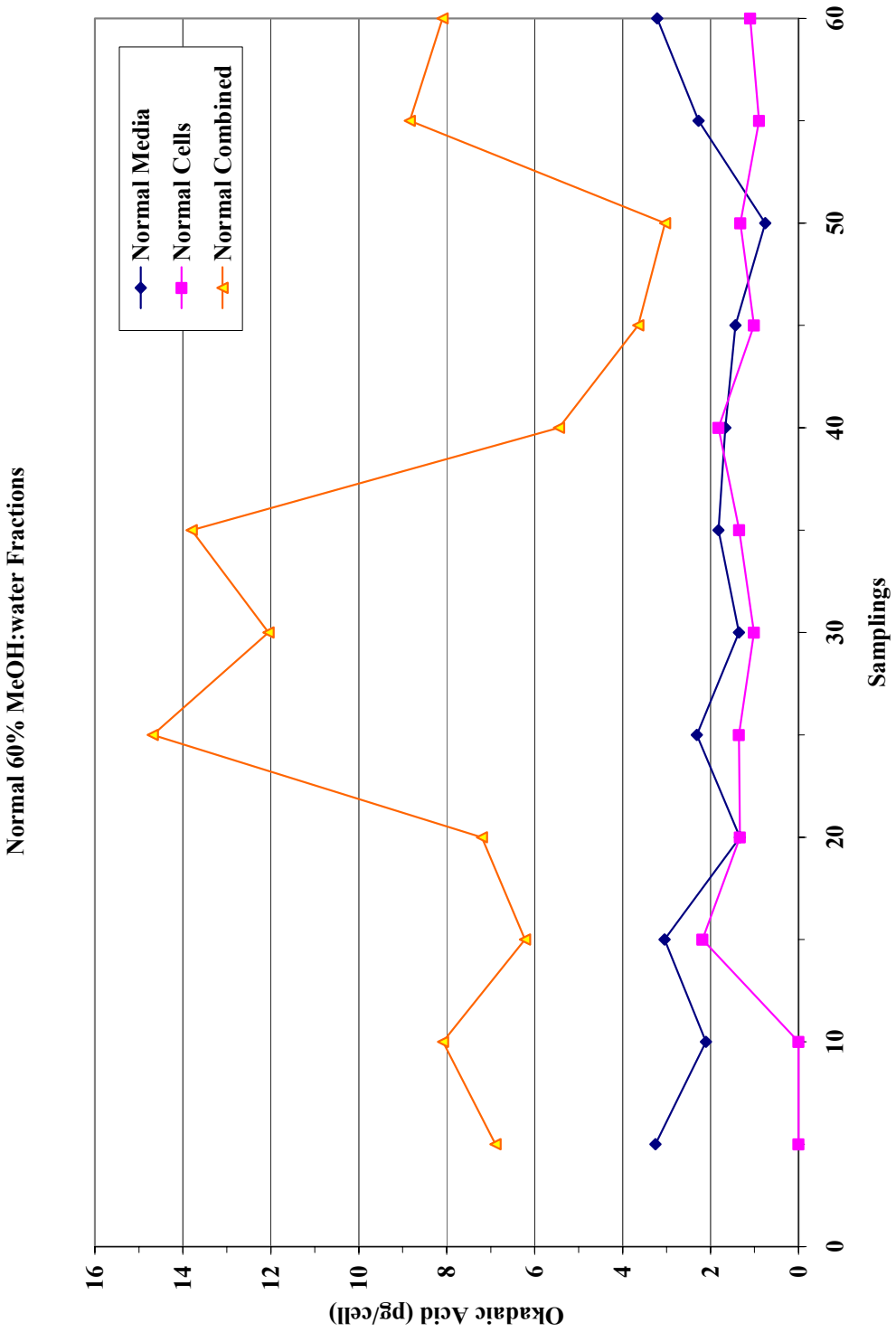

Figure 10. The 60% MeOH:water Normal fractions, the normal media, normal cells, and normal combined (cells and media), analyzed for okadaic acid (pg/cell) during the growth period of 2 months using ELSD.

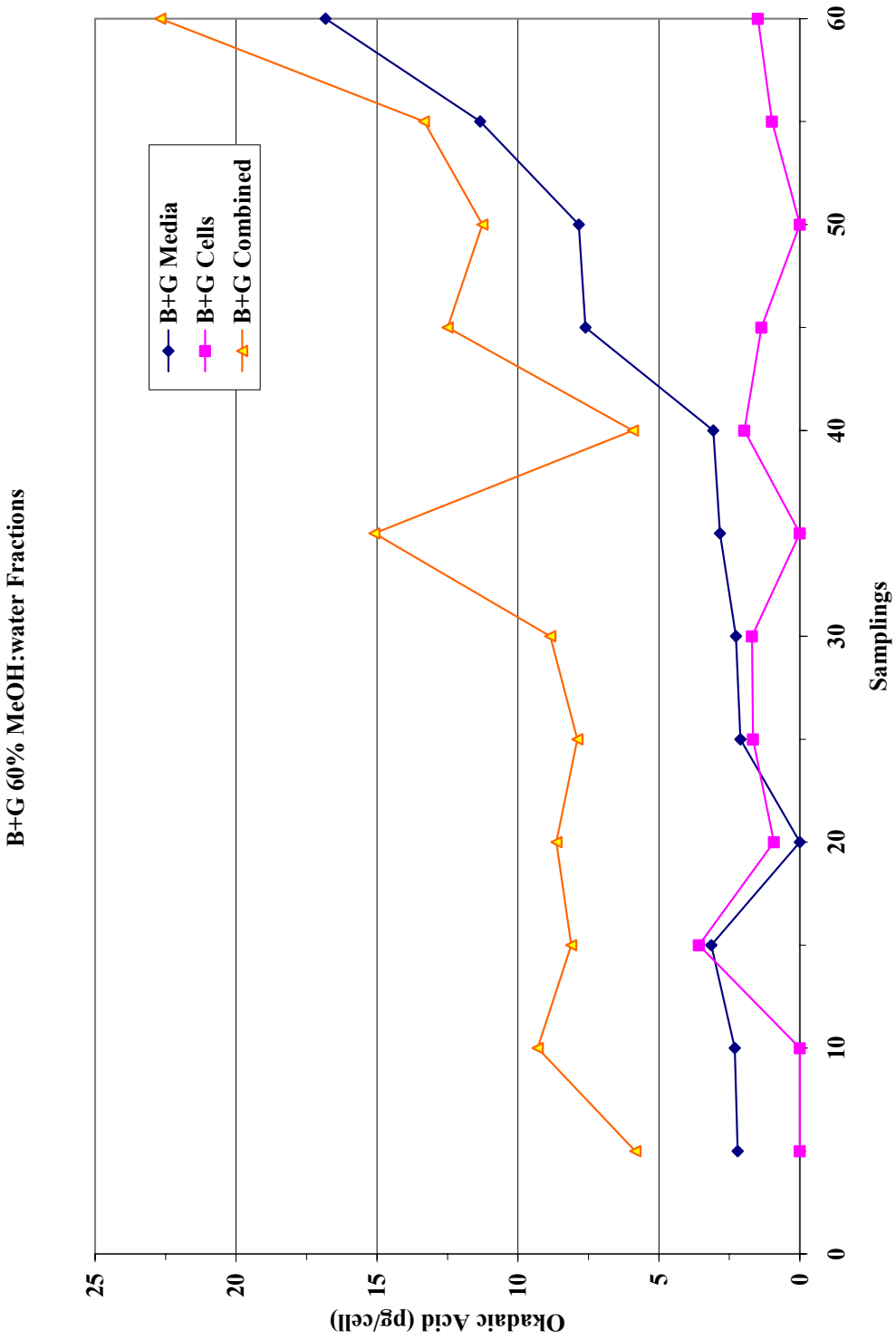

Figure 11. The 60% MeOH:water fractions, the B+G media (bicarbonate and glycine added), B+G cells, and B+G combined (cells and media), analyzed for okadaic acid (pg/cell) during the growth period of 2 months using ELSD.

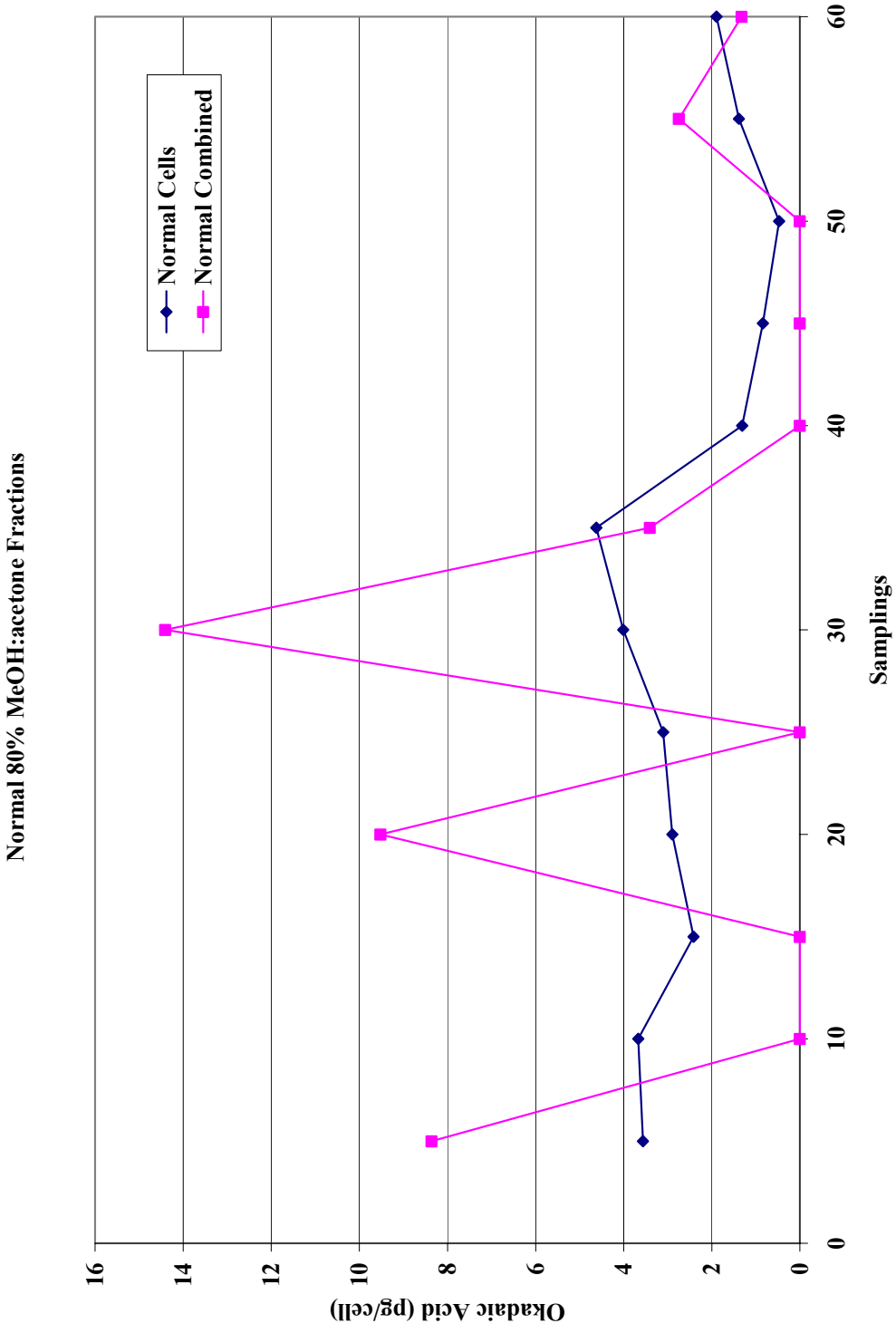

Figure12. The 80% MeOH:acetone fractions, the normal cells, and normal combined (cells and media), analyzed for okadaic acid (pg/cell) during the growth period of 2 months using ELSD.

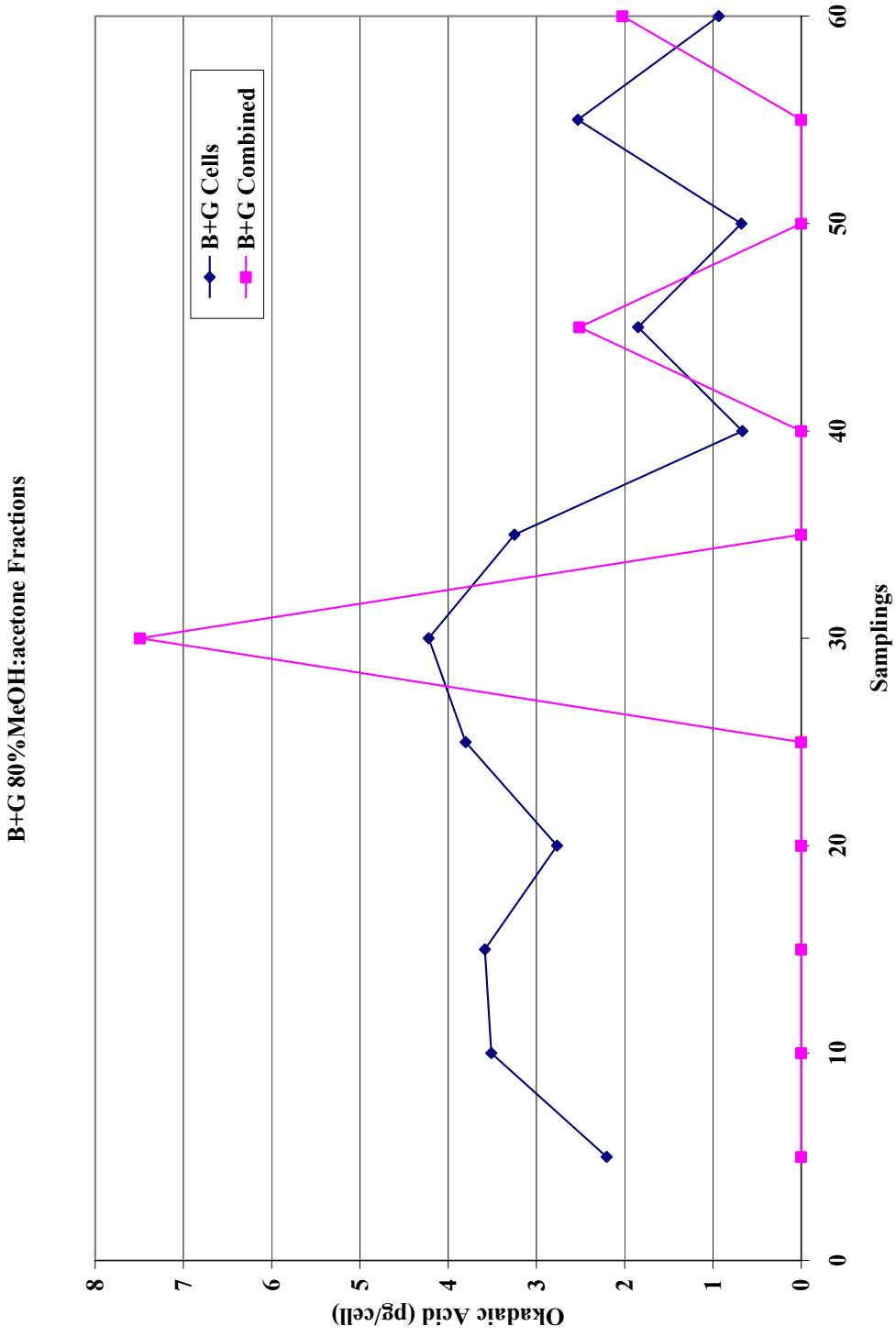

Figure 13. The 80% MeOH:acetone fractions, the B+G (bicarbonate and glycine supplement) cells, and B+G combined (cells and media), analyzed for okadaic acid (pg/cell) during the growth period of 2 months using ELSD.

Figure 9 shows the how using the MS as a back up to prove the presence of okadaic acid also can work to support the data produced by the ELSD. The normal flask indicates that the best time for isolation of okadaic is at 1 month as seen in figure 10 with an approximate concentration of 13 pg/cell. In figure 11 the trend continues to increase in okadaic acid over time with a concentration of 20 pg/cell at the end. The 80% MeOH:acetone fractions in figures 12 and 13 prove the existence of a limited amount of okadaic acid in these fractions.

DISCUSSION

Evaporative Light Scattering Detection (ELSD) Method

From the results obtained here, the evaporative light scattering (ELS) detector appears to be an economical and efficient chemical detector to find and identify DSP toxins. The ELS detector works by the scattering of a light beam by a nebulized analyte that passes across the beam. The signal is based on the presence and amount of a substance, not on the actual chemical or physical properties of the analyte (Cardenas et al., 1999). When used in conjunction with an HPLC system, it can provide information on the presence of individual toxins based on their retention times. To our knowledge there is no report of the application of an ELS detector to detect and quantify DSP toxins. As shown in this work, the method does not require an exorbitant level of sample clean up, one simple column will suffice, and the level of detection is comparable or better than other LC-coupled detectors. Comparably, the ELSD does not have the flaws of time consumption in sample preparation or the unreliability of some of the methods, and it is relatively inexpensive, $7,000 -$12,000. This detector is more than capable of filling the role as a scanning technique for the detection of DSP toxins in contaminated shellfish. Like UV detection of a naturally occurring chromophore, the ELS detector one of the most straightforward

methods available, in that it does not require any chemical reactions, or special enzymes or proteins, and can be operated successfully with minimum training. In this work it has been shown that this relatively simple, inexpensive detector that has detection limits that rival almost all the other methods for the detection of DSP toxins. From the calibration curves it was possible to define the limit of detection for okadaic acid on the ELS detector, at 25 ng of toxin on column. The useful information came about while testing a spiked shellfish sample. Most assays quote their limit of detection in mass of toxin/100g of shellfish tissue, by being able to detect the okadaic acid spike, the ELSD data for an actual shellfish sample was determined to be 7 μg/100 g of shellfish material. The important idea to remember is that 7 μg/100 g is not the limit of detection for the ELSD, it was simply how much of a spike was found. The actual limit of detection for the ELSD on shellfish material would be much lower. This value does supply a way of comparing the ELSD with the other methods though. The fluorescent derivative method has a limit of 1 μg/100 g shellfish (Gonzalez et al., 2000), ELISA 10 μg/100 g of shellfish (Mountfort et al., 1999), mouse bioassay 20 μg/100 g of shellfish (Mountfort et al., 1999), and the protein phosphatase assay 1 μg/100 g of shellfish (Mountfort et al., 1999). With the exception of the PP assay, the ELS detector is more sensitive than the other methods without even probing its lower limits.

The utility of the ELS detector permitted an investigation of the effect on toxin production, of adding B+G to laboratory cultures of P. lima. It was ultimately found that the highest yield of okadaic acid in a *Prorocentrum lima* culture is actually dependant on the time of harvest, figures 10 and 11. The highest concentration,13 pg/cell, was found at 1 month normal media cultures, figure 10, while in the B+G cultures the concentration was highest,20 pg/cell, at 2 months figure 11. Interestingly the trend indicates that toxin concentration is continuing to

increase at 2 months when the study stopped. It indicates that if the cultures were allowed to grow longer they would yield even more okadaic acid. Another benefit to using the B+G cultures is that the majority of the okadaic acid is found in the media. Utilizing the media allows for an easier and faster isolation of okadaic acid because there are far less interfering compounds in the media, as opposed to the cells. The combined (cells and media) samples provided the perfect forum to compare the normal and B+G culture treatments. This reinforces the data that the normal flasks contain the most okadaic acid at month and the B+G cultures at 2 months, or later. In the beginning of the growth curve the okadaic concentrations were nearly the same in both treatments, but at the end there was a reasonably large difference. At the end of 2 months the normal culture combined samples contained 7.75 pg/cell of okadaic acid, where as the B+G combined samples yielded 23 pg/cell, over twice as much okadaic acid. Mass spectral analysis corroborated the existence of okadaic acid determine by ELSD and provided supporting evidence for the concentration of okadaic acid throughout the growth curve as seen figure 9**.**

In the chromatographic clean-up of samples for all analyses, the 60% MeOH:water fraction the SPE column was used since it had been established to contian the vast majority of okadaic acid. To ensure recovery of all the toxin, the column was eluted with 80% MeOH:acetone, which removed all remaining compounds. There was no okadaic acid found in the 80%MeOH:acetone fractions from the media samples. However okadaic acid was detected in the 80% MeOH:acetone fractions of the cells and combined, for both the normal and B+G, figures 12 and 13. The data was relatively scattered, but indicates a small, but significant portion (2-5 pg/cell on average) is eluting outside of the 60% MeOH:water fraction. I believe this could be solved chromatographically. Instead of jumping from 60% MeOH:water right to 80% MeOH:acetone, an elution of 80% MeOH:water should be added. Then the 80% MeOH:water

and 60% MeOH:water could be combined. This would allow nearly all of the okadaic acid to be grouped in 1 fraction. Interestingly a previous study had been done examining the effect of adding a variety of amino acids, except glycine, to cultures to improve the production of toxins, but they found no change in toxin production (Souto et al., 2001).

The final project involved testing a variety of unknown microalgal samples supplied by Dr. C. Tomas. The experiment was initiated to determine the utility and speed of the ELSD method for the detection and quantification of DSP toxins in unknown phytoplankton. Four unknown and blinded microalgal samples were tested, PM, SC, PH, and PL. The SC sample (*Scrippsiella trochoidea*), was submitted as a negative control and indeed no okadaic acid was found. The PL sample (Prorocentrum lima) acted as a positive control and okadaic acid was found at a concentration of 5.73 pg/cell. The phytoplankton sample PH, (*Prorocentrum "hawaii"*) had not been examined for DSP toxin production before, and the ELS data clearly show that it is a strong DSP producer and contained okadaic acid at a concentration of 5.39 pg/cell. The fourth sample PM (*Prorocentrum minimum*), has been found to produce okadaic acid, but in very small concentrations, namely 0.39 pg/cell.

Because of the sensitivity of the ELS detection method, it was possible to analyze and detect okadaic acid in these microalgal samples using only a minimum volume of culture (approximately 25 mL). In addition, the method proved to be reliable and easy to perform. This method wouldn't be very useful if it took a week or more to run samples.

CONCLUSION

Numerous methods have been described to detect toxins that are detrimental to human health. The DSP toxin group, which includes the parent toxin okadaic acid, are a family of

toxins that have had a severe effect on human health and the shellfish industry. Due to the chemical properties of this group of toxins their detection is difficult, so a variety of methods have been investigated to tackle this problem. Currently the mouse bioassay, is the most universally accepted method. While the mouse bioassay provides information on the general toxicity of a sample, quantitative data is suspect due to the presence of other interfering factors and there is an increasing desire to move away from an assay method that uses animals. The ELISA and PP assays have impressive limits of detection, but both require very specific biological molecules for their use. For example, the ELISA needs a series of toxin specific antibodies, and the PP assay requires specific proteins. Both of these biochemical methods are sensitive to matrix effects and problems of cross reactivity with all the DSP toxins are a major drawback when using ELISA methods.

Alternative chemical detection methods have required the preparation of fluorescent derivatives, which involves a time-consuming chemical reaction using highly reactive and often unstable reagents. The yield of such reactions is often not high, and toxins can have more than one fluorescent group added which makes the data inaccurate (Rawn et al., 2005; Quilliam et al., 1998). Mass spectrometry is a powerful method for the detection of DSP toxins, and has an LOD slightly better than the ELS detector, and can provide additional structural information such as molecular weight and characteristic fragment ions. However, due to the expense of such an instrument and the need for qualified, trained operators, it is not generally available to many monitoring and regulatory laboratories. In contrast, the data obtained here indicate that the ELS detector has a competitive LOD, is relatively inexpensive to purchase, and easy and economical to operate.

Throughout this study the main goals were to determine the utility of employing an ELS detector to detect DSP toxins in phytoplankton and shellfish samples. Each project illustrated a facet of this detector's capabilities. The calibration curves provided ELSD data that could be graphed and a line fitted to permit easy conversion of an ELSD signal to ng/μL. The spiked mussel sample showed that the ELSD can work on complicated samples and still detect okadaic acid, and do so better than nearly all other methods. ELSD data was also used to track the production of okadaic acid by cultures of P. lima during a 2 month period. Finally, the method was shown to be a useful and rapid approach in monitoring the presence of DSP toxins in various phytoplankton, and revealed the presence of okadaic acid in *Prorocentrum "hawaii"*, for the first time.

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