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Basis for a New Procedure To Eliminate Diarrheic Shellfish Toxins from a Contaminated Matrix

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The natural contamination of shellfish with diarrheic shellfish toxins (DSP) has important public health implications. To avoid the economic effects of toxic episodes on shellfish farmers and the related industry, research on artificial methods alternative to the natural detoxification of shellfish is needed. Because the usual thermal processes are not efficient, alternative technologies have to be studied. Here preliminary results are presented about the lability of the DSP toxin okadaic acid in a supercritical atmosphere of carbon dioxide with acetic acid. Most of the toxin is eliminated (up to 90%), and the biological activity against its target enzyme is also severely affected (up to 70% reduction). Detoxification of contaminated shellfish requires a partial dehydration, and the detoxification yield is lower than that obtained with free toxin. Mass spectrometry experiments suggest that acetylation of the toxin molecule is not the basis of the inactivating mechanism, but a conformational change is suggested. This is the first report of the use of supercritical fluids to inactivate toxins.

KEYWORDS: Diarrheic shellfish toxins; food detoxification; supercritical fluid

INTRODUCTION

Since 1978, when diarrheic shellfish poisoning (DSP) was first reported, toxic episodes in almost every part of the world have been described (*1*, *2*). The poisoning is caused by okadaic acid and other liposoluble polyethers from marine dinoflagellates that accumulate in edible shellfish and then reach human consumers. In addition to the public health concerns, DSP has become one of the main challenges for shellfish farmers due to the long-lasting presence of toxic episodes. The interruption of shellfish extraction severely distorts the activity of the canning and transforming industry, sometimes compromising their economical viability (*3*).

DSP toxins display a remarkable thermoresistance, and the thermal treatment of shellfish is not an efficient way to reduce the toxicity. Hence, the only current approach to the removal of toxins from shellfish is natural detoxification, whereby the shellfish are maintained in the sea for several weeks once the toxic episode has disappeared (*4*). The problem is that the natural detoxification process is slow and depends on the metabolic activity of the mollusks, which is inhibited by several environmental conditions such as low temperature.

These circumstances prompt researchers to look for alternative methods to detoxify DSP-contaminated shellfish. Nevertheless, there have not been many efforts along those lines, with only one reported study evaluating the effectiveness of the depuration procedure to reduce the DSP toxicity (*5*). Unfortunately, the usual depuration procedure applied to remove the microbiological load of shellfish is not an effective method for the removal of DSP toxicity (*6*). Further studies on the effect of ozone-containing water reported an enhanced effectiveness of depuration to eliminate DSP toxins (*5*). The reported data were scarce, and significant variability—depending on the toxic profile—was shown; further results were not reported again on this interesting topic.

To summarize, the common industrial processes carried out in the shellfish-processing industry—depuration, boiling, and thermal sterilization—are not useful for the reduction of DSP toxicity. Hence, it would seem to be necessary to do research on alternative technologies that could potentially be used as viable procedures to allow the safe exploitation of DSP-contaminated shellfish.

Supercritical carbon dioxide was recently reported to be a highly efficient sterilizing medium in food and medicinal material (*7*−*14*). The inactivating effect was shown in bacteria, viruses, and even heat-resistant proteins.

In this paper we show preliminary results concerning the elimination of the DSP toxin okadaic acid and its biological toxic activity after exposure to a supercritical mixture of carbon dioxide with acetic acid.

The decrease of the okadaic acid content was monitored by high-performance liquid chromatography (HPLC) with fluorometric detection, a physicochemical method that takes into

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account the concentration of okadaic acid. In addition, the effect of the treatment on the toxic activity was unequivocally studied with the fluorometric protein phosphatase inhibition assay (*15*). Likewise, mass spectrometry techniques (*16*) were used to check for chemical changes related to the mechanism of inactivation of the toxin.

MATERIALS AND METHODS

Reagents. Okadaic acid (OA), free acid (≈98% pure), was purchased from Alexis Corp. (Läufelfingen, Switzerland). Ampules of solid OA were stored at −80 °C, and diluted methanolic solutions (0.1 mg of OA/mL) were kept at −20 °C. Liquefied carbon dioxide (99.996% purity) was provided by Praxair. Glacial acetic acid (99% pure), methanol, and ethyl acetate of analytical grade were from Panreac (Barcelona, Spain). Certified okadaic acid for calibration was from the Institute for Marine Biosciences, National Research Council of Canada, Halifax, Canada (reference OACS-1). 1-(Bromoacetyl)pyrene (BAP) (Aldrich, Milwaukee, WI) was stored at −20 °C, as were the acetonitrile solutions of BAP (0.2% w/v) and 10% diisopropylethylamine (DIPA) (Sigma). HPLC grade acetonitrile (Panreac) and Milli-Q water (Millipore Corp.) were used in the HPLC mobile phase. Bulk silica gel 60 (40−63 μm) for column chromatography (Merck, Darmstadt, Germany), stored at room conditions, was used in the solid phase extraction (SPE) cleanup of toxin derivatives before HPLC analysis.

Processing Purified Toxin with Supercritical Carbon Dioxide/Acetic Acid. Methanolic solutions of the DSP toxin okadaic acid were placed in the cell of a supercritical fluid extraction system (Suprex PrepMaster/Accutrap, Suprex Corp., Pittsburgh, PA). The stainless steel cell was filled with steel beads making up a porous bed. Nitrogen was slowly released at the bottom of the cell while the toxin was gradually added to completely evaporate the methanol. After the cell had been filled with dense carbon dioxide, a flow of carbon dioxide/acetic acid was passed through the cell until a stationary concentration of acetic acid was reached (3−8% volume was assayed): the toxin carried by the supercritical mixture was recovered in methanol for later analysis. This will be called the "extract". Then, both temperature and pressure were set to the established value to keep the toxin under this atmosphere. Finally, the cell was thoroughly rinsed with methanol to collect the remaining substances for analysis. This sample containing the nonextracted toxin will be called the "rinse". Both collected solutions were analyzed by HPLC-FLD and by the fluorometric protein phosphatase inhibition assay. HPLC-FLD determines the concentration of okadaic acid, whereas the enzymatic assay accounts for the toxic DSP activity of the extracts. An alkaline hydrolysis (*17*) of these samples was carried out to check for hydrolyzable OA derivatives produced by the treatment with acetic acid in supercritical carbon dioxide. Results were compared with and without alkaline hydrolysis.

Processing Toxic Shellfish with Supercritical Carbon Dioxide/Acetic Acid. Fresh mussels naturally contaminated with okadaic acid were partially dehydrated by freeze-drying (20 °C, 0.000131 bar). Partially dried shellfish was introduced in a 10 mL supercritical fluid extraction cell. As with purified toxin, after the cell had been filled with dense CO_2, a continuous extraction was made at a flow rate of 1 mL/min with supercritical carbon dioxide/acetic acid during 15 min to reach the desired concentration of acetic acid (5−8% acetic acid was assayed on a volume/volume basis). The shellfish was maintained under the supercritical mixture at 40 °C, and a pressure of 200 bar was applied for 30 min−5 h. The mussels were weighed and analyzed for their toxin content before and after processing.

Extraction of Mussel Samples. Initial solid−liquid extraction with 4 mL of 80% methanol per 1 g of shellfish hepatopancreas homogenate was carried out as usual from intact shellfish (*18*). Mussel samples were weighed before and after the freeze-drying step to estimate water loss. To maintain the reproducibility of the extraction, the estimated amount of water removed was added again. An aliquot (2 mL) of the aqueous−methanolic crude extract was transferred to a test tube fitted with a screw cap and washed with *n*-hexane, 2 × 2 mL, by vortex mixing for 30 s and centrifuged (1500*g*/1 min). The upper layer was discarded each time, and water (2.8 mL) and ethyl acetate (3 mL) were added to the residual solution and vortex-mixed for 2 min. After centrifugation (1500*g*/1 min), the upper ethyl acetate layer was transferred to a test tube. The ethyl acetate extraction was repeated once, and both ethyl acetate extracts were combined and made up to 10 mL. The liquid−liquid extraction is a quantitative procedure as was previously shown (*19*). This ethyl acetate extract was used for HPLC determination, whereas the assay of inhibition of protein phosphatases was carried out with the primary methanolic extracts.

Determination of Okadaic Acid. The toxin was determined by HPLC with fluorescence detection after the fluorescent labeling of the toxin with 1-bromoacetylpyrene, using a validated analytical method (*19*). The HPLC system was calibrated with certified OA purchased from the Institute for Marine Biosciences, National Research Council of Canada, Halifax, NS, Canada (reference OACS-1). The calibration range was from 1 to 40 ng of OA injected on-column. Some of the samples were diluted to allow their determination within the calibration range. Determination of the samples was performed as follows: Aliquots of 0.1 mL were taken from the collection vials (or diluted samples), transferred to polypropylene microtubes, and dried to be esterified with 1-bromoacetylpyrene in acetonitrile with diisopropylethylamine as catalyst. Then, a cleanup sample process was undertaken by solid phase extraction with silica gel. The chromatographic system consisted of a single HPLC pump (Kontron 420, Milan, Italy) set at a flow rate of 1.1 mL/min, a variable-wavelength fluorescence detector (Shimadzu RF-535) set to 356 nm excitation and 440 nm emission, and an HPLC autosampler with a loop of 20 μL (Kontron 360). A Hypersil-ODS (5 μm, 4 × 250 mm, Tracer Analytica, Barcelona, Spain) cartridge column eluted with acetonitrile/water (85:15, v/v) was used. Data collection and analysis were done using the Kontron chromatographic data system 450-MT2-V3.0.

Determination of DSP Toxicity. The fluorescent protein phosphatase inhibition assay (FPPIA) was run as previously described (*15*), which is known to provide accurate quantitative results (*20*, *21*). 6,8-Difluoro-4-methylumbelliferyl phosphate (50 μM) was used as fluorogenic substrate. The enzymatic assays were performed in 96-well assay plates. Each well contained 5 μL of $NiCl_2$ (40 mM), 5 μL of BSA (5 mg/mL), 0.025 unit of enzyme in 10 μL volume (the commercial enzyme solution was diluted to a 1/80 rate with 50 mM Tris-HCl + 0.1 mM $CaCl_2$, pH 7.0), and 50 μL of 50 mM Tris-HCl + 0.1 mM $CaCl_2$, pH 7.0. The mixture was stabilized for 10 min at 37 °C. Blanks were similarly prepared but without enzyme; four blanks were run in each plate. Then, 10 μL of calibrating solutions or sample extract (diluted with 50 mM Tris-HCl + 0.1 mM $CaCl_2$, pH 7.0) was added. Three wells were used for every sample (triple replicates/plate). The reaction mixture was incubated for 10min at 37 °C to allow the enzyme−toxin interaction. At that time, the fluorescent substrate was added in 120 μL of 50 mM Tris-HCl, pH 7.0. After 10 min of incubation at 37 °C, the yielded fluorescence was read in a microplate-reader fluorometer. OA concentrations in samples were calculated by interpolation of the activity values, with respect to controls, from the linear portion of the calibration curve. Protein phosphatase 2A isolated from human red blood cells was obtained from Upstate Biotechnology; certified solutions of OA were purchased from the Institute for Marine Biosciences, National Research Council of Canada (reference OACS-1).

Mass Spectrometry. Mass spectral analyses were performed on a magnetic mass spectrometer (Micromass AutoSpec). A detectable signal for the molecular ion of okadaic acid could not be obtained with electron ionization (EI) or chemical ionization (CI) techniques. Hence, liquid secondary ion mass spectrometry (L-SIMS) experiments were finally selected to check for chemical changes such as acetylation or molecular fragmentations during the supercritical processing of OA. The scan mass spectra range was from m/z 100 to 2000, and the potential was set at 8000 V. L-SIMS spectra are very similar to fast-atom bombardment (FAB) spectra, but cesium ions are used for high-energy collisions. Both ionization methods are suitable to avoid the extensive fragmentation of the molecular ion in the mass spectra of nonvolatile substances to determine the molecular weight of chemical species. Glycerol was chosen as matrix material. Positive and negative ion mass spectra were obtained.

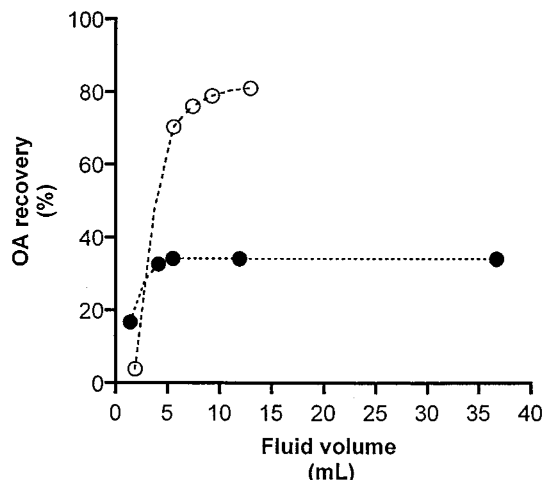

Figure 1. Recovery plot of the supercritical fluid extraction of okadaic acid with carbon dioxide/acetic acid [●; $P = 315$ bar; $T = 60$ °C; acetic acid = 3% (v/v)] and carbon dioxide/methanol [○; $P = 300$ bar; $T = 40$ °C; methanol = 7.5% (v/v)]. In both experiments the following conditions were the same: OA = 0.04 mg; flow = 1 mL/min; cell = 1.9 mL.

Table 1. Effect of Different Treatments with Supercritical Carbon Dioxide/Acetic Acid on the Elimination of Pure Okadaic Acid (As Monitored by HPLC)

pressure (bar)	temp (°C)	acetic acid (% v/v)	time exposed (h)	amount of toxin (μg)	toxin elimination (%)
315	60	3	0.5	40	65
200	40	5	0.5	40	90
200	40	8	0.5	40	63
300	40	5	15	20	89
300	40	5	3	40	88
300	40	5	4	87	55

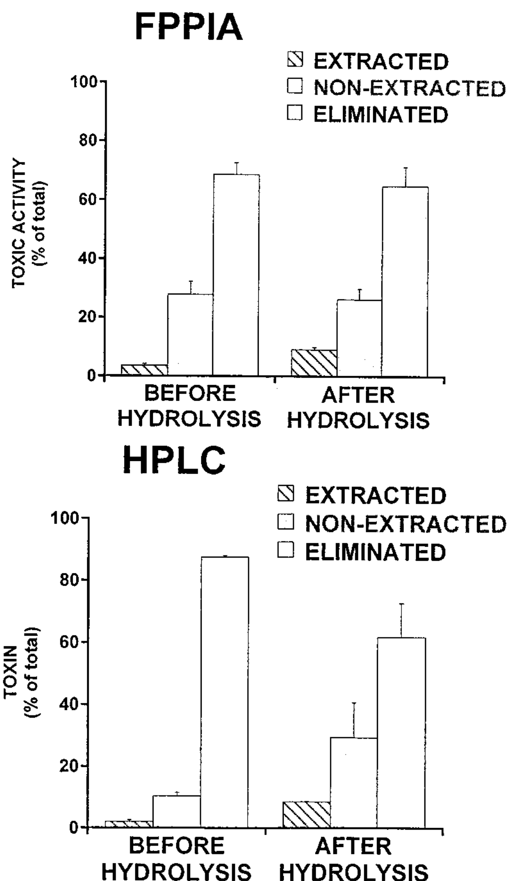

Figure 2. Effect of processing with supercritical carbon dioxide/acetic acid on the amount of okadaic acid (HPLC) and its phosphatase inhibitory activity (FPPIA). Processing conditions: 5% acetic acid in supercritical carbon dioxide at 40 °C and 300 bar for 150 min.

RESULTS AND DISCUSSION

Supercritical carbon dioxide modified with methanol or water allows the extraction of the diarrheic shellfish toxin okadaic acid (*22*). Nevertheless, we have found that when acetic acid was added as modifier to supercritical carbon dioxide, the exposed toxin disappeared. Because the chromatographic behavior of the toxin depends on its chemical properties, this observation points to some chemical changes of the toxin molecule. This surprising fact is shown in the recovery plot corresponding to an extraction experiment with CO_2 containing 3% acetic acid, where the maximum recovery of OA was <40% as compared with the almost quantitative extraction of the toxin with methanol-modified carbon dioxide (Figure 1).

Then, some experiments rinsing the system with methanol after the toxin had been exposed to the carbon dioxide−acetic acid supercritical mixture were undertaken. The results (Table 1), unequivocally demonstrate that the amount of OA decreases after the treatment. It is worth noting that the effect of acetic acid over the toxin is highly enhanced in supercritical conditions because the treatment with liquid pure acetic acid did not cause a significant reduction of the amount of toxin. Although nearly 90% OA reduction was observed in some experiments with a supercritical mixture containing 5−8% acetic acid, no more than 25% reduction was observed for samples of OA exposed to liquid glacial acetic acid for up to 18 h. Some intensive properties of supercritical fluids, such as activity and energy, are heterogeneous due to the clustering effect displayed by a supercritical fluid (*23*, *24*), and this may be the reason for the enhanced effect of acetic acid according to the severe changes of chemical equilibrium and kinetics in supercritical conditions (*25*).

Nevertheless, the most interesting fact is that the acidic supercritical mixture causes a significant reduction of the toxic activity of okadaic acid. The inhibition of certain protein phosphatases constitutes the basic mechanism of action of OA and related substances (*26*−*29*). Therefore, we monitored the biological toxic activity by means of the fluorometric protein phosphatase inhibition assay, an in vitro test for DSP toxins (*15*).

The results of processing the purified toxin with carbon dioxide−acetic acid (95:5; v/v) at 40 °C and 300 bar for 150 min are shown in Figure 2. The percentage of elimination was obtained by subtracting from the total the values found in the extract and the rinse. HPLC measurements did show that ~90% of the toxin was eliminated. To check for OA derivatives not detected by the HPLC method that could still display DSP toxicity, an alkaline treatment was performed (*17*). We have found that some of the toxin was recovered after alkaline treatment, with a final percentage of toxin elimination close to 70%. Interestingly, in that case, the loss of biological activity was also close to 70%, and contrary to the OA concentration, the measured toxicity did not vary with previous alkaline treatment of the extract and the rinse. Because the experiment was done with pure toxin and no matrix or other substances were present, the result is very significant for the assessment of the effect of the acidic supercritical mixture on the reduction of the DSP toxicity.

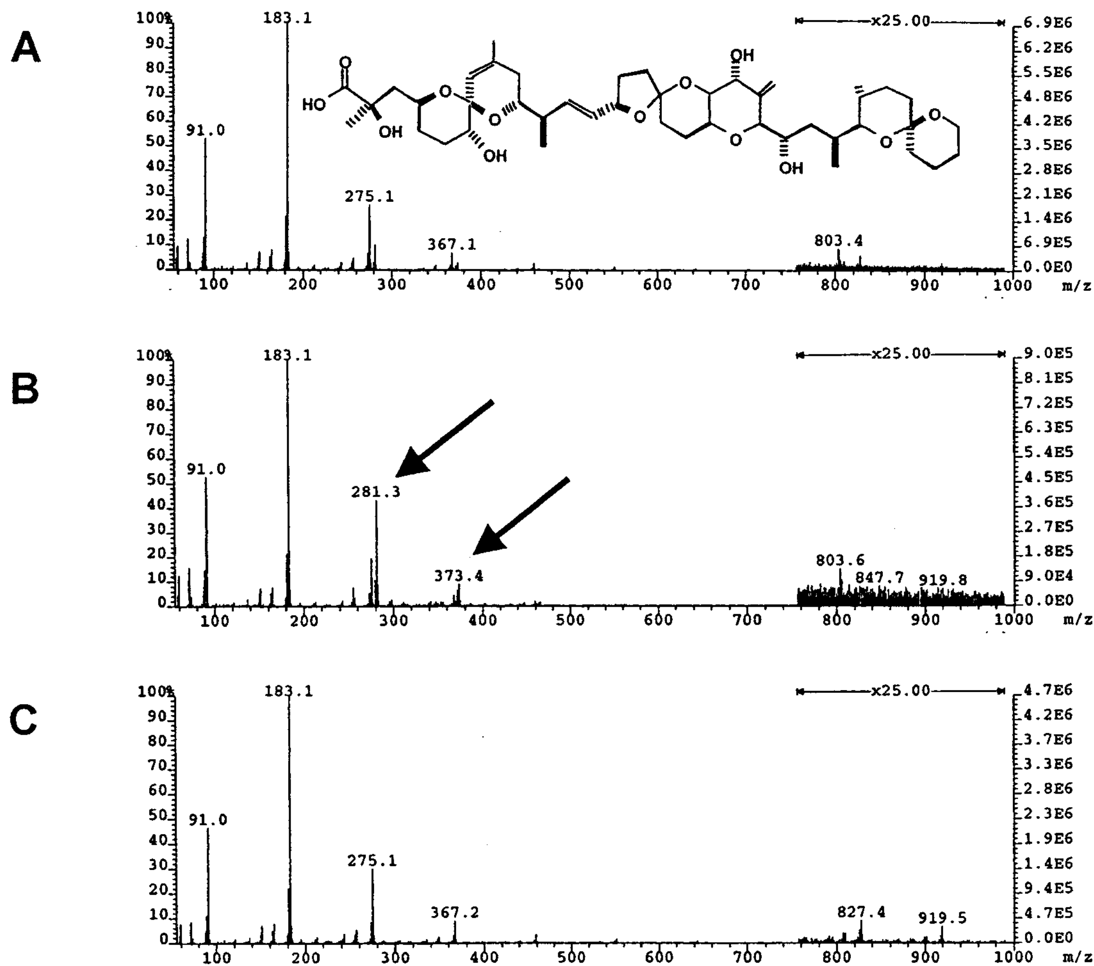

Figure 3. Negative ion L-SIMS spectra of okadaic acid (A), the rinse solution (B), where the new peaks are marked with arrows, and (C) the glycerol matrix.

Mass spectrometry was used to check for chemical transformations of the toxin molecule because only low amounts were available, preventing the use of other methods such as NMR. The simplest modifications to check for are those affecting molecular weight, such as potential reactions of esterification or breakdown of the toxin molecule. This is why ionization methods providing low fragmentation spectra were selected. Chemical ionization (CI) and electron ionization (EI) did not produce a detectable molecular ion peak for okadaic acid in the positive ion mode; hence, L-SIMS ionization was used as described under Materials and Methods. Extensive fragmentation has already been previously reported for CI and EI ionization methods (*16*). Information about molecular weight of any toxin derivative is of high value to study the hypothesis of inactivation of okadaic acid by acetylation with acetic acid.

Mass spectra from OA were compared with those belonging to samples of the extract and also with the methanolic rinse carried after the supercritical process. Positive ion L-SIMS spectra of pure OA displayed a significant protonated molecular ion peak at *m/z* 805. Peaks with higher *m/z* values corresponding to the sodium (*m/z* 827) or potassium (*m/z* 844) adducts were also observed. It has been reported that positive FAB spectra usually show several peaks with *m/z* 787, 769, and 751 resulting from losses of water from the protonated molecular ion (*16*, *30*). However, only the peak at *m/z* 787 was detected in our spectra.

Apart from the peaks belonging to the glycerol matrix, the negative ion L-SIMS spectrum of OA is dominated by the $[M - H]^-$ anion at *m/z* 803, similar to the negative ion FAB spectrum (Figure 3A) (*16*).

After the treatment of okadaic acid with carbon dioxide/acetic acid (95:5; v/v) at 40 °C and 300 bar for 4 h, as detailed in the last row of Table 1, the spectra of both the extract and the rinse were compared with that of OA. The spectra demonstrate that the inactivation was not based on an acetylation of the toxin with acetic acid, because peaks with *m/z* values higher than those of OA were not shown in the spectra (neither positive nor negative) of the extract and rinse solutions.

However, although the spectrum of the extract was not qualitatively different from the spectrum of okadaic acid, some changes were observed in the rinse's spectrum: a new peak with an *m/z* value of 281 appeared in the negative ion L-SIMS spectrum of the methanolic rinse (Figure 3B). In the positive ion spectrum the peak appeared at *m/z* 282. Both positive and negative ion spectra show the corresponding glycerol adduct at 92 units higher. It is worth pointing out that a peak with an apparent mass of 240 amu is shown in the EI and CI spectra of OA (*16*). It belongs to the C27−C38 fragment that constitutes a "tail", outside the cyclic conformation of the rest of the molecule (*16*, *31*, *32*). Interestingly, the new peak found in the spectrum of inactivated toxin arises 42 units up, and this suggests the addition of an acetic acid molecule to this fragment (60 amu of acetic acid minus 18 amu from one water molecule). HPLC-MS-MS (*33*, *34*) studies are necessary to thoroughly elucidate this issue related to the mechanism of inactivation.

Samples of mussels naturally contaminated with okadaic acid

Table 2. Effect of Freeze-Drying of Different Samples Containing Okadaic Acid on the Elimination of Pure Okadaic Acid (As Monitored by HPLC)

sample	processing	initial OA content ($n = 2$)	OA eliminated (%)
mussel hepatopancreas	freeze-dried to 43% fresh wt	3.3 μg/g (wet basis)	82
mussel hepatopancreas	freeze-dried to 25% fresh wt	2.2 μg/g (wet basis)	55
mussel hepatopancreas	freeze-dried to 5% fresh wt	1 μg/g (wet basis)	89
ethyl acetate extract	dried, dissolved in water,	70 ng/500 μL	58
OA in methanol	and freeze-dried	2135 ng/100 μL	67

were also processed with the above-mentioned supercritical mixture. The process described in this paper was not able to reduce the OA content in fresh mussels, even after 12 h of exposure to a supercritical mixture containing 10% of acetic acid. Nevertheless, as is said above, the supercritical treatment diminished the OA of an extract obtained from contaminated mussels. This ethyl acetate extract was obtained following the extraction procedure for HPLC determination. This observation led us to consider that the conditioning of the meat of the mussel through partial dehydration could facilitate access of the supercritical mixture into the matrix of contaminated mussels. This is why fresh mussels were partially freeze-dried as described under Materials and Methods. The toxin content of partially freeze-dried mussel hepatopancreas containing 1 μg of OA/g was reduced to 51−57% after 190 min of exposure to the supercritical mixture.

Moreover, a significant and consistent decrease of the OA content was found during the freeze-drying of contaminated mussel hepatopancreas. To minimize any oxidative reaction, the extraction and determination were done immediately after the dehydration. A similar reduction was shown on standard solutions of okadaic acid and mussel ethyl acetate extracts when they were dried, resuspended in water, and freeze-dried. Regardless of the experimental evidence, it is not easy to theoretically understand how the freeze-drying process could affect the OA content. Results of the reduction percentage of OA monitored by HPLC in different samples are shown in Table 2.

To summarize, okadaic acid and related polyethers are thermally stable toxins, but a significant lability was demonstrated under this acidic supercritical treatment. The discovery of the toxin inactivation is in line with the recent report of the inactivation of bacteria, viruses, and even heat-resistant proteins in supercritical carbon dioxide (*8*, *9*, *13*). This method has been proposed as an interesting sterilization alternative in the food industry, and recent papers suggest that the mechanism of inactivation depends on the closeness to the critical point and the pH (*35*, *36*). Furthermore, inactivation was shown to depend on the chemical nature of the fluid, and this could be the reason for the DSP inactivation effect of this supercritical mixture containing acetic acid (*13*). MS experiments demonstrate that the inactivation does not rely on the acetylation of the toxin molecule, so the new peaks shown in the MS spectra point to a conformational change causing toxin inactivatio; the toxin's biological activity depends on its tridimensional conformation (*37*, *38*). A breakdown of the toxin molecule is not expected from the new peak in the MS spectra of the rinse: a new chemical species should differ by 2 units in the positive and negative ion modes, and we have found these peaks at m/z 282 and 281.

Extensive and long-lasting studies are necessary to fully understand and validate any DSP shellfish detoxification procedure. However, here is the first experimental evidence that the denaturalizing and inactivating effect of some food-compatible supercritical mixtures could be useful in the inactivation of DSP toxins, opening the possibility to investigate alternative low-temperature treatments to allow the safe exploitation of DSP-contaminated shellfish. In addition to the nontoxic and food-compatibility properties of carbon dioxide and acetic acid, one additional advantage of the treatment with supercritical fluids is that they are easily removed from the product through depressurization.

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