Solubility of the Diarrheic Shellfish Toxin Okadaic Acid in Supercritical CO_2

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ABSTRACT: The solubility of okadaic acid (OA) in supercritical CO_2 was measured using a flow-type apparatus with sequential sampling during dynamic nonrecirculating experiments at saturation conditions. Methanol and water were used as solvent modifiers of CO_2. Collected OA was measured by high-performance liquid chromatography with fluorimetric detection after derivatization with 1-bromoacetylpyrene to obtain the labeled ester of the toxin. Solubility results were obtained with methanol concentrations ranging from 0 to 8.5% volume in the CO_2 density range of 0.495 to 0.913 g/mL at 40, 60, and 73°C. Measured solubility of OA ranged from 0 to 15×10^{-6} mol/L, increasing with methanol concentration and fluid density and diminishing with temperature. Experiments with water-modified CO_2 up to 0.3% volume (near saturation) were done at 60°C; solubilities of OA up to 5×10^{-6} mol/L were measured. This is the first approach to handle the liposoluble diarrheic shellfish toxins with supercritical CO_2. The study, with pure OA, provides useful information regarding the effects of pressure, temperature, and addition of modifiers on its solubility. Obtained results show that the toxin can be solubilized in this media and potential applications are suggested and being currently investigated.

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KEY WORDS: Diarrheic shellfish poisoning, entrainer, food analysis, food processing, liposoluble toxins, okadaic acid, solubility, supercritical fluid.

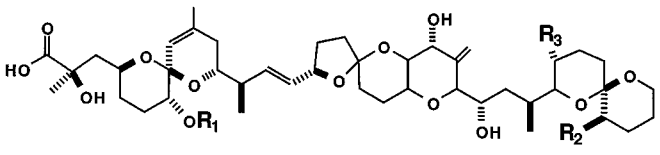
Since the first report on its occurrence in Japan (1), the human syndrome Diarrheic Shellfish Poisoning (DSP) has become a worldwide issue of public health and a significant economical problem in the shellfish industry (2,3). The poisoning is caused by a group of liposoluble polyethers from marine phytoplanktonic dinoflagellates, namely okadaic acid (OA) and its derivatives. Unpredictable phytoplankton blooms lead to the accumulation in filtering edible shellfish of these diarrhogenic and tumor-promoting substances that can reach the human consumer (4,5).

The toxin OA is a polyether derivative of a C_{38} fatty acid ($C_{44}H_{68}O_{13}$) with a melting point of 170°C and a molecular weight of 805 (6). Despite its liposolubility the molecule shows a significant hydrophilicity due to the presence of a carboxylic acid group, four hydroxyl groups, and seven ether oxygens. Scheme 1 shows the chemical structure of the DSP toxins.

OA is an expensive substance. It is required as a standard in analytical procedures for the determination of the toxins in shellfish (7,8), and it is also currently used as a molecular probe in biomedical research (9). Supercritical fluid technology is a promising field in the food and pharmaceutical industry but, as far as we know, supercritical fluid extraction (SFE) of this toxin has never been addressed. Supercritical fluids display interesting transport properties to carry out extraction processes. In addition, the solubilizing power of a supercritical fluid is easily adjustable between that of liquids and gases (10).

Potential benefits that could be expected from the application of this separation technique are: (i) elimination of organic solvents in the purification procedure from natural sources to obtain pure toxin, (ii) automation of the sample preparation stage prior to the analytical determination of the toxin in shellfish, and (iii) development of an industrial method for the detoxification of contaminated shellfish, which decreases the economic impact of toxic episodes in producing areas. Nevertheless, the determination of basic data of phase equilibrium of the toxin in supercritical media is fundamental in the application of this technology, because the design and optimization of SFE processes depend on solubility data.

Prior to conducting experiments with toxin-amended or naturally contaminated shellfish, it was necessary to measure the solubility of the pure toxin in supercritical media. Supercritical CO_2 has many desirable properties for effecting SFE such as it is nontoxic, environmentally clean, cheap, nonflam-



	R_1	R_2	R_3
Okadaic acid (OA)	H	H	Me
Dinophysistoxin-1 (DTX-1)	H	Me	Me
Dinophysistoxin-2 (DTX-2)	H	Me	H
Dinophysistoxin-3 (DTX-3)	Acyl	(H o Me)	

SCHEME 1

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mable, and easy to remove by vaporization (10). Another important characteristic of CO_2 is that the critical point is achieved at low temperature and pressure values. These are the reasons why CO_2 is unique among the candidate fluids, and why we measured the solubility of OA in pure supercritical CO_2. CO_2 modified with small amounts of two polar modifier solvents was also used with the aim of selectively enhancing the solubility of this polar molecule. Experiments were done in a continuous flow-through apparatus at saturation conditions, with sequential sampling. Off-line determination of toxin content of the obtained solutions was done by high-performance liquid chromatography (HPLC).

EXPERIMENTAL PROCEDURES

Reagents. OA for solubility measurement experiments (≈98% pure) was purchased from Alexis Corp. (Läufelfingen, Switzerland). Ampoules of solid OA were stored at −80°C and diluted methanolic solutions (20 µg OA/mL) were stored at −20°C. Liquefied CO_2 (99.996% purity; Praxair, Madrid, Spain), and methanol (HPLC-grade; Panreac, Barcelona, Spain) were fed to the SFE system. Nitrogen (E-48) was purchased from Praxair. Certified OA for HPLC calibration was from the Institute for Marine Biosciences, National Research Council of Canada (Halifax, Canada) (reference OACS-1).

Apparatus. A Suprex PrepMaster/Accutrap SFE system (Suprex Corp., Pittsburgh, PA) with a dual head reciprocating pump and an automated variable restrictor was used in the experiments. The modifier solvent was introduced in-line with an HPLC pump (Kontron 422; Milano, Italy) connected to the system through a magnetic mixer (Kontron M491; Rotkreuz, Switzerland). The Accutrap module allows off-line collection in a thermoregulated solid trap consisting of inert steel beads (0.5 mm diameter), where the extracted toxin was collected and subsequently recovered with a methanol rinse solution. The extraction cell was an empty HPLC column (1.7 mL) filled with inert steel beads (0.5 mm diameter) making up a porous bed.

Procedure. Aliquots of 0.1 mL of the methanolic solution of OA were subsequently placed in the cell while nitrogen was slowly released at the bottom of the cell to completely evaporate the methanol. The process was carefully repeated several times until an amount of approximately 40 µg (0.0322 mol) of toxin had been adsorbed onto the porous metallic bed of the extraction cell. Since the SFE system measures the flow of CO_2 as pumped volume in the CO_2 pump, leaks in the pump can lead to actual flow lower than measured; hence, at the start of every experiment the system was set to desired temperature and pressure (with pure CO_2), and checked for leaks during 15 min. Average values greater than 0.15 mL/min were not accepted and average leak was subtracted from the theoretical flow value to obtain the real flow value. Next, a dynamic step was started by flowing CO_2 through the system; once the gas was detected at the outlet of the system by its bubbling in water (usually about 1 min after the valve V1 was opened), the modifier pump was set to the desired constant flow. The total flow rate through the cell was 1 mL/min. Saturation was warranted since previous experiments (static extraction steps up to 24 h) were made to ensure the saturation of the fluid at the flow selected. After every 1.5 mL of fluid flowing through the system, the pumps were stopped and extracted OA was washed from the trap with methanol (2 × 1 mL) and collected in 2-mL glass vials topped with a septum (Fig. 1). This dynamic step was repeated several times (seven to nine) until the toxin was completely removed (higher fluid volume was used, 10 mL, in the two last steps of every experiment). Several fractions were collected per experiment to maximize the analytical information. The toxin was recovered after each experiment to be reused later on. Temperature at the variable restrictor was set to 40ºC, and the trap was at 25ºC. The main set of experiments, using methanol as entrainer, were carried out in the next sequence. First, a subset of experiments were performed at 40ºC (just above the critical temperature of methanol/CO_2 mixtures) while varying the fluid density at a fixed concentration of methanol (5%, vol/vol). Then, a second round of assays at 40ºC was run at a constant CO_2 density of 913 g/L while varying the concentration of methanol. The picture was completed with experiments that combined conditions of fluid

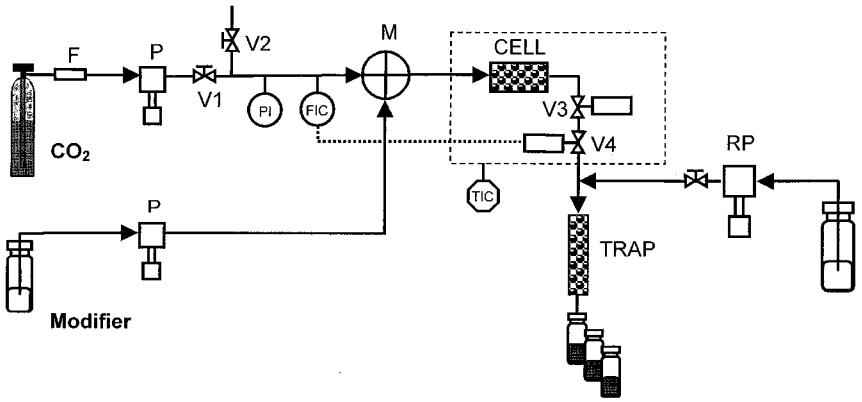

FIG. 1. Diagram of the supercritical fluid extraction instrumentation. F, filter; FIC, flow indicator and controller; M, magnetic mixer; P, pump; PI, pressure indicator; RP, rinsing pump; V, valves.

density, modifier concentration, and temperature selected to spread out the significance of the solubility behavior. These subsets were designed to estimate the effect of each of the tested parameters alone. Simultaneous estimation of the effect of the three factors by means of statistical experiment design was avoided because a linear and homogeneous contribution of the three factors was not expected.

Off-line determination of toxin. The toxin was determined by HPLC with fluorescence detection after the fluorescence labeling of the toxin with 1-bromoacetylpyrene (11). Nevertheless, the HPLC system was calibrated weekly with certified OA (reference OACS-1). The calibration range was from 1 to 40 ng 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 clean-up sample process was undertaken by solid phase extraction with silica gel. The chromatographic system consisted of a single HPLC pump (Kontron 420) set at a flowrate of 1.1 mL/min, a variable-wavelength fluorescence detector (Shimadzu RF-535; Kyoto, Japan) 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, vol/vol) was used. Data collection and analysis were done using the Kontron chromatographic data system 450-MT2-V3.0.

RESULTS AND DISCUSSION

Preliminary experiments showed that the diarrheic shellfish toxin, OA, was not soluble in pure supercritical CO_2. Toxin was never detected (detection limit = 10^{-9} mol/L) in the pressure range of 90 to 450 bar and in the temperature range from 40 to 150ºC. Although the toxin is a fatty acid derivative, it shows a significant hydrophilicity due to its hydroxyl groups and the carboxylic function. Accordingly, with this relative hydrophilic nature, a value of 0.20 for the log *P* value of the toxin in *n*-octanol/water was experimentally measured in our laboratory.

Methanol and water were used as polar modifier solvents added to carbon dioxide to achieve the solubilization of the toxin. Methanol is the most used polar modifier because of its high miscibility range with supercritical CO_2 (12). Water was selected because of its natural presence in shellfish. Water has a significant modifier ability although its solubility in supercritical CO_2 is very low (13,14).

Solubility data (Table 1) come from the average solubility observed in the saturated fractions of every experiment. The first fractions corresponding to the equilibration step (while entrainer concentration achieved its corresponding steady-state value) were not considered. The flow of supercritical CO_2 was continuously monitored to assess the concentration of the modifier solvent, since its final value resulted from the relative flow of supercritical CO_2 and that of the modifier itself. Since instantaneous variations in the flow of CO_2 typically approached 10–15% of the set flow, the concentration of modifier was assumed to be the main source of experimental error, due to the equipment limitations. Minimization of the response delay in the flow controller was achieved by reducing the dead volume from the sample cell to the flow controller removing the intermediate filters of the equipment.

TABLE 1
Solubility Data of the Diarrheic Shellfish Poisoning Toxin Okadaic Acid in Modified CO_2

Modifier (%, vol/vol)	Pressure (bar)	Temperature (K)	Solubility × 10^6 (mol/L)[a]	Number of sat. fractions
Methanol				
0	90	313	<d.l.	
0	200	313	<d.l.	
7.5	300	313	8.96 ± 1.4	*n* = 3
6	300	313	8.19 ± 2.2	*n* = 3
5	300	313	3.74 ± 0.7	*n* = 3
8.5	300	313	15.15 ± 2.9	*n* = 3
5	90	313	1.24 ± 0.1	*n* = 5
5	150	313	1.80 ± 0.1	*n* = 5
5	200	313	3.76 ± 0.3	*n* = 3
5	100	313	0.91 ± 0.1	*n* = 4
7.5	100	313	4.35 ± 0.9	*n* = 3
8.5	100	313	3.99 ± 0.9	*n* = 3
0.0	300	313	<d.l.	
8.5	200	313	11.05 ± 1.3	*n* = 4
8.5	315	333	2.30 ± 0.1	*n* = 7
5	315	333	1.81 ± 0.3	*n* = 3
8.5	390	346	3.17 ± 0.4	*n* = 3
Water				
0.1	315	333	1.08 ± 0.1	*n* = 3
0.3	315	333	5.4 ± ??	*n* = 1

[a]Solubility is expressed as the average concentration observed in the the saturated fractions ± the standard error. Abbreviations: sat., saturated; d.l., detection limit; ??, unknown.

Experiments with higher concentrations of methanol than reported in Table 1 were performed, although saturated fractions were not obtained due to the limited availability of this expensive toxin. Solubility determination experiments with water-modified supercritical CO_2 were done at the lowest concentration allowable in our system, 0.1% volume, and also at 0.3%, a concentration close to saturation. A significant enhancement in OA solubility was observed after the addition of water irrespective of the low miscibility of water in CO_2. Nevertheless, the higher miscibility of methanol in supercritical CO_2 allowed the toxin solubility to rise to higher levels than observed in water-modified supercritical CO_2.

Experiments carried out with methanol/CO_2 showed the effect of pressure and temperature on toxin solubility (Fig. 2). While OA solubility increased with pressure at constant temperature as expected, a rise in temperature in the range from 40 to 73ºC caused a decrease in solubility. This unforeseen effect of temperature might be due to the exothermic formation of complexes between OA and methanol. A negative contribution of temperature to solubility was previously reported in a system where strong complexes of cosolvent and

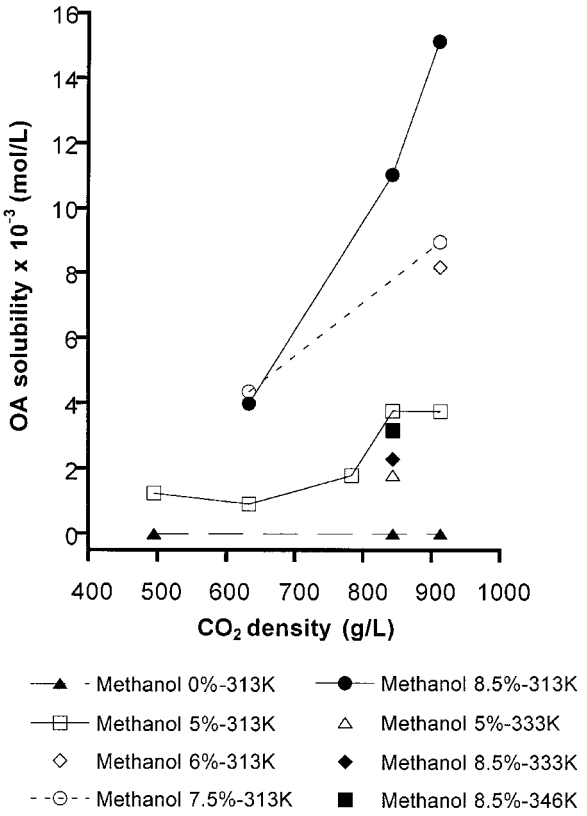

FIG. 2. Solubility of okadaic acid (OA) in methanol-modified supercritical CO_2.

solute were formed (15). The formation of toxin–methanol complexes was not demonstrated in this paper, but a significant effect of cosolvent is clearly suggested because the toxin solubility was negligible without the cosolvent in supercritical CO_2.

Other substances with chemical structures close to OA are the polyether antibiotics monensin, lasalocid, salinomycin, and narasin. Solubility determinations in a recirculating system showed significant variation between the four antibiotics; all the antibiotics were soluble in pure supercritical CO_2 except lasalocid. Differences were discussed in light of different conformations, a feature that could also explain the lower solubility of OA. The reported structure of the toxin in water and in apolar solvents (17) was different from the cyclic structure of these antibiotics, which allow them to enhance its hydrophobic-like properties and exhibit its characteristic ionophoric activity.

The knowledge of the solubility pattern of the toxin in supercritical CO_2 establishes the start-point for development of a purification method of the toxin. The negligible solubility in pure supercritical CO_2 opens the possibility of previously removing any other substance of lipophilic nature from crude contaminated-shellfish extracts or from toxic phytoplankton. Also, other polar substances that are soluble in pure CO_2 at higher temperatures can be removed. The toxin could then be selectively extracted with methanol-modified supercritical CO_2 at low temperatures. The selective extraction of OA could also provide the basis of developing an automated sample-preparation method for its determination by HPLC. In addition to purification, the required fluorescent labeling of the toxin by esterification with 1-bromoacetylpyrene can also be quantitatively carried out in supercritical CO_2, since an esterification yield of 97% has been observed.

The suitability of this technology to remove the DSP toxicity from shellfish should be studied to check the viability of this selective extraction with food-compatible solvents such as water-saturated supercritical CO_2 or ethanol-modified supercritical CO_2 from partially freeze-dried shellfish. Obviously, the inability of pure CO_2 to extract the toxin poses an additional problem because only food-compatible cosolvents are allowed.

We conclude that OA can be solubilized in modified CO_2. In addition, the dependence of its solubility on fluid density, temperature, and addition of modifiers is shown. However, further investigations on the suggested applications of this technology are required.

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