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ORIGINAL PAPER

Feasibility of gamma irradiation as a stabilisation technique in the preparation of tissue reference materials for a range of shellfish toxins

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Abstract The effect of γ-irradiation on concentrations of hydrophilic and lipophilic phycotoxins has been investigated by use of HPLC–UV and LC–MS. Pure toxins in organic solvents and toxins in mussel (*Mytilus edulis*) tissues were irradiated at three different doses. In solution all toxin concentrations were reduced to some extent. Most severe decreases were observed for domoic acid and yessotoxin, for which the smallest dose of irradiation led to almost complete destruction. For pectenotoxin-2 the decrease in concentration was less severe but still continuous with increasing dose. Azaspiracid-1 and okadaic acid were the least affected in solution. In shellfish tissue the decrease in toxin concentrations was much reduced compared with the effect in solution. After irradiation at the highest dose reductions in concentrations were between ca. 5 and 20% for the lipophilic toxins and there was no statistical difference between control and irradiated samples for azaspiracids in tissue. Irradiation of shellfish tissues contaminated with domoic acid led to a more continuous decrease in the amount of the toxin with increasing dose. The effect of irradiation on the viability of microbial activity in shellfish tissues was assessed by using total viable counting techniques. Microbial activity depended on the type of shellfish and on the pretreatment of the shellfish tissues (with or without heat treatment). As far as we are aware this is the first investigation of the effectiveness of irradiation as a technique for stabilising tissue reference materials for determination of phycotoxins. Our results suggest that this technique is not effective for materials containing domoic acid. It does, however, merit further investigation as a stabilisation procedure for preparation of shellfish tissue materials for some lipophilic toxins, in particular azaspiracids.

Keywords Algal toxins · Domoic acid · Okadaic acid · Azaspiracids · Pectenotoxin · Yessotoxin

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Introduction

The accumulation of naturally occurring algal toxins in molluscan shellfish is a substantial potential threat to human health and as a result many countries have established monitoring programmes involving routine analysis of shellfish for the presence of these toxins. Reference materials (RMs) are required for development, validation, and quality control of the different analytical methods used in regulatory monitoring; currently only a few tissue RMs are available.

Stability is an extremely important requirement of RMs, and in particular of certified reference materials (CRMs). When preparing RMs, a stabilisation step or measure is usually included either during or after preparation of a material. Microbial spoilage is a particular concern for biological RMs, and because it is extremely difficult to ensure aseptic conditions during the preparation of such materials it is preferable to have a stabilisation step after a material has been divided or bottled in portions suitable for distribution and use.

In the preparation of CRMs for the algal toxins domoic acid (DA) and okadaic acid (OA) heat treatment using a steam retort after bottling has been reported [1]. This method has, however, recently been shown to cause degradation and rearrangement of azaspiracids (AZAs) [2], toxins for which there is currently no CRM available. As a result of this heat degradation, alternative stabilisation techniques are required.

Gamma irradiation is an ideal procedure for stabilisation against microbial degradation, because it can be performed on packaged material through the container walls [3]. Irradiation is traditionally used to prevent microbial spoilage of food products, and it has been used to stabilise seawater RMs for nutrients [4], but there are few literature reports of its use for preparation of biological RMs. The technique is typically applied in the preparation of RMs to be certified for inorganic substances, because many organic compounds are destroyed by such treatment.

While freezing is the usual storage condition for wet tissue homogenates, it is often necessary that RMs, in particular CRMs and materials to be used in proficiency testing schemes, have to be shipped long distances globally, when transport conditions and durations are difficult to guarantee. Because of this difficulty it is important that every possible step is taken during preparation of a material to ensure stability even at elevated temperatures in the short-term.

The objective of this study was to investigate the feasibility of using irradiation as a stabilisation technique in the preparation of RMs for shellfish toxins. The effect of different irradiation doses on a range of phycotoxins in solution and in mussel tissues (*Mytilus edulis*) was investigated. The toxins investigated included the lipophilic toxins AZA1, OA, pectenotoxin-2 (PTX2), and yessotoxin (YTX) and the hydrophilic toxin DA. All these toxins are found above the regulated levels in a variety of shellfish from European waters. The effect of the different doses on the microbial activity of shellfish tissues was also examined, to determine if there was a dose that efficiently eliminated microbial activity but did not substantially degrade the analytes of interest.

Materials and methods

Chemicals

Methanol and acetonitrile were obtained as Pestiscan-grade solvents from Labscan (Dublin, Ireland). Water for extraction and for the mobile phase was obtained from a reverse-osmosis purification system (Barnstead). Trifluoroacetic acid (TFA), formic acid, and ammonium formate were obtained from Sigma–Aldrich.

Standards

Calibration standards

DA, OA and PTX2 calibration standards were prepared from certified calibration solutions produced by the NRC in Canada (NRC CRM-DA-d, NRC CRM-OA, NRC CRM-PTX2). The AZA calibration standards used were dilutions of an AZA1 lot isolated under supervision of Dr M. Satake at Sendai University Japan during 2001 from mussel samples originating from Ireland (Killary Harbour 1996, Bantry 2000). The YTX calibration standards used were dilutions of a purified YTX lot supplied by Dr M. Satake.

Standards for irradiation trials and spiking of tissues

DA solution A DA solution for the study was prepared from the CRM. A dilution was prepared in 10% (*v*/*v*) aqueous acetonitrile and checked by weight, using a calibrated balance. The solution was divided into 0.5 mL portions in amber glass ampoules and flame sealed under nitrogen immediately after preparation.

Combined toxin solution A combined OA, PTX2, AZA1, and YTX solution was prepared, in methanol, using OA and PTX-2 CRM calibration solutions. The AZA1 used was purified and isolated at the Marine Institute as part of the ASTOX project [5]. Purified YTX was isolated and supplied by the Norwegian Veterinary Institute. The solution was divided and stored under nitrogen in the same way as the DA solution.

Shellfish samples for toxin analysis

A DA laboratory reference material (LRM) was prepared in-house from whole mussel tissue. The homogeneity of this material had previously been assessed (2.4% between bottle CV, $n=13$) [6]. Another mussel tissue LRM containing AZA1, 2, and 3, OA, and dinophysistoxin-2 (DTX2) was also used (CV=5.0% and 5.1%, $n=14$, for AZA and OA equivalents, respectively) [7]. In addition a separate material was prepared from cooked whole-mussel flesh naturally contaminated with YTX and spiked with PTX2 and AZA1. All tissue samples were hermetically sealed in polypropylene tubes under argon.

Samples for assessing microbial activity

To assess microbial activity shellfish samples separate from those for toxin analysis were prepared. The whole flesh of fresh mussels (*Mytilus edulis*) and oysters (*Crassostrea gigas*) was separately collected, homogenised, and dispensed into capped vials (10-g portions). To mimic the

effect of a heating step, which is common when shellfish are being processed, triplicate control samples (no irradiation) of the fresh shellfish tissues were placed in a boiling water bath for 5 min. Additional control samples that received no heat treatment were also used.

Irradiation of samples

Materials which had been stored at −20 °C since preparation were sent on dry ice to the Isotron irradiation facility in Ede, The Netherlands. Five replicates of each standard and tissue material, including the materials for biological testing, were treated at doses of 6 kGy, 12 kGy, and 24 kGy (γ-irradiation, source cobalt-60). The materials for each dose were put into separate plastic bags with two dosimeters. The three plastic bags were then put in a cylinder (dimensions 30 cm×13 cm, $H \times D$), designed for use in the test scale irradiator (JS6500 Nordion Tote Box Irradiator) to minimise variation in the irradiation dose. When the first dose of 6 kGy had been delivered irradiation was stopped, a plastic bag was removed from the container and irradiation was resumed. The same process was repeated after administration of 12 kGy. Control materials were also dispatched, and kept at the same temperature, but were not irradiated. After treatment all the samples were relabelled to facilitate blind testing and returned on dry ice to the Marine Institute in Ireland for analysis. The irradiated and control samples were again stored at −20 °C until analysis.

Extraction

Tissue samples (4 g) containing DA were extracted twice with 50% aqueous methanol and the extract was diluted to 50 mL. This procedure has previously been described in detail [8]. A double-extraction procedure was also applied for samples containing AZA, OA, YTX, and PTX2. Samples (2 g) were extracted with 9 mL 100% MeOH as the solvent in each step. The extracts were centrifuged at 4,000 rpm for 10 min, combined in a 25 mL volumetric, and diluted to volume with MeOH (100%). For analysis extracts were filtered through 0.2 μm filters (Schleicher and Schuell) into HPLC vials.

Instrumental analysis

HPLC–UV-DAD analysis of DA

The method used closely followed the procedure published by Quilliam et al. [9], without the clean-up step and with other modifications [8]. A Shimadzu HPLC system with a photo-diode-array detector (PDA) was used. HPLC was performed on a reversed-phase C-18 Vydac column (250 mm×4.6 mm, 10-μm particles).

LC–MS analysis of DSP/AZP toxins

Analysis was performed with a Waters 2795 HPLC coupled to a Micromass Q-TOF Ultima (quadrupole-time-of flight hybrid), equipped with a z-spray ESI source. The Q-TOF was used in TOF-MS–MS mode. A binary mobile phase was used, with A (100% water) and B (95% acetonitrile) each containing 2 mmol L^{-1} ammonium formate and 50 mmol L^{-1} formic acid. For AZAs and PTX2 a C_{18} ACE (30 mm×2.1 mm) column was used with an isocratic run of 60% B for 7 min and analysis of both in positive-ionisation mode. For OA a C_{18} ACE (30 mm×2.1 mm) column was used with an isocratic run of 55% B for 6.5 min and the MS in negative-ionisation mode. Finally, for YTX a C_8 BDS Hypersil column (50 mm×2.1 mm) was used with gradient elution, starting with 30% B at time zero, increasing linearly to 90% B at 8 min, then 90% B for 2.5 min, then decreasing again to 30% B over 0.5 min which was held again for 3 min until the next run. YTX was analysed in negative-ionisation mode as adapted from Quilliam et al. [10].

Biological testing

Before analysis the samples were incubated for 1 day, 5 days, or 7 weeks at 25 °C, depending on the severity of previous treatment. The irradiated, heat-treated, and control samples were tested by total viable count (TVC) (The method used was comparable with ISO method 4833). The TVC procedure involved incubation for 5 days at 25 °C on agar plates prepared with "Plate Count Agar" (Oxoid). The detection limit was 10 viable cells g^{-1}. Agar plates with no detectable bacterial colonies were incubated for another 3 days and checked again.

Results

Actual doses received by test materials in the test scale irradiator were measured in two positions (Table 1). Repeatability of doses was adequate.

Average results from microbiological testing (n=3 for each treatment/incubation combination) are listed in Table 2. Bacterial counts were much higher for oysters than for mussels, confirming previous trials (data not shown). Simple heating of fresh samples was sufficient to eliminate microbial activity in mussels. For oysters, however, positive bacterial counts were obtained for all samples that were only heat-treated. For both mussels and oysters all three doses of irradiation investigated were effective in sterilising the

Table 1 Range of doses (kGy) measured in test scale irradiator during treatment

	Planned	Measured		Average
		1st	2nd	
Control	0	0	0	0
Dose 1	6	7.2	7.8	7.5
Dose 2	12	11.9	12.8	12.4
Dose 3	24	21.2	25.9	23.6

tissues. There was one positive count, of three replicates, for the 12-kGy dose and the oyster samples; this may, however, also be attributed to a leaking container or laboratory contamination. No colonies were detected after further incubation (3 days) of previously clear plates.

Table 3 shows the actual toxin concentrations measured in the control samples of all the materials. For illustration, these control levels were used to normalise the results obtained after the different treatments, for comparison of tissue and solution samples.

In solution, DA was severely affected by the treatment with no DA being detected in samples given any of the irradiation doses (Fig. 1). In tissue samples treated with 6 kGy there was ca. 40% reduction in DA concentration. There was ca. 80% reduction in samples exposed to 12 kGy, and no DA was detected in tissue samples exposed to the top dose of 24 kGy.

For AZA1 irradiated in MeOH, increasing doses led to decreasing concentrations (Fig. 2). In comparison with the controls there was ca. 50% reduction in concentration for samples given the highest dose. The differences between the average concentrations recorded for the samples treated with different doses were all statistically significant, except between the 6 and 12 kGy samples. For AZA1 in tissue no reduction in concentration was observed for the different doses. AZA2 and AZA3 were also present in the tissue samples and, similarly to AZA1, no significant reduction in the measured concentrations were observed (data not shown). In the mussel material spiked with AZA1 there was no degradation at any of the doses.

Table 2 Determination of TVC in treated fresh shellfish samples after incubation at 25 °C for 1 day, 5 days, and 7 weeks (units expressed as CFU g^{-1})

	Treatment	24 hours	5 days	7 weeks
Oyster	Control	95,667		
	Control (heated)	14		
	6 kGy	<LOD		
	12 kGy	<LOD	31[a]	
	24 kGy		<LOD	<LOD
Mussel	Control	14,667		
	Control (heated)	<LOD		
	6 kGy	<LOD		
	12 kGy	<LOD	<LOD	
	24 kGy		<LOD	<LOD

[a] One sample out of three gave a positive count after incubation for 5 days

Table 3 Concentration of analytes in control solution and control tissue samples

Analyte	Conc. in solution (ng mL^{-1})	Conc. In mussel tissue (μg g^{-1})
AZA1	43.3	0.21, 0.17[a]
OA	51.5	0.14
PTX2	59.9	0.05
YTX	59.5	0.65
DA	10.4[b]	11.2

[a] Concentration in spiked material
[b] Concentration in μg mL−1

There were significant reductions in concentration for OA irradiated in MeOH (Fig. 3). In comparison with the controls there was an approximate 30% reduction in OA concentration for the samples given the highest dose. In the tissue there was a gradual reduction in OA concentration with increasing dose, but the differences between the samples subjected to the progressively increasing doses were not statistically significant. In comparison with the controls, however, the 20% reduction of OA in the samples treated with 24 kGy, was statistically significant. DTX2, a later eluting isomer of OA also present in the tissue, was affected in the same way as OA (data not shown).

PTX2 irradiated in MeOH was significantly affected (Fig. 4). Compared with the control samples reductions were ca. 30, 50 and 75% in samples treated with doses of 6, 12, and 24 kGy respectively. For the spiked tissue samples none of the doses led to any significant reductions (Fig. 4).

Analysis of YTX in MeOH solution showed it was extremely unstable when irradiated, with only trace amounts of the analyte being detected in the samples

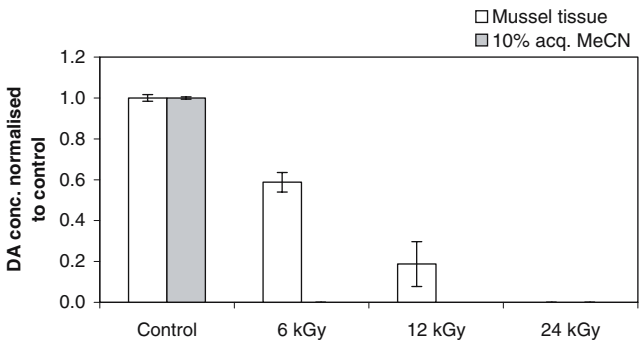

Fig. 1 DA concentrations measured in a tissue matrix and in solution after irradiation. *Error bars* represent ±1 SD (n=5). DA was not detected in any of the irradiated 10% aqueous MeCN solutions

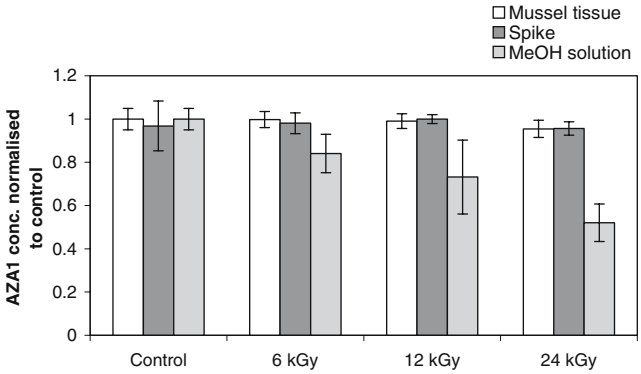

Fig. 2 AZA1 concentrations measured in MeOH, in naturally contaminated tissue, and in spiked tissue after irradiation. *Error bars* represent ±1 SD (n=5)

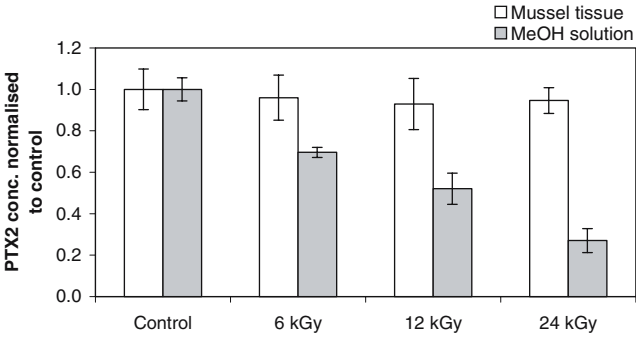

Fig. 4 PTX2 concentrations measured in MeOH and tissue after irradiation. *Error bars* represent ±1 SD (n=5)

subjected to the lowest dose of 6 kGy (Fig. 5). In the mussel tissue samples, reduction was only significant (ca. 15%) at 24 kGy.

Discussion

The microbiological tests showed that irradiation at all the doses was an effective sterilisation method, although there was one outlier at the intermediate dose for the oyster samples after incubation for 5 days. The effect of simple heat treatment, which is common when processing shellfish, was imitated by use of a water bath. No microbial activity was detected in the heated mussel samples; heat treatment during processing is not an adequate stabilisation procedure, however, because it is not practical to perform the entire subsequent preparation of an RM under aseptic conditions.

The results of this study show that gamma irradiation has different effects on different toxin groups—some have complete lack of resistance, whereas for others increased degradation was observed with increasing doses. A common feature for all groups examined was a protective effect of the tissue matrix against the gamma rays. The level of protection was highly variable, however, depending on the toxin.

As part of these studies degradation products were not examined. Such products can be numerous, and are typically present in very small quantities. This will be the focus of future studies, and will be facilitated by use of more highly concentrated samples. In the studies discussed here no additional peaks were observed in chromatograms, and there was no indication of interferences formed by the treatment.

Irradiation would not be a suitable stabilisation technique in the preparation of DA tissue RMs, because it is clear that degradation of DA increases with increasing dose. In treatment of a full-scale material in a commercial sized irradiation unit heterogeneity could, therefore, be introduced as a result of uneven distribution of radiation across a batch. Heat treatment has been shown to be a suitable stabilisation technique for DA, however, and although this still results in reductions in concentration, these are much less significant and are consistent between samples of the same treatment group [8]. These results thus confirm that heat treatment remains the best technique for the stabilisation of wet tissue RMs for DA.

AZA1 was much more resistant to the irradiation treatment. In the tissue samples no statistically significant difference was detected between any of the treatment

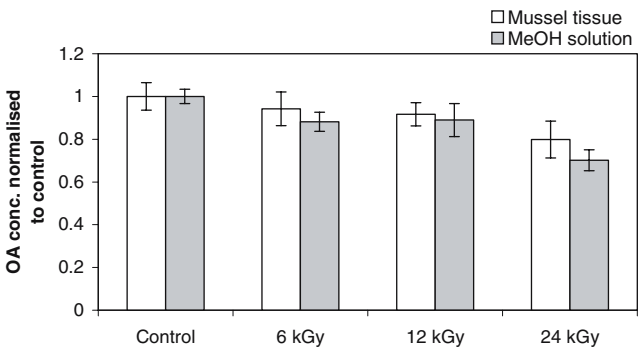

Fig. 3 OA concentrations measured in MeOH and tissue after irradiation. *Error bars* represent ±1 SD (n=5)

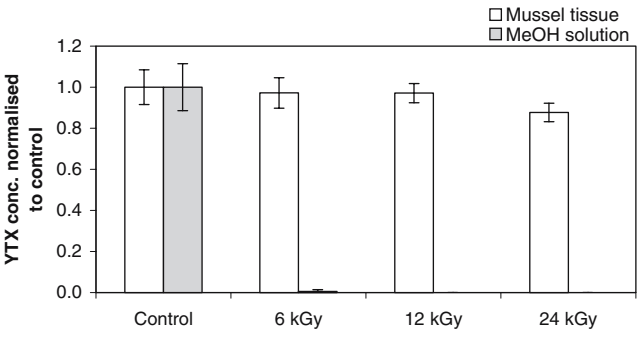

Fig. 5 YTX concentrations measured in MeOH and tissue after irradiation. *Error bars* represent ±1 SD (n=5). YTX was not detected in the MeOH solutions treated with 12 or 24 kGy

groups. In tissue spiked with AZA1 no significant reductions were observed, showing that even when the toxin is not naturally present the matrix still has a protective effect. Because there were no differences between the different treatment groups, irradiation should be regarded as a suitable method for stabilisation of AZA tissue RMs after they have been dispensed. The technique may be particularly useful, because previous studies have shown that heat stabilisation using a steam retort reduces concentrations of in AZA1 and 2 and completely degrades AZA3 [2].

OA was affected by irradiation both in solution and in tissue. The protective effect of the matrix meant that reductions in concentration in tissues samples subjected to the highest dose were lower than for the equivalent solutions. Whereas in the tissue samples there was some degradation of OA and none of AZA, it is interesting to note that the largest reduction in the MeOH solution (ca. 30%) was still less than that of AZA1 in the corresponding samples (50%). There was some degradation in the tissue samples; concentrations were ca. 20% lower in the samples treated with 24 kGy. Although there were reductions in OA concentrations, irradiation may still be a suitable stabilisation procedure for preparation of OA tissue RMs, because the reductions observed were consistent between samples of the same treatment group. However, consideration of increased variability with use of a large irradiation unit is required.

Reduced concentrations of PTX2 in MeOH solution with increasing dose was clearly evident. Similarly to AZA1, no significant decreases were observed in the tissue samples. This may also be important because currently no matrix CRM is available for PTXs and previous studies have shown that heating may not be a desirable stabilisation technique for PTX2 (MA Quilliam, personal communication, 2006).

Results for YTX in solution were quite similar to those of DA with only trace amounts of the analyte being detected in the samples subjected to the lowest doses. In tissue, however, reduction in the concentration of YTX was significant only at the highest dose. Irradiation may be a suitable stabilisation method for YTX materials, although further investigations would be required to confirm this and consideration of increased variability with large-scale preparations would be required.

With irradiation the aim is to microbiologically stabilise a material. The dose of irradiation used to achieve this is important, however, because the technique is known to generate free radicals. Previous studies have shown that the oxidative rancidity of lamb meat increased with increasing dose of radiation [11] but use of small quantities of antioxidants have been shown to inhibit this lipid peroxidation [12]. Parallel studies have shown that use of antioxidants alone can improve the stability of DA in mussel tissue RMs [13] and they have been used in the preparation of the matrix CRMs available for shellfish toxins [1].

Until this point we have discussed the risk of degradation of toxins as a result of microbiological activity. It is possible, however, for toxins to be degraded or rearranged by enzymes. In this work no studies were performed to determine if the viability of enzymes was reduced by the irradiation. In the harvesting of shellfish tissues to be used in the preparation of RMs, however, it is usual practice to process the materials by steam cooking. This would drastically reduce enzymatic activity in the tissues, making it difficult to discern any effect of irradiation on enzyme activity.

In summary irradiation may be a useful stabilisation technique in the preparation of tissue RMs for all the toxins examined except DA. Reductions in concentrations as the result of a stabilisation step would be acceptable if there was no effect on the homogeneity of a material. This is very difficult to guarantee with irradiation, because the exact dose received depends on location in the irradiation chamber and the distance from the source. In this study the doses were evenly distributed among the samples (Table 1), and this is supported by the standard deviations for the different treatment groups of the analytes which were affected by the treatment. It is, however, important to note that these experiments were conducted using a test scale irradiator, and the precision of dose achieved here would be difficult to reproduce with a production-scale material in a commercial irradiation unit. It may, therefore, be prudent to consider irradiation as a stabilisation technique only for those analytes that are not affected at higher doses than are necessary to eliminate microbial activity. Studies evaluating the stability of irradiated tissues are in progress in the authors' laboratory.

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