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ORIGINAL PAPER

Effect of addition of antibiotics and an antioxidant on the stability of tissue reference materials for domoic acid, the amnesic shellfish poison

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Abstract Five separate reference materials (RMs) were prepared from a mussel (*Mytilus edulis*) tissue containing domoic acid (DA) from scallop hepatopancreas (*Pecten maximus*). Homogenates were separately spiked with antibiotics, an antioxidant, or a combination of both. Control materials did not contain any additives and were prepared from lightly cooked and autoclaved mussel tissues. Stability studies were run over a 148-day period at three different temperature conditions: −20 °C, +4 °C and +40 °C. DA contents in all materials were characterised by HPLC-UV. Homogeneities were demonstrated at the beginning of the study, with coefficients of variance of less than 4% (n=9). DA was stable at −20 °C in all materials. The control materials showed significant degradation after two days at +40 °C, and after eight days at +4 °C. Each of the materials containing additives demonstrated better stability during the initial period of the study. In addition there was no significant degradation in any of the materials with additives stored at +4 °C over the duration of the study. The material containing a combination of the antibiotics and the antioxidant displayed the best stability of all the materials. There was no significant reduction in DA concentration at all temperature conditions after eight days, and after 32 days the decrease at +40 °C was still <20 %. Following this, a DA laboratory reference material (LRM) was prepared and, based on previous results, spiked with both the antioxidant and antibiotics. A short-term stability study on this material gave similar results to the corresponding material in the additives study. This study shows that combined use of the additives investigated in the preparation of a mussel tissue reference material for DA ensures analyte stability for a period of up to eight days at temperatures of up to +40 °C, a condition that is particularly important when shipping test materials globally.

Keywords Reference materials · Domoic acid · Stability · Additives · Antioxidant · Antibiotics

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Introduction

The availability and use of reference materials (RMs) are key elements in the quality control procedures of official control laboratories concerned with demonstrating the adequacy of their tests. For RMs, stability is of the utmost importance, as analysts need to be able to use RMs whenever they are required [1]. Stability is especially important for certified reference materials (CRMs), as they have fixed certified values, and any potential for variation in these values as a result of instability is not acceptable. For biological matrices in particular, any kind of deterioration during the shelf life of a material should be avoided [2].

There are a number of techniques employed to promote and enhance the stability of RMs. The choice of procedure used, however, depends on the analyte of interest, and the representativeness of the material, not being adversely affected.

Removal of water during the preparation of RMs by freeze-drying is a stabilisation method routinely employed by the Institute of Reference Materials and Measurements (IRMM) in the preparation of biological CRMs [1]. Heat treatment is another common stabilisation technique with

wide application. The National Research Council of Canada (NRCC) has used heat stabilisation in the preparation of biological CRMs for shellfish toxins [3, 4]. While this is an efficient way of eliminating bacterial growth, heat treatment also results in significant changes to the nature of the matrix. Therefore, alternative stabilisation techniques may sometimes be required. Another method of stabilisation is gamma irradiation, although the technique is typically only applied in the preparation of RMs to be certified for inorganic analytes [5], as many organic compounds are destroyed by such treatment. Additives are another way of enhancing the stability of materials. The antioxidant ethoxyquin has been used in the preparation of mussel tissue CRMs for marine biotoxins [3, 4] and also in carp (*Cyprinus carpio*) CRMs for organochlorine compounds [6]. Butylated hydroxytoluene (BHT), another antioxidant, has been used in the preparation of mussel tissue RMs for organic contaminants [7]. While there is reference to the use of antioxidants in RM preparations, little information is available on their effectiveness.

In this study, the use of additives to improve the stability of domoic acid (DA) in mussel tissue RMs was investigated. The amnesic shellfish poisoning (ASP) toxin DA is a naturally occurring hydrophilic compound. A number of diatoms and macro alga have been shown to produce DA, but the *Pseudo-nitzschia* group of diatoms has been highlighted as the main producer [8, 9]. This toxin is frequently found at levels well above the regulatory limit of 20 mg/kg [10] in shellfish from Irish waters, as well as internationally.

Although initial autoclaving could destroy most bacteria in shellfish tissues, subsequent material handling and dispensing of aliquots is not usually carried out aseptically. Therefore, bacterial growth cannot be excluded in dispensed aliquots at temperatures above freezing, which RMs are frequently exposed to during transport. Consequently, we decided to investigate the stabilising properties of antibiotics. Ethoxyquin and a combination of three antibiotics, namely ampicillin, erythromycin and oxytetracycline, were used in the study. Autoclaved mussel homogenates containing DA were spiked with the antioxidant, the antibiotics, and a mixture of both. Control materials not containing any of the additives were prepared from autoclaved tissue and tissue which had been only lightly steamed. The stability of DA in the various materials was examined at different temperatures and over time.

Methods and materials

Chemicals and standards

Ethoxyquin, oxytetracycline, ampicillin, erythromycin and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). DA calibration solutions were prepared from certified standards (NRC Canada, CRM-DA-d). Methanol, acetonitrile and ethanol were obtained as HPLC-grade solvents from Labscan (Stillorgan, Ireland). A reverse osmosis system (Barnstead Int., Dubuque, IA, USA) supplied water for the extraction solvent and mobile phase.

Preparation of feasibility study materials

Blue mussels (*Mytilus edulis*) from Bantry Bay on the southwest coast of Ireland were obtained during 2004. The mussels were lightly steamed to facilitate removal of the flesh from the shell. A stock material was prepared by mixing ~1.5 kg of whole mussel flesh with scallop (*Pecten maximus*) digestive glands highly concentrated in DA (~1000 mg/kg). Less than 2% digestive glands were required to achieve a suitable DA concentration. The stock tissue was homogenised using a Waring (Hartford, CT, USA) blender and divided into five equally sized portions. Four of the portions were combined and stabilised using an autoclave at 121 °C and 15 psi for 15 min (ST19 Express Autoclave, Dixons, Wickford, UK). The stabilised material was redivided into four lots. The moisture content of each was adjusted to 85% to reflect that of a natural mussel matrix, and during this step the additives were introduced. One portion was spiked with the antioxidant (ethoxyquin), a second was spiked with a mixture of the three antibiotics (oxytetracycline, ampicillin, erythromycin), and a third was spiked with a combination of the antioxidant and the antibiotics. Each additive was added at a concentration of 0.02%. As a control against the treated RMs, two materials without additives were prepared. One control material was prepared from the remaining autoclaved lot, while a second control material was prepared from the lot that had not been stabilised. *Note* that when all of the additives used were spiked into a crude mussel extract contaminated with DA, there was no interference in the separation or detection of the compound by HPLC-UV.

The individual materials were homogenised using an Ultraturrax (IKA Werke, Janke & Kunkel, Staufen, Germany). A Gilson (Middleton, WI, USA) pipette was used to dispense the materials as 4 g aliquots into 5 ml glass tubes (Teklab Ltd., Durham, UK). Mixing was maintained during the dispensing step. The tubes were sealed using wadded screw caps. For each material, a minimum of 70 aliquots were dispensed. The materials were stored at −20 °C until characterisation.

Aliquots of each material were taken for homogeneity testing (n=9) and the remaining aliquots were divided equally between the temperature conditions selected for the study; −20 °C, +4 °C and +40 °C. Three aliquots of each

material were extracted and analysed from each condition at each of the following time points; 2, 8, 16, 32, 64 and 148 days. Stability studies of all the differently treated RMs were run in parallel, and analyses of all materials were carried out on the same day for each time point.

Preparation and characterisation of ASP LRM 05-1

Subsequent to the additives study, a LRM was prepared in-house [Burrell S, Deedigan L, McCarron P (2005) Unpublished report on the preparation of ASP LRM 05-1. Marine Institute, Galway, Ireland] for quality control in routine monitoring for DA in shellfish harvested in Irish waters. The material was prepared using mussels naturally incurred with DA at ~10 mg/kg and additionally fortified with DA from scallop hepatopancreas, to bring the total concentration up to ~20 mg/kg. A combination of the antioxidant and antibiotics were spiked into the material at the same concentrations used in the previous study. The material was dispensed in 4 g aliquots (n=675). The homogeneity of ASP LRM 05-1 was analysed and a short-term isochronous [11] stability study was carried out over a 32-day period at −20 °C, +4 °C and +40 °C soon after the preparation.

Extraction

Extraction was performed following a study by Harkin et al. [12]. Aliquots (4 g) were extracted twice in 50 ml centrifuge tubes with 16 ml volumes of 50:50/MeOH:H_2O. The first extraction was performed using a multitube vortex mixer (V400, Alpha Labs, Eastleigh, UK) for 1 min at full power. After centrifugation (CR4-22, Jouan, Thermo Electron Corp., San Jose, CA, USA) at 3500 rpm for 15 min, the supernatant was transferred to a 50 ml volumetric flask. The second extraction of the remaining pellet was carried out using an Ultraturrax homogeniser at 11,000 rpm for 1 min, and after centrifugation the supernatant was added to that of the first extraction in the 50 ml volumetric flask. The combined supernatants were then made up to 50 ml using the same extraction solvent. Overall, this gave a solvent-to-sample ratio of 12.5:1.

Aliquots of the final extracts were filtered through 0.2 μm filters (Schleicher & Schuell, Whatman, Brentford, UK) into HPLC vials. These filtered extracts were analysed directly without further clean-up.

Instrumental analysis

DA was determined as the sum of DA and epi-DA, closely following the procedure published by Quilliam et al. [13], without the clean-up step and with some minor modifications, as accredited in the authors' laboratory [14]. A Shimadzu (Kyoto, Japan) HPLC system with a photodiode array (PDA) detector was used. The HPLC column was a reverse-phase C-18 Vydac (Hesperia, CA, USA; 250 mm× 4.6 mm, 10 μm). The HPLC programme was isocratic followed by a column flush at higher percent acetonitrile, as described in McCarron and Hess [15]. Validation of the method showed within-batch repeatability to be ≤3.0% for replicate analyses of an LRM (n=14), and the between-batch reproducibility to be 9.9% for the analysis of 30 LRMs in consecutive batches.

Results

Each material was satisfactorily homogeneous for DA, making them suitable for the analytical study (Table 1). The coefficient of variance for DA concentrations in all of the aliquots of the five materials together was ~4 % (data not shown), showing that, even though the individual materials were prepared separately, the preparation method used was reproducible.

Figures 1 and 2 display the results of stability studies on each of the materials. No degradation was observed at −20 °C in any of the materials over the study period (Table 2). The changes in average values for the different days were due to between-run variability, and the coefficients of variance for the average of the daily averages ranged from 5.3 to 6.8%, which was less than the between-batch reproducibility of the method. All data has been normalised to the average values of the aliquots stored at −20 °C for each day of the study to compensate for the between-run variability. If the stability studies had been run isochronously, between-run variability would have been excluded. However, as there were five separate materials, the isochronous design was not feasible due to the number of extractions and instrumental analyses necessary; but, since all of the materials were extracted and analysed together at each of the individual time points, the data are comparable.

Table 1 Homogeneity of prepared materials characterised on day zero of stability study (n=9)

Material	Average DA conc. (μg/g)	SD	% CV
Unstabilised control	11.1	0.4	3.5
Control	10.9	0.3	3.1
Antioxidant	11.7	0.5	3.9
Antibiotics	11	0.2	2.1
Antibiotics & antioxidant	11.6	0.3	2.5
ASP-LRM*	21.9	0.4	1.7

*n=26

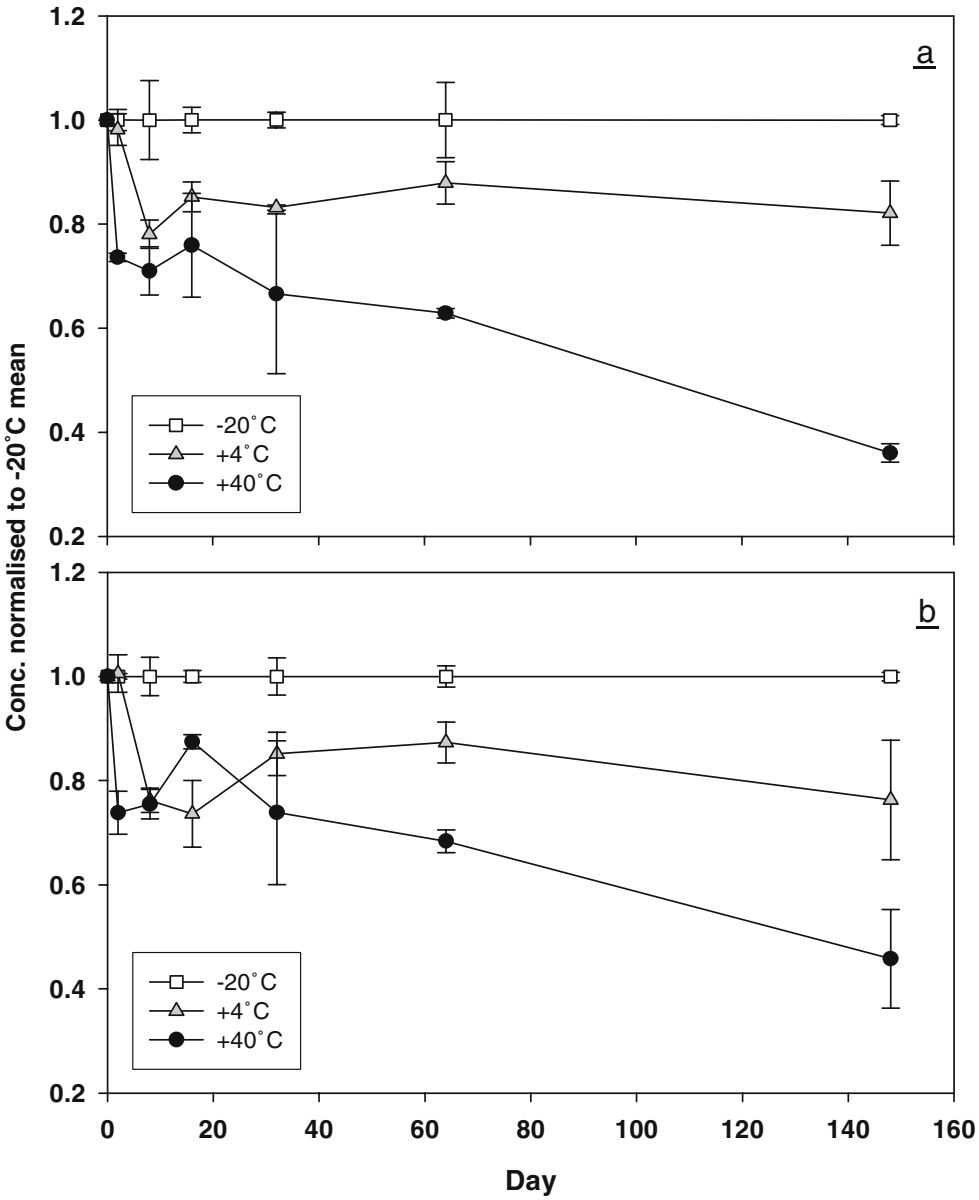

Fig. 1a, b Stability graphs of control materials expressed relative to the average of the −20 °C values for each day. **a** Control material without heat treatment; **b** control material autoclaved prior to homogenisation. Error bars represent ± 1SD (n=3)

Control materials (without additives)

There were no significant changes in concentration after two days for the unstabilised and autoclaved control materials at +4 °C (Fig. 1a,b). However, at day 8 the average concentrations dropped and then fluctuated without major reduction for the remainder of the study. For both control materials there were significant decreases in DA concentration at +40 °C after two days. Following this, slow degradation with some variation was observed up to the end of the study period. For aliquots stored at +40 °C there was a considerable between-sample variability on some of the days, as is illustrated by the large error bars for the replicates (± 1SD, n=3). A similar situation was observed at +4 °C.

Antioxidant and antibiotic materials

The average DA concentrations for both the antioxidant and antibiotic materials at +4 °C fell slightly on some of the days (Fig. 2a,b). However, in the control materials there was a considerable reduction in the DA observed at +4 °C after eight days, which persisted for the remainder of the study, indicating that ethoxyquin and the mixture of antibiotics separately improved the stability of DA in the mussel materials. Significant degradation of DA occurred at +40 °C in both materials. However, the sudden drop in concentration observed at this condition for the control materials was not apparent. The decreases were more gradual for both the antioxidant and antibiotic materials. This further demonstrates the positive influence the

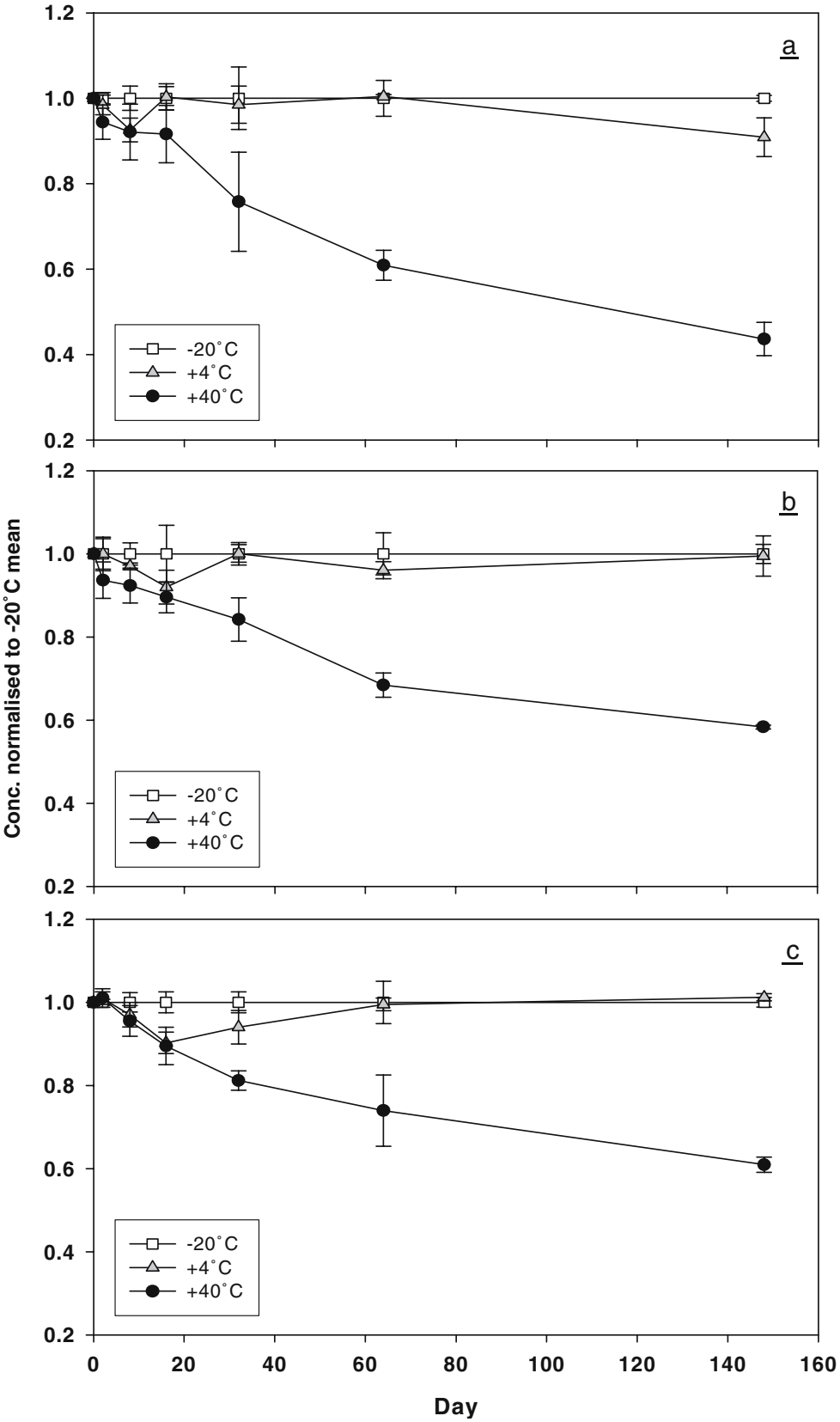

Fig. 2a–c Stability graphs of materials containing additives expressed relative to the average of the −20 °C values for each day. **a** Antioxidant is only additive; **b** antibiotics are only additive; **c** both antioxidant and antibiotic were added. Error bars represent ± 1SD (n=3)

Table 2 Average DA concentrations in the −20 °C samples taken on each day of the study for the different materials (n=3)

Day	Control without heat treatment		Control		Antioxidant		Antibiotics		Antioxidant and antibiotics	
	DA conc (μg/g)	SD	DA conc (μg/g)	SD	DA conc (μg/g)	SD	DA conc (μg/g)	SD	DA conc (μg/g)	SD
2	10.4	0.2	10.7	0.1	11.8	0.2	11	0.5	11.8	0.1
8	10.8	0.8	11.2	0.4	12.1	0.3	11.3	0.3	12	0.3
16	10.6	0.3	11.1	0.1	11.3	0.3	10.8	0.7	12	0.3
32	11.1	0.2	10.8	0.4	11.1	0.8	10.3	0.3	11.6	0.3
64	12.2	0.9	12.8	0.3	13.1	0.6	12.2	0.6	13.2	0.7
168	11.6	0.1	11.7	0.1	12.8	0.1	11.6	0.3	12.9	0.2
Average	11.1		11.4		12		11.2		12.2	
SD	0.69		0.78		0.82		0.66		0.65	
%CV	6.2		6.9		6.8		5.9		5.3	

additives had on the stability of DA. At +40 °C the material containing ethoxyquin degraded the least over days 2, 8 and 16, but the average values dropped below those of the antibiotic material after days 32, 64 and 148.

Combination material

The material containing both the antioxidant and the antibiotics displayed the best stability of all the materials prepared. There was no significant reduction in DA concentration at +4 °C over the entire study, and after eight days at +40 °C (Fig. 2c). The combination of additives in this material seemed to act in an additive or synergistic way. Again, for this material, the degradation of DA at +40 °C was quite gradual over the duration, and while significant reduction in DA concentration was apparent after 148 days, the DA concentration remained higher over the whole study duration in this material than for any of the others (Table 3).

ASP LRM 05-1

The LRM showed good homogeneity for DA, with a coefficient of variance of 1.7 % (n=26) (Table 1). After homogeneity testing, a short-term stability study was performed using the same temperature conditions as in the additive study, and with very similar time points. Figure 3 compares the stability study results of the LRM with those of the material that contained a combination of the antioxidant and the antibiotics after eight days. There was good similarity between the results, with no significant change in DA concentration in either of the materials at any of the conditions at eight days. For both materials, DA was stable at −20 °C and at +4 °C, with <20% reduction at +40 °C over a 32-day period.

Table 3 Degradation of domoic acid in the reference materials stored for 148 days at +40 °C (harshest study conditions)

Material	% DA degraded	%CV
Unstabilised control	64	4.9
Control	54.2	21
Antioxidant	56.4	8.9
Antibiotics	41.6	0.8
Antioxidant and antibiotics	39	3

Discussions

While all the materials were prepared from lightly steamed mussels, autoclaving seemed to improve the stability of DA. This is indicated by a comparison of the unstabilised and autoclaved control materials. The normalised DA values of the unstabilised material stored at +40 °C are consistently lower than those of the cooked material (Table 3) (*Note:* the difference was only statistically significant by t-test on day 64). This suggests that in mussel tissue RMs, DA is less likely to degrade when a heat stabilisation step is included, even if the remainder of the preparation procedure is not aseptic, as was the case in this study.

The individual antioxidant and antibiotic materials showed stability for DA at +4 °C over the course of the study, and the onset of degradation at +40 °C was not as immediate as in the control materials. At +40 °C the antioxidant material was more stable than the antibiotic material after 16 days, but the antibiotic material showed less degradation over the remainder of the study (Fig. 2a,b). This may indicate that mussel tissue RMs for DA are more susceptible to oxidative degradation in the short term, but are more at risk from microbiological degradation over increasing periods of time. Thus, it was the combination of the antioxidant and the antibiotics into a single material which produced the best results. Although the antibiotic treatment showed slightly more efficient protection of DA,

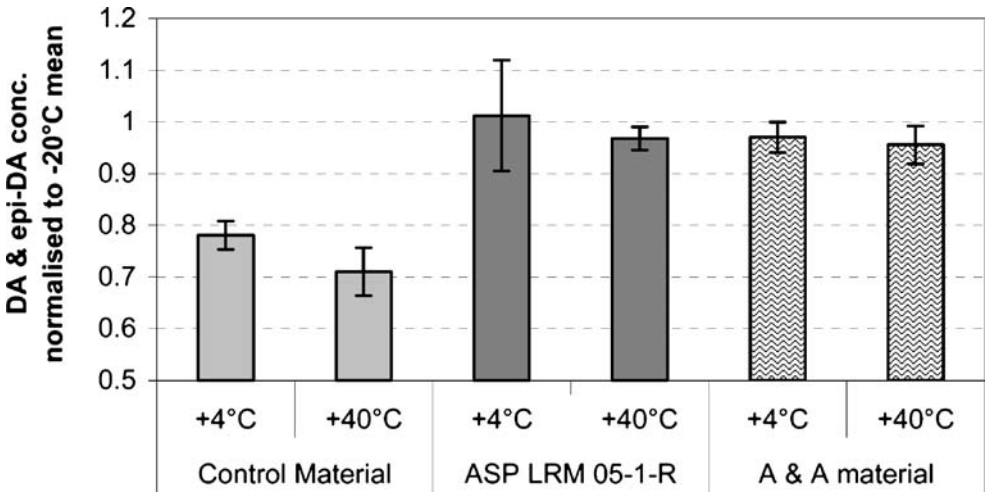

Fig. 3 Normalised DA concentrations in control material, ASP LRM 05–1 and combination material after eight days at +4 °C and +40 °C. Error bars represent ± 1SD (n=3)

the degradation pattern for DA is very similar to the material with antioxidant. Therefore, it cannot be excluded that the compounds added as antibiotics also possess antioxidant properties.

The stability study on ASP LRM 05-1 verified the results of the initial study. It is interesting to note that the mussel tissue used to prepare the LRM was in part (~50%) naturally contaminated with DA, whereas all DA present in the additives study was spiked. The results may suggest that DA is no more stable in a naturally incurred matrix than in a spiked one (Fig. 4).

The decreased concentration in both control materials after two days at +40 °C implies that mussel tissue RMs for DA may be susceptible to degradation during transport if not stored under suitably low temperature conditions. This is important information for the production of biological CRMs, and for materials prepared for use in proficiency testing schemes, as it is often necessary to ship these long distances globally, when the transport conditions and durations are difficult to guarantee. The results show that a combined use of the additives investigated in the preparation of a mussel tissue RM for DA ensures analyte stability for a period of up to eight days at temperatures up to +40 °C, which would provide a safeguard from degradation during transport.

Significant degradation of DA took place at +40 °C in all the materials. Even though the additives seemed to postpone this in the short term, the degradation was evident over the entire study. Temperature conditions such as this are typically included to add an accelerated dimension to stability studies, from which information on suitable conditions of transport for such materials can be derived. These high-temperature conditions are also included to generate data from which a shelf life or an expiry date for the material can be assigned. Shelf lives are typically calculated by extrapolation using the Arrhenius equation. Security factors are usually included as it is very difficult to extrapolate for the behaviour of molecules in varying RM matrices [1].

While the use of antioxidants in RM and CRM preparations has been reported [3, 4], there is little or no

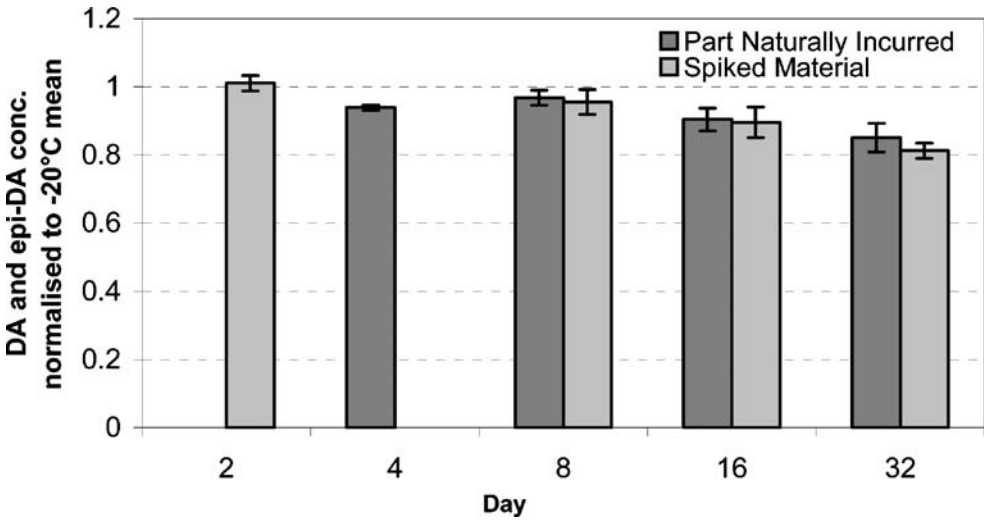

Fig. 4 DA concentration in spiked and part naturally incurred materials at +40 °C. Error bars represent ± 1SD (n=3)

literature on the use of antibiotics. The results presented in this study show that microbial activity may play a significant role in the degradation of DA in mussel RMs, at temperatures above freezing. This can be seen in Table 3, where it is clear that DA concentrations were reduced more over the duration of the study at +40 °C in the materials without antibiotics. These differences are statistically significant. Additionally, aliquots of each material stored at +40 °C were examined two months into the study, and phase separation comprising irregularly shaped gas pockets was only observed in the materials that did not contain antibiotics, suggesting some form of biological activity not present in the materials with antibiotics.

Heat treatment and freeze-drying are methods typically used to inhibit microbial activity, but these techniques may not always be desirable. For instance, heat stabilisation can cause significant alteration of a matrix and may result in the destruction of some analytes. While freeze-drying is a technique commonly used in biological RM preparations, emphasis is increasingly being placed upon the preparation of RMs that are more representative of natural samples. Because of the minimal impact that additives would generally have on analytes of interest, as well as on the physical representativeness of materials, the authors feel that the use of additives should be considered in combination with, or as a feasible alternative to, more traditional stabilisation techniques, and further research on the topic is warranted.

This study examined the influence of additives on the stability of an analyte in RMs. However, during the preparation of RMs, the stability of the matrix is also of great importance, and the use of additives is effective at maintaining this [1]. The results show that the use of additives is a simple method of enhancing the stability of RMs prepared in house, when the preparation of freeze-dried materials or stabilisation by irradiation may not be practical options.

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