J Radioanal Nucl Chem (2009) 282:957–962
DOI 10.1007/s10967-009-0236-y

Perna perna mussel reference material: short term stability assessment

E. G. Moreira · M. B. A. Vasconcellos · M. G. M. Catharino · V. A. Maihara · M. Saiki

Received: 28 June 2009 / Published online: 16 July 2009

Abstract Instrumental neutron activation analysis was used at a 2-month long isochronous short term stability test performed on a *Perna perna* mussel candidate reference material. The assessment of the analysis of variance test, as well as, the normalized results to the control temperature showed no systematic changes in the concentration of Ag, As, Br, Co, Cr, Cs, Fe, Eu, La, Na, Rb, Se, Sc, Th and Zn during the test period. The result showed that the candidate reference material may be transported under normal transport conditions without significant changes in composition for the determined elements.

Keywords Reference material · Mussel · *Perna perna* · INAA · Stability test

Introduction

Due to their intimate contact with sea water, mussels may accumulate toxic trace elements which are a concern from a nutritional point of view. However, mussels are resistant to deleterious effects, remaining alive. This fact is used in water quality monitoring studies, as toxic elements bioaccumulate in the tissues of the animals [1]. To improve the confidence on biomonitoring measurement results and to make it possible to compare results from different places in time, various quality assurance procedures such as the use of certified reference materials are needed [2].

The short term stability study, one of the steps in the characterization of new reference materials, is used to verify if normal transport conditions will modify the status of the property values of interest [3]. Temperature variation is a key factor in biological material degradation and hence vials of the material are kept at different temperatures and analyzed as a function of time to check for possible property value variations. In this study a new Brazilian mussel candidate reference material was submitted to an isochronous short term stability test after bottling and gamma ray sterilization. In the isochronous design, element determination is performed only after all samples are submitted to the various temperatures and time periods of the test. In this case, the measurements may be accomplished in a situation closer to repeatability conditions than in a traditional design, resulting in more precise conclusions from the measurements [4].

Vials of the material were taken for testing for various time periods up to two months, at several temperatures (−20, 20, 40, and 60 °C). After the test periods, Ag, As, Br, Co, Cr, Cs, Fe, Eu, La, Na, Rb, Se, Sc, Th and Zn were determined by instrumental neutron activation analysis, INAA. To verify if the material may be transported under normal conditions, a comparison of element concentration for the various temperatures to the ones obtained at the control temperature (−20 °C) was performed. The lowest temperature was taken as the control temperature for the test, as it is considered that biological reference materials are stable for element content at this temperature. An analysis of variance, ANOVA, approach was performed for testing for each element, taking into account the variability within each vial (four replicates) and between vials.

E. G. Moreira (✉) · M. B. A. Vasconcellos · M. G. M. Catharino · V. A. Maihara · M. Saiki
Instituto de Pesquisas Energéticas e Nucleares, IPEN – CNEN/SP, Av. Prof. Lineu Prestes, 2242, São Paulo, SP 05508-000, Brazil
e-mail: emoreira@ipen.br

To supplement the ANOVA test, the mean results for the various temperatures were normalized for every element to the results obtained at the control temperature (−20 °C), according to Eq. 1.

$$R_{\mathrm{T}} = \frac{X_{\mathrm{T}}}{X_{-20\,^{\circ}\mathrm{C}}} \quad (1)$$

In the case of stability, the ratio R_{T} should be equal to 1, considered the uncertainty of the measurements which may be estimated from the coefficient of variation, CV, of the measurements obtained at each temperature [5], as given by Eq. 2.

$$U_{\mathrm{T}} = \frac{\sqrt{\left(\mathrm{CV}_{\mathrm{T}}^{2} + \mathrm{CV}_{-20\,^{\circ}\mathrm{C}}^{2}\right)} \times R_{\mathrm{T}}}{100} \quad (2)$$

It should be noted that as the different measurements were performed under the same conditions, Type B uncertainty contributions from X_{T} and $X_{-20\,^{\circ}\mathrm{C}}$ cancel in an estimation of the uncertainty in R_T and only the Type A contributions will remain (basically the repeatability of the two measurements).

Experimental

Isochronous layout

The preparation of the candidate reference material was described elsewhere [6]. Figure 1 presents the 2-month long isochronous design used in this study. Shadowed spaces represent the testing period for each X vial at the various temperatures until time 0, i.e., the time for analyses. A stratified random scheme was used for selection of the thirteen vials used. Vials were kept at −20 ± 2 °C (Continental FC 26 Freezer); 20 ± 2 °C (climatized room); 40 ± 3 °C (Fanem 315 SE oven) and 60 ± 3 °C (Fanem Orion 515C oven) for periods that varied from 0.5 to 2.0 months. After the test periods all vials were stored at −20 °C till analysis.

Samples and elemental standard preparation

About 0.150 g of samples was weighed in properly cleaned polyethylene bags using a Shimadzu AEM-5200 analytical balance. Elemental standards were prepared by pipetting Spex standard element solutions onto Whatman paper filters, using variable volume pipettes (Eppendorf or Jencons). For some elements, the original solution was diluted in volumetric flasks prior to pipetting. After drying, paper filters were kept in polyethylene vials with the same geometry as for the samples. Four sample aliquots were taken from each vial for analysis.

Irradiation and element determination

After the test periods, sample aliquots and elemental standards were irradiated simultaneously for 8 h at 10^{12} n cm^{-2} s^{-1} thermal neutron flux of the IEA-R1 Nuclear Research Reactor at IPEN-CNEN/SP. ^{76}As, ^{82}Br, ^{140}La and ^{24}Na radionuclides were measured for 1.5 h, after a 7-day decay period, while ^{110m}Ag, ^{60}Co, ^{51}Cr, ^{134}Cs, ^{59}Fe, ^{152}Eu, ^{86}Rb, ^{75}Se, ^{46}Sc ^{233}Pa (for Th) and ^{65}Zn radionuclides were measured for 10 h, after a 15-day decay period. Gamma ray measurements were performed using a Canberra GC2018 HP Ge detector coupled to a Canberra DSA-1000 multichannel analyzer. Gamma ray spectra were collected and processed using a Canberra Genie 2000 version 3.1 spectroscopy software. Element content calculations were carried out using a Microsoft Excel spreadsheet. Sample aliquots were irradiated and measured in random in order to avoid interferences from any possible trends that might arise in the results during the measurement campaign.

Fig. 1 Isochronous design for the short term stability test

Temperature / time	2.0 months	1.5 months	1.0 month	0.5 month	0 month
-20 °C	X				
20 °C	X				
		X			
			X		
				X	
40 °C	X				
		X			
			X		
				X	
60 °C	X				
		X			
			X		
				X	

Results and discussion

Table 1 presents the INAA measurement results obtained for the mussel candidate reference material vials submitted to the various temperatures and time periods of the short term stability test. The results are presented on a wet mass basis, as no correction for residual moisture was performed. Table 2 summarizes the ANOVA test output obtained for the mean concentration of four replicates for the thirteen vials for each element. The null hypothesis of the ANOVA test, H_0, is that there is no difference among the mean concentration of the vials. If F, the calculated statistic of the test, is lower than the critical F_c value, there is no evidence to reject H_0. Hence, the reference material may be considered stable for the test conditions. As a general trend, it was observed that the within vial variability (represented by its mean square, MS) was greater or the same order of magnitude than the between vials variability. This was an indication that the observed variability is due to the precision of the INAA method used rather than to any significant differences among the vials. Except for Se, $F < F_c$ at the 95% confidence level for all the elements, indicating that there was no significant differences among the mean concentrations obtained for the vials, regardless of the period and temperature at which the vials were kept. In the case of Se, if a 99% confidence level is considered, the results may be considered equal, as at this less restrictive level $F < F_c$ ($F_c = 2.678$). These findings are corroborated by the obtained p-values which, with the exception of Se, were all greater than the level of significance $\alpha = 0.05$.

Table 3 presents the normalized results of the short term stability test as defined in the introduction above. Apart

Table 1 Element concentration in mg kg^{-1} obtained by INAA for the short term stability test (wet mass basis)

Vial	Temp. (°C)	Time, month	Ag	As	Br	Co	Cr	Cs	Eu	Fe
104	−20	2.0	2.36 ± 0.21	17.2 ± 4.5	206 ± 9	0.845 ± 0.059	1.09 ± 0.18	0.106 ± 0.019	0.0522 ± 0.0060	603 ± 49
150	20	0.5	2.33 ± 0.16	17.8 ± 4.6	219 ± 27	0.852 ± 0.029	1.24 ± 0.15	0.109 ± 0.011	0.0533 ± 0.0075	617 ± 37
123	20	1.0	2.32 ± 0.12	14.2 ± 2.8	199 ± 19	0.855 ± 0.042	1.26 ± 0.12	0.108 ± 0.013	0.0520 ± 0.0064	606 ± 28
81	20	1.5	2.36 ± 0.13	14.7 ± 2.1	218 ± 21	0.878 ± 0.052	1.17 ± 0.19	0.109 ± 0.012	0.0522 ± 0.0042	597 ± 31
13	20	2.0	2.37 ± 0.15	16.8 ± 2.4	205 ± 31	0.839 ± 0.112	1.17 ± 0.11	0.103 ± 0.015	0.0517 ± 0.0039	586 ± 24
166	40	0.5	2.34 ± 0.25	16.9 ± 4.1	218 ± 16	0.849 ± 0.030	1.13 ± 0.17	0.108 ± 0.017	0.0510 ± 0.0043	598 ± 58
111	40	1.0	2.44 ± 0.29	16.6 ± 5.4	216 ± 27	0.877 ± 0.056	1.18 ± 0.12	0.109 ± 0.011	0.0530 ± 0.0038	613 ± 10
51	40	1.5	2.36 ± 0.09	14.2 ± 1.8	208 ± 17	0.826 ± 0.008	1.14 ± 0.07	0.112 ± 0.006	0.0535 ± 0.0044	586 ± 41
7	40	2.0	2.23 ± 0.10	16.4 ± 3.9	209 ± 25	0.817 ± 0.018	1.07 ± 0.14	0.106 ± 0.015	0.0527 ± 0.0040	602 ± 18
170	60	0.5	2.41 ± 0.36	14.5 ± 1.3	198 ± 4	0.866 ± 0.053	1.16 ± 0.26	0.110 ± 0.016	0.0519 ± 0.0064	607 ± 21
86	60	1.0	2.32 ± 0.30	15.9 ± 5.0	219 ± 19	0.860 ± 0.038	1.18 ± 0.17	0.112 ± 0.006	0.0525 ± 0.0045	599 ± 39
65	60	1.5	2.41 ± 0.05	17.1 ± 5.7	216 ± 33	0.879 ± 0.077	1.17 ± 0.13	0.110 ± 0.008	0.0537 ± 0.0064	608 ± 14
15	60	2.0	2.34 ± 0.27	15.6 ± 4.3	218 ± 15	0.862 ± 0.091	1.23 ± 0.17	0.104 ± 0.010	0.0555 ± 0.0034	599 ± 18

Vial	Temp. (°C)	Time, month	La	Na (%)	Rb	Sc	Se	Th	Zn
104	−20	2.0	0.73 ± 0.06	1.92 ± 0.09	4.50 ± 0.61	0.191 ± 0.012	4.43 ± 0.33	0.263 ± 0.027	118.2 ± 7.3
150	20	0.5	0.78 ± 0.10	1.99 ± 0.11	4.46 ± 0.69	0.187 ± 0.006	4.35 ± 0.18	0.260 ± 0.025	117.4 ± 10.4
123	20	1.0	0.69 ± 0.08	1.82 ± 0.14	4.64 ± 0.49	0.188 ± 0.013	4.56 ± 0.10	0.250 ± 0.017	116.2 ± 5.4
81	20	1.5	0.70 ± 0.17	2.03 ± 0.19	4.74 ± 0.82	0.193 ± 0.010	4.59 ± 0.32	0.262 ± 0.027	116.4 ± 2.2
13	20	2.0	0.77 ± 0.18	1.86 ± 0.52	4.29 ± 0.65	0.191 ± 0.011	4.47 ± 0.29	0.277 ± 0.069	112.9 ± 8.4
166	40	0.5	0.73 ± 0.03	1.95 ± 0.15	4.53 ± 0.45	0.189 ± 0.006	4.43 ± 0.24	0.251 ± 0.011	115.3 ± 11.1
111	40	1.0	0.70 ± 0.17	1.97 ± 0.39	4.76 ± 0.36	0.195 ± 0.015	4.42 ± 0.19	0.271 ± 0.019	119.9 ± 1.5
51	40	1.5	0.74 ± 0.04	1.91 ± 0.24	4.73 ± 0.51	0.188 ± 0.007	4.29 ± 0.34	0.254 ± 0.022	114.4 ± 9.4
7	40	2.0	0.72 ± 0.09	1.92 ± 0.18	4.27 ± 0.39	0.182 ± 0.011	4.14 ± 0.15	0.240 ± 0.022	115.3 ± 8.0
170	60	0.5	0.68 ± 0.13	1.88 ± 0.10	4.65 ± 0.74	0.195 ± 0.012	4.40 ± 0.22	0.267 ± 0.027	119.7 ± 8.1
86	60	1.0	0.71 ± 0.14	1.94 ± 0.11	4.65 ± 0.55	0.188 ± 0.018	4.45 ± 0.14	0.251 ± 0.027	115.4 ± 7.4
65	60	1.5	0.76 ± 0.20	1.95 ± 0.26	4.91 ± 0.60	0.197 ± 0.010	4.54 ± 0.40	0.270 ± 0.021	117.0 ± 3.7
15	60	2.0	0.75 ± 0.13	2.03 ± 0.25	4.54 ± 0.47	0.198 ± 0.027	4.30 ± 0.28	0.265 ± 0.057	117.6 ± 4.2

Mean result and confidence interval at 95% confidence level for $n = 4$

Table 2 ANOVA table for the short term stability test

Element	Variation source	MS	F	p-value	F_c^a
Ag	Between	0.0105	0.586	0.840	2.010
	Within	0.0179			
As	Between	6.113	1.000	0.467	2.010
	Within	6.110			
Br	Between	236	1.249	0.287	2.010
	Within	189			
Co	Between	0.00151	1.128	0.366	2.010
	Within	0.00134			
Cr	Between	0.0123	1.175	0.334	2.010
	Within	0.0105			
Cs	Between	0.0000298	0.459	0.927	2.010
	Within	0.0000649			
Eu	Between	0.00000662	0.620	0.812	2.010
	Within	0.00001068			
Fe	Between	340	0.749	0.696	2.010
	Within	454			
La	Between	0.00395	0.611	0.820	2.010
	Within	0.00647			
Na	Between	0.0212	0.537	0.877	2.010
	Within	0.0395			
Rb	Between	0.136	1.032	0.440	2.010
	Within	0.132			
Sc	Between	0.0000787	1.120	0.373	2.010
	Within	0.0000703			
Se	Between	0.0615	2.312	0.024	2.010
	Within	0.0266			
Th	Between	0.000443	1.073	0.408	2.010
	Within	0.000413			
Zn	Between	16.2	0.768	0.678	2.010
	Within	21.1			

[a] F_c for $\alpha = 0.05$; $v_1 = 12$; $v_2 = 39$

from As (at 60 °C and 0.5 month exposure period) and Cr (at 20 °C and 1.0 month exposure period), the obtained ratios 1 are comprised between the respective $R_T - U_T$ and $R_T + U_T$ ranges, and hence, no instability can be concluded. The observed variations for As and Cr seem to be more related to a possible lack of precision of the method than to any noticeable degradation of the material. As an illustration, Fig. 2 shows the R_T values for selected elements (uncertainties are not shown for clarity). The results obtained for Ag and Fe is the pattern for most elements. Even though Se failed the ANOVA test at 95% confidence level, no significant degradation was observed from its plot. The plot for As shows that this element was the most adverse where stability is concerned. Even though As may be unstable in reference material due to degradation or losses of its organic species [7], the pattern of the graph, including the behavior for the vials kept at 60 °C led the authors to the conclusion that the observed variations are related to poor precision and not to any degradation during the test period.

Conclusion

With the application of the INAA method to the isochronous short time stability test it was possible to confirm that there was no systematic changes in concentration for 15 elements during the time period of this study. It was concluded that the candidate mussel reference material is stable enough to be transported under normal transport conditions without any significant changes in composition for the determined elements.

Table 3 Normalized results of the short term stability test obtained by INAA

Element	Temperature (°C)	$R_T \pm U_T$			
		0.5 month	1.0 month	1.5 months	2.0 months
Ag	20	0.991 ± 0.070	0.985 ± 0.064	1.002 ± 0.061	1.004 ± 0.070
	40	0.991 ± 0.087	1.034 ± 0.095	0.999 ± 0.061	0.946 ± 0.059
	60	1.024 ± 0.111	0.986 ± 0.098	1.021 ± 0.058	0.992 ± 0.092
As	20	1.03 ± 0.24	0.82 ± 0.17	0.85 ± 0.16	0.97 ± 0.18
	40	0.98 ± 0.22	0.96 ± 0.25	0.83 ± 0.15	0.95 ± 0.21
	60	0.84 ± 0.15	0.92 ± 0.24	0.99 ± 0.26	0.90 ± 0.22
Br	20	1.059 ± 0.087	0.964 ± 0.065	1.057 ± 0.071	0.992 ± 0.099
	40	1.053 ± 0.058	1.045 ± 0.087	1.009 ± 0.059	1.011 ± 0.082
	60	0.958 ± 0.028	1.060 ± 0.065	1.048 ± 0.104	1.058 ± 0.054
Co	20	1.009 ± 0.050	1.013 ± 0.055	1.040 ± 0.060	0.993 ± 0.094
	40	1.005 ± 0.050	1.038 ± 0.062	0.978 ± 0.044	0.967 ± 0.045
	60	1.025 ± 0.060	1.019 ± 0.053	1.041 ± 0.073	1.021 ± 0.081
Cr	20	1.14 ± 0.15	1.16 ± 0.14	1.07 ± 0.16	1.07 ± 0.13
	40	1.04 ± 0.15	1.09 ± 0.13	1.05 ± 0.12	0.98 ± 0.13
	60	1.06 ± 0.19	1.08 ± 0.15	1.07 ± 0.13	1.13 ± 0.15
Cs	20	1.02 ± 0.13	1.01 ± 0.14	1.03 ± 0.14	0.97 ± 0.14
	40	1.02 ± 0.15	1.03 ± 0.13	1.05 ± 0.13	1.00 ± 0.14
	60	1.03 ± 0.15	1.05 ± 0.12	1.03 ± 0.13	0.98 ± 0.13
Eu	20	1.023 ± 0.117	0.998 ± 0.106	1.002 ± 0.089	0.991 ± 0.086
	40	0.979 ± 0.088	1.017 ± 0.087	1.025 ± 0.091	1.011 ± 0.088
	60	0.995 ± 0.106	1.006 ± 0.091	1.030 ± 0.108	1.065 ± 0.088
Fe	20	1.024 ± 0.065	1.006 ± 0.059	0.989 ± 0.060	0.971 ± 0.055
	40	0.991 ± 0.079	1.017 ± 0.053	0.974 ± 0.065	0.998 ± 0.054
	60	1.007 ± 0.056	0.994 ± 0.065	1.008 ± 0.062	0.994 ± 0.054
La	20	1.07 ± 0.10	0.95 ± 0.09	0.95 ± 0.15	1.05 ± 0.17
	40	1.00 ± 0.06	0.96 ± 0.15	1.01 ± 0.07	0.98 ± 0.10
	60	0.93 ± 0.12	0.97 ± 0.13	1.04 ± 0.18	1.02 ± 0.13
Na	20	1.035 ± 0.046	0.948 ± 0.053	1.057 ± 0.067	0.965 ± 0.171
	40	1.014 ± 0.056	1.026 ± 0.129	0.992 ± 0.084	1.000 ± 0.065
	60	0.976 ± 0.041	1.009 ± 0.046	1.016 ± 0.090	1.054 ± 0.085
Rb	20	0.99 ± 0.13	1.03 ± 0.11	1.05 ± 0.14	0.95 ± 0.12
	40	1.01 ± 0.11	1.06 ± 0.10	1.05 ± 0.11	0.95 ± 0.10
	60	1.03 ± 0.14	1.03 ± 0.12	1.09 ± 0.12	1.01 ± 0.11
Sc	20	0.982 ± 0.044	0.984 ± 0.059	1.010 ± 0.053	1.003 ± 0.054
	40	0.989 ± 0.044	1.022 ± 0.064	0.986 ± 0.046	0.956 ± 0.052
	60	1.022 ± 0.058	0.986 ± 0.070	1.033 ± 0.054	1.036 ± 0.099
Se	20	0.983 ± 0.053	1.029 ± 0.051	1.036 ± 0.067	1.009 ± 0.063
	40	1.001 ± 0.059	0.997 ± 0.055	0.967 ± 0.067	0.934 ± 0.049
	60	0.993 ± 0.057	1.004 ± 0.052	1.025 ± 0.075	0.970 ± 0.061
Th	20	0.990 ± 0.087	0.952 ± 0.072	0.995 ± 0.090	1.055 ± 0.178
	40	0.955 ± 0.066	1.032 ± 0.079	0.967 ± 0.081	0.911 ± 0.079
	60	1.017 ± 0.090	0.955 ± 0.088	1.027 ± 0.082	1.009 ± 0.150
Zn	20	0.993 ± 0.067	0.983 ± 0.048	0.985 ± 0.040	0.955 ± 0.058
	40	0.975 ± 0.070	1.014 ± 0.040	0.967 ± 0.062	0.976 ± 0.057
	60	1.013 ± 0.058	0.976 ± 0.055	0.990 ± 0.043	0.995 ± 0.044

R_T and U_T calculated according to Eqs. 1 and 2

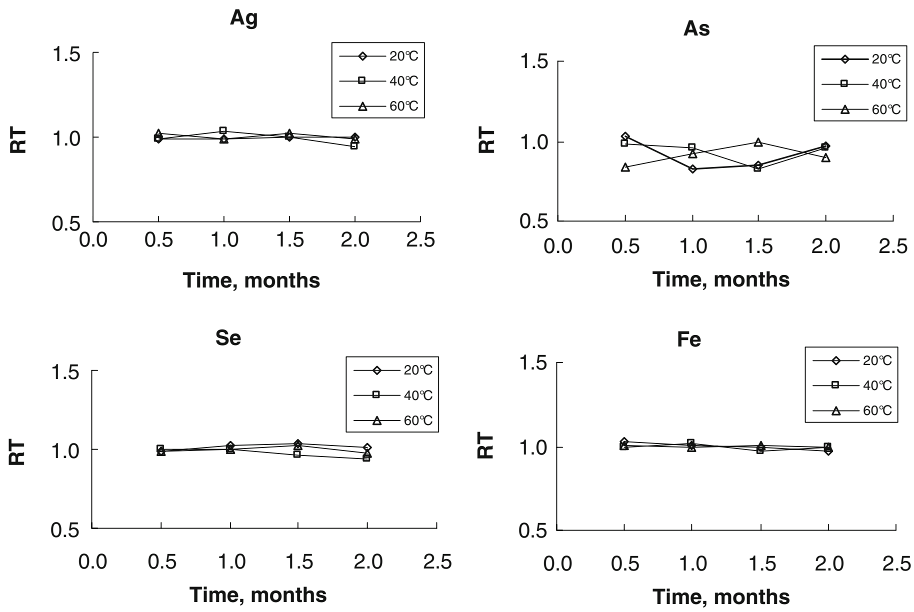

Fig. 2 R_T ratios for the short term stability test of selected elements

Acknowledgements This study was undertaken with financial support from FAPESP and CNPq.

References

1. Gosling E (2003) Bivalve molluscs—biology, ecology and culture. Fishing New Books, Oxford
2. Zschunke A (ed) (2000) Reference materials in analytical chemistry—a guide for selection and use. Springer, Berlin
3. ISO, International Organization of Stantardization (2006) Certification of reference materials—general and statistical principles—ISO Guide 35. 3rd edn, Geneva
4. Lamberty A, Schimmel H, Pauwels J (1997) Fresenius J Anal Chem 360:359
5. IAEA, International Atomic Energy Agency (2003) World-wide intercomparison exercise for the determination of trace elements and methylmercury in fish homogenate. IAEA-407, Monaco
6. Moreira EG, Vasconcellos MBA, Maihara VA, Catharino MGM, Saiki M (2007) J Braz Soc Ecotoxicol 2:61
7. Greenberg RR (2005) SIM 8.16P—toxic metals in seafood—evaluation of measurement results, toxic metals in seafood workshop. SIM General Assembly, Trinidad Tobago