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1 **Assessment of metal, metalloid and radionuclide bioaccessibility from mussels to**
2 **human consumers, using centrifugation and simulated digestion methods coupled**
3 **with radiotracer techniques**

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25 ABSTRACT: The dietary bioaccessibility of 7 elements (²⁴¹Am, Cd, Co, Cs, Mn, Se and Zn) in
26 the Mediterranean mussels *Mytilus galloprovincialis* (Lamarck, 1819) was assessed for human
27 consumers. In this respect we assessed and compared the proportion of elements associated with
28 the cellular cytosolic (“soluble”) fraction vs. the bioaccessible fraction derived, respectively,
29 from (1) differential centrifugation method and (2) simulated digestion method. Comparisons
30 were carried out on both raw and cooked mussels. Results showed that (1) the centrifugation
31 method systematically underestimated (up to a factor 4) element bioaccessibility in raw mussels
32 compared to the *in vitro* digestion method (e.g., 10 vs. 42% for ²⁴¹Am), and (2) the cooking
33 process (5 min at 200°C) lead to concentrating the elements in mussel tissues (e.g., by a factor 2
34 for Zn) and reducing their bioaccessibility. Overall, the simulated *in vitro* digestion method
35 appears as a powerful tool for seafood safety assessment and cooking could contribute in
36 reducing substantially the global trace element intake from mussel tissues (up to 65% for Cd and
37 Cs).

38
39
40 **Keywords:** Bioavailability; seafood; digestion simulation; differential centrifugation; seafood
41 safety

42

43 **Statement (according to requirements indicated in “EES Instructions to Authors”).**

44

45 Funding sources that supported the work described in the manuscript were from IAEA-MEL
46 regular budget.

47

48 The present work was mainly carried out using *in vitro* techniques; however it also involved the
49 use of marine bivalves. All experimental studies were conducted in accordance with national and
50 institutional guidelines for the protection of animal welfare.

51

52 **1. Introduction**

53

54 Among seafood, bivalves generally display a high capacity in bioaccumulating metals, which
55 suggests that the risk for their human consumers may not be negligible (e.g., Chauvelon et al.,
56 2008; Metian et al., 2008). This risk depends primarily on the dietary bioavailable fraction of
57 metal (viz., the fraction that is actually assimilated from the food and can reach the systemic
58 circulation of an organism), that depends itself on the metal speciation within the seafood soft
59 tissues. Before the 1990's, it was generally assumed that the bioavailable fraction was reliably
60 assessed by the metal content of the cytosolic ("soluble") fraction in the cells. Later on, studies
61 on the bioavailability of metals focusing on the "insoluble" fraction of the cells showed that the
62 use of the cytosolic fraction alone underestimated the fraction of the metals that was bioavailable
63 to the higher trophic levels. Nowadays, it is considered that the metals contained in both cytosolic
64 and organelles fractions better reflect the bioavailable fraction (Wallace and Lopez, 1996;
65 Wallace and Luoma, 2003). More recently, simulated digestion methodologies were developed to
66 provide a more realistic assessment of the dietary bioavailable fraction of contaminants (Oomen
67 et al., 2003; Versantvoort et al., 2005; Amiard et al., 2008).

68 The objective of the present study was to assess the dietary bioaccessible fraction (viz., the
69 fraction resulting from the digestive process that can potentially be assimilated by the organism)
70 of selected elements from mussels to human consumers, using two different methods, i.e.,
71 differential centrifugation and *in vitro* simulated digestion. Both methods were coupled with
72 highly sensitive radiotracer techniques by using the corresponding γ -emitting radiotracers of the
73 selected elements, i.e., four metals (Cd, Co, Cs, Mn, Zn), one metalloid (Se) and one artificial
74 radionuclide (^{241}Am). It is noteworthy that γ -emitting radiotracers of Co and Cs are also
75 radionuclides commonly associated with nuclear industry wastes and fallout from nuclear

76 weapon testing. The present work also investigated the effect of cooking (raw vs. cooked
77 mussels) on the metal content in the mussel soft tissues and on the resulting change in dietary
78 bioaccessibility.

79

80 **2. Material and Methods**

81

82 Individuals of the Mediterranean mussel, *Mytilus galloprovincialis* (n = 32; 4-6 cm length), were
83 collected along the coast of the Principality of Monaco and exposed for 3 weeks to the 7 selected
84 radiotracers (^{54}Mn , ^{60}Co , ^{65}Zn , ^{75}Se , ^{109}Cd , ^{134}Cs and ^{241}Am) dissolved in natural 0.45- μm filtered
85 seawater (closed-circuit 70-l aquarium; salinity: 36 p.s.u.; temperature: $21 \pm 0.5^\circ\text{C}$; pH: 8.0 ± 0.1 ;
86 light/dark cycle: 12h/12h). Activity of the radiotracers measured in seawater over the exposure
87 duration was: 0.23 kBq $^{54}\text{Mn l}^{-1}$, 0.48 kBq $^{60}\text{Co l}^{-1}$, 0.58 kBq $^{65}\text{Zn l}^{-1}$, 0.31 kBq $^{75}\text{Se l}^{-1}$, 1 kBq
88 $^{109}\text{Cd l}^{-1}$, 0.58 kBq $^{134}\text{Cs l}^{-1}$ and 0.14 kBq $^{241}\text{Am l}^{-1}$. These radiotracer additions corresponded to
89 stable metal concentrations of 1.1 pmol Mn l^{-1} , 0.6 pmol Co l^{-1} , 30 pmol Zn l^{-1} , 7 pmol Se l^{-1} , 0.3
90 pmol Cd l^{-1} , 11 pmol Cs l^{-1} , i.e., concentrations more than two orders of magnitude lower than the
91 background concentrations in open seas (Ward, 2000). During this period, scallops were fed
92 twice daily a phytoplankton diet (*Isochrysis galbana*; $5 \cdot 10^4$ cells ml^{-1}) and the seawater and spike
93 were renewed every day for one week then every two days (Hédouin et al., 2006). After the
94 exposure, mussels were placed in clean flowing seawater for 2 days in order to remove the
95 radiotracers loosely bound to the mussels and to clear the gut contents (Metian et al., 2007).

96 A subsample of the mussels (n = 16) was then collected and kept raw whereas the remaining
97 individuals (n = 16) were placed individually in 50-ml glass beakers, covered and heated for 5
98 min at 200°C on a hot plate. This temperature of cooking was selected in order to reflect a

99 intermediate temperature between relative low cooking temperatures (~100°C in boiling water)
100 and high ones (250 to 350°C with oven or barbecue).

101 In order to determine the possible effect of cooking on the radiotracer contents in the mussels,
102 tissues and fluids of 8 raw and 8 cooked mussels were radioanalyzed. Gamma-counting was
103 carried out according to the method described in Rodriguez y Baena et al. (2006), using a high-
104 resolution γ -spectrometry system consisting of four coaxial Germanium (N- or P-type) detectors
105 (EGNC 33-195-R, Canberra[®] and Eurysis[®]) connected to a multi-channel analyzer and a
106 computer loaded with a spectra analysis software (Interwinner[®] 6).

107 For the remaining mussels (n = 8 for both cooked and raw individuals), the whole soft tissues
108 were treated (1) by differential centrifugation according to the method described in Bustamante
109 and Miramand (2005) to isolate the cellular cytosolic fraction by ultracentrifugation (28,000 G
110 for 1 h, using a Sorvall RC28S ultracentrifuge) or (2) according to the *in vitro* simulated
111 digestion method as described by Versantvoort et al. (2005). Briefly, this latter method consists
112 of a three-step procedure which simulates quite closely the human digestive processes occurring
113 in the mouth, stomach and small intestine (Versantvoort et al., 2005; Brandon et al., 2006;
114 Amiard et al., 2008). The food matrix was first minced at 4°C and exposed to artificial saliva at
115 pH 6.8 for 5 min. Artificial gastric juice at pH 1.3 was then added for 2 h and finally a mixture of
116 artificial duodenal juice, bile and HCO₃ at pH 8.1–8.2 was added for a further 2 h. The incubation
117 temperature was 37.2 ± 0.2°C. Chemicals and enzymes used are the same as described in
118 Versantvoort et al. (2005) and Amiard et al. (2008) and were purchased from Sigma[®]. After the
119 *in vitro* digestion, the resulting chyme was centrifuged at 1,572 G for 15 min at 37.2 ± 0.2°C.
120 According to Versantvoort et al. (2005), the elements in the supernatant are representative of
121 those occurring in the food (i.e. the mussels) and that are bioaccessible to humans (i.e. the

122 fraction that can be absorbed by the human gut enterocytes). Pellets and supernatants from both
123 treatments (centrifugation and simulated digestion) were radioanalyzed in order to determine the
124 activity of the 7 radiotracers.

125

126 **3. Results**

127

128 The overall results of the subcellular fractioning and simulated *in vitro* digestion methods showed
129 that the bioaccessible fraction of the metals in mussels varied from 10% (^{241}Am in raw mussels)
130 to 92% (^{134}Cs in raw mussels) (Table 1). However, for all the studied elements, the bioaccessible
131 fraction determined using the *in vitro* digestion method was always higher (by a factor of up to 4)
132 than the one assessed using the differential centrifugation method (Table 1).

133 Examination of Table 2 indicates that the cooking process resulted in concentrating
134 systematically all elements but ^{134}Cs in the mussel soft tissues (by 20 to 70%) and in releasing a
135 significant amount of all elements but ^{241}Am in the cooking juice.

136 It was also observed that although cooking increased the element concentration in the mussel
137 flesh, the elements remaining in the cooked flesh were less bioaccessible than when occurring in
138 raw tissues, in particular for Cd, Se, and Zn (Table 1).

139

140 **4. Discussion**

141

142 The results of our study show clearly that the bioaccessible fraction determined using the
143 simulated *in vitro* digestion method was systematically higher (by a factor of up to 4) than when
144 assessed using the differential centrifugation method (see Table 1). In particular, the latter
145 method considerably underestimated the bioaccessibility of ^{241}Am , Co, Mn, Se and Zn. This

146 observation was not that surprising as, from the theory, it was expected that the differential
147 centrifugation method would provide a lower bioaccessibility estimate since it is only related to
148 the cytosolic subcellular fraction (Wallace and Luoma, 2003). In contrast, by definition, the
149 simulated *in vitro* digestion method would allow assessing the global metal fraction which is
150 bioaccessible, regardless of the subcellular partitioning of the elements. By using a simplified
151 empirical digestion simulation (acetic acid solution at pH 4), Bragigand et al. (2004) had already
152 shown that some metals (Ag, Cd, Cu and Zn) could be bioavailable from the insoluble subcellular
153 fraction of oyster cells. The *in vitro* digestion previously developed by Oomen et al. (2003) and
154 Versantvoort et al. (2005) and used in the present study is a step forward to evaluate
155 bioaccessibility of metals from seafood products by humans as it mimics quite closely the
156 conditions occurring all along the human digestive tract (constant temperature, succession of
157 enzymatic activities and pH, corresponding to each digestive step occurring from the mouth to
158 the intestine).

159 The analysis of radiotracer content in raw and cooked mussels demonstrated that the cooking
160 process resulted in concentrating by 20 to 70% most of the studied elements due to the loss of
161 moisture as well as in releasing metals into the cooking juice. In particular, the cooking juice
162 displayed metal concentrations higher by up to one order of magnitude than in the inter-valve
163 fluid of raw mussels (see Table 2), corresponding to similar differences in terms of metal load
164 (both cooking juice and inter-valve fluid were of similar volume).

165 Nevertheless, the comparison of our data with those previously published showed that a trend can
166 hardly be generalized regarding the cooking effect on the metal content in seafood. Indeed, while
167 some elements were found to concentrate in seafood after cooking (e.g. total and inorganic As in
168 fish and molluscs; Devesa et al., 2001), other elements were not (e.g. Cd, Cu, Pb and Zn in the
169 fish *Tilapia nilotica*; Atta et al., 1997). Furthermore, the element concentration in tissues after

170 cooking appears to depend on (1) the species considered (e.g. ^{134}Cs is not concentrated in cooked
171 mussel tissues whereas it is in fish tissues; present study and Burger et al., 2004, respectively),
172 (2) the element considered and its chemical speciation (Devesa et al., 2001), and (3) the type of
173 cooking (Ersoy et al., 2006). Although the aforementioned factors are generally reported as
174 influencing the cooking effect, it has to be noted that the species-dependence factor could be
175 partly due to the difference in tissue consideration when different species are investigated.
176 Indeed, studies dealing with bivalves as ours generally consider the whole soft tissues as edible
177 target, whereas fish related studies generally consider only the muscle tissues (fillet). Therefore,
178 differences in the nature of the tissues (e.g., protein composition and moisture content) and in
179 metal interactions with the cellular components in different tissues could lead to contrasting
180 results. This is particularly true when storage organs such as liver / hepatopancreas or kidneys are
181 considered. Indeed these organs are the main sites where detoxification processes take place,
182 which usually result in an increase of excretion capacity or, more generally, in an increase in
183 sequestration capacity and thus in different binding strength of the metals with cellular
184 components (e.g., Metian et al., 2005).

185 Finally, although cooking resulted in an increase in element concentration in whole mussel flesh,
186 it also appeared that the elements remaining in the cooked flesh were significantly less
187 bioaccessible than those occurring in the raw tissues. This was particularly obvious for Cd, Se
188 and Zn (see Table 1).

189 Considering the simulated *in vitro* digestion method and taking into account all the parameters
190 (i.e., change in weight and in metal concentration of the cooked flesh, change in bioaccessible
191 fraction), we have assessed that the metal intake for a consumer eating mussels would be reduced
192 by 25% for Mn, 35% for Zn, 40% for Co, 50% for Se and 65% for Cd and ^{134}Cs if the mussels
193 are previously cooked and the cooking juice discarded before consumption. This decrease in

194 metal intake would be even more important if the bioaccessibility assessed via the subcellular
195 fractioning method was considered (as the decrease in bioaccessibility between raw and cooked
196 mussels was more marked). However, the information provided by the latter method is probably
197 not directly comparable between raw and cooked mussels. Indeed, cooking of the flesh results in
198 changes/damages of the cellular structure (e.g., protein agglutination), which will affect
199 substantially the cytosol (both in its nature and occurrence) as well as the results of a
200 centrifugation approach and their meaning. Hence the use of the centrifugation fractioning
201 method is most probably relevant and informative only when the raw product is considered.

202

203 **5. Conclusion**

204

205 The simulated *in vitro* digestion method represents a powerful tool for the safety assessment of
206 commercially important seafood products. Our study indicates that the bioaccessible fraction of
207 metals in mussel soft tissues for human consumers depends on the metal chemical properties (e.g.
208 distribution among cellular components and binding properties) and on the preparation of this
209 seafood (in the present case, raw vs. cooked at 200°C for 5 min). The present work also showed
210 that the cooking process generally concentrated the elements in mussel soft tissues, decreased
211 their bioaccessibility for the consumers and released a significant part of the elements into the
212 cooking juice. Results indicated that, providing the cooking juice is discarded, consumption of
213 cooked mussels can contribute in reducing significantly the global intake of trace elements from
214 seafood in humans (by as much as 65% for Cd and ¹³⁴Cs). Further studies are required on seafood
215 containing norm-exceeding levels of trace elements in order to assess whether the risk for
216 consumers could be decreased in cooked products down to levels allowing safe marketing. This

217 could be of particular interest in the case of Cd for which the maximum concentration allowed in
218 marketed bivalves is low ($1 \mu\text{g g}^{-1}$ wet wt; EC, 2006).

219

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221

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229

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231

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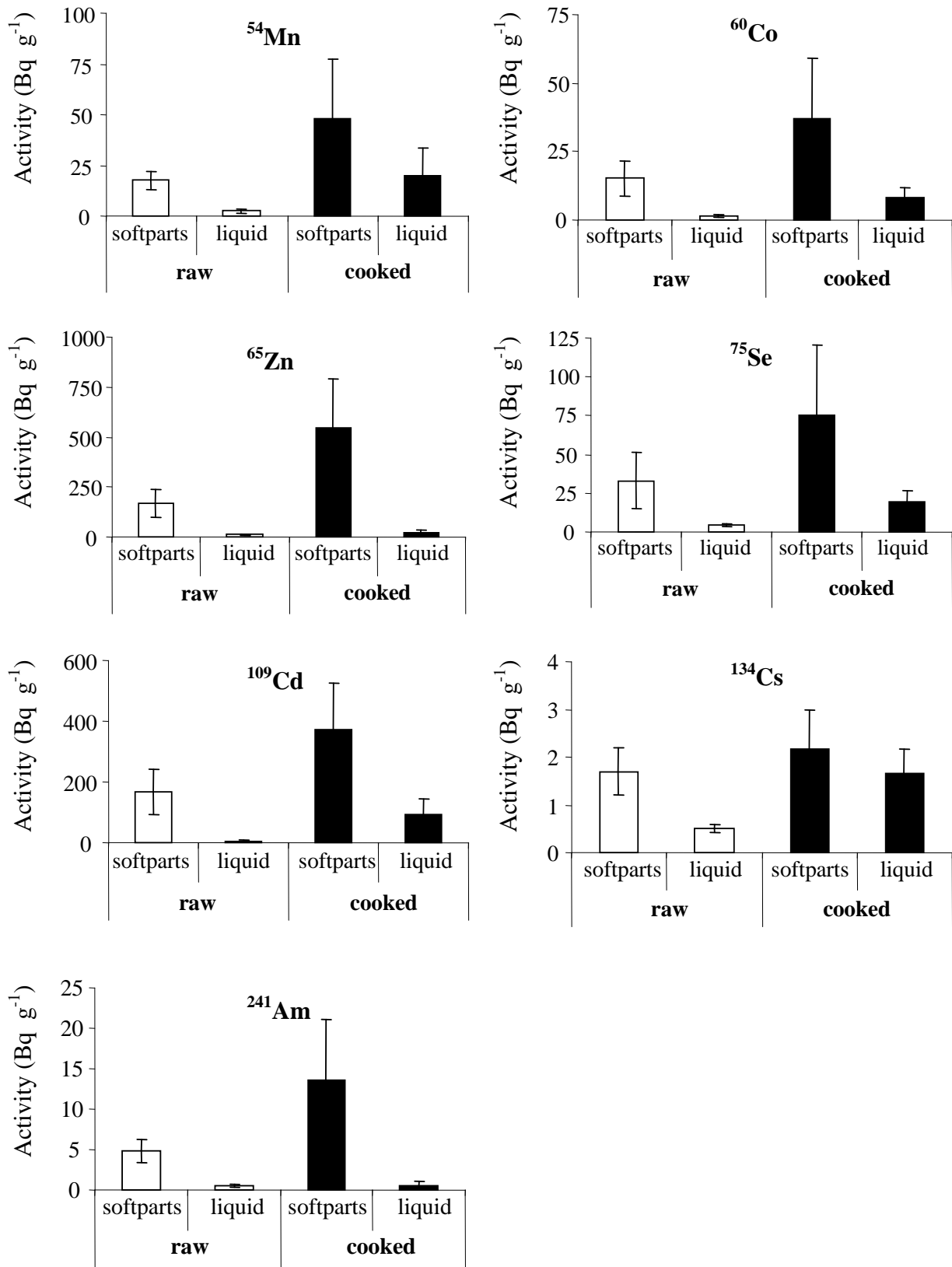


Figure 1. Repartition of radiotracer activities (Bq g⁻¹) between tissues and liquid of the mussel *Mytilus galloprovincialis* before and after a cooking process.

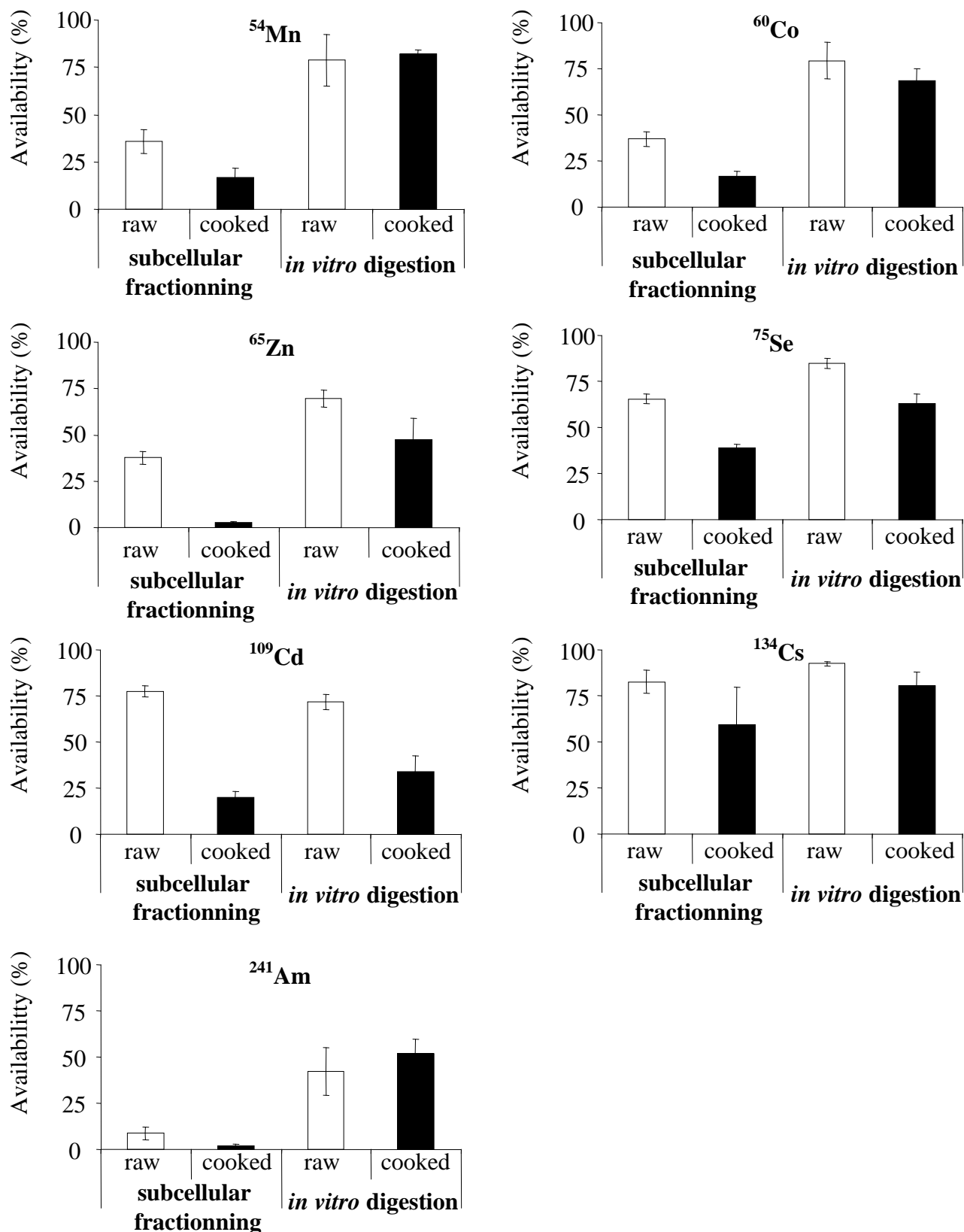


Figure 2. Radiotracer availabilities (% of initial activity in tissues) for mussel consumers following either subcellular or digestion methods (with or without the cooking process).

Table 1. Radiotracer bioaccessibility (%; mean \pm SD, n = 8) from mussel soft tissues (raw or cooked) to human consumers, assessed using either differential centrifugation fractioning method or simulated *in vitro* digestion method.

| Mussel preparation | Method for assessing bioaccessibility | ^{54}Mn | ^{60}Co | ^{65}Zn | ^{75}Se | ^{109}Cd | ^{134}Cs | ^{241}Am |
|---------------------------|--|------------------------------------|------------------------------------|------------------------------------|------------------------------------|-------------------------------------|-------------------------------------|-------------------------------------|
| Raw | fractioning | 36 \pm 6 | 37 \pm 4 | 38 \pm 4 | 65 \pm 3 | 77 \pm 3 | 82 \pm 6 | 10 \pm 3 |
| | digestion | 79 \pm 13 | 79 \pm 10 | 70 \pm 5 | 85 \pm 3 | 72 \pm 4 | 92 \pm 1 | 42 \pm 13 |
| Cooked | fractioning | 17 \pm 5 | 17 \pm 3 | 3 \pm 1 | 39 \pm 2 | 20 \pm 3 | 59 \pm 21 | 2 \pm 1 |
| | digestion | 82 \pm 2 | 68 \pm 7 | 47 \pm 12 | 63 \pm 5 | 34 \pm 9 | 80 \pm 8 | 52 \pm 8 |

Table 2. Radiotracer activity (Bq g⁻¹ wet wt; mean ± SD, n = 8) in mussel soft tissues and fluid before and after cooking. Fluid: inter-valve fluid (in raw mussels) or cooking juice (in cooked mussels).

| Mussel preparation | Compartment | Weight (g wet wt) | ⁵⁴Mn | ⁶⁰Co | ⁶⁵Zn | ⁷⁵Se | ¹⁰⁹Cd | ¹³⁴Cs | ²⁴¹Am |
|---------------------------|--------------------|--------------------------|------------------------|------------------------|------------------------|------------------------|-------------------------|-------------------------|-------------------------|
| Raw | soft tissues | 2.4±0.7 | 41±10 | 34±9 | 379±74 | 73±27 | 366±64 | 4±0.6 | 11±2 |
| | fluid | 5.4±1.9 | 13±7 | 7±4 | 60±26 | 23±5 | 30±13 | 3±0.7 | 3±2 |
| Cooked | soft tissues | 1.3±0.5 | 54±21 | 42±17 | 648±152 | 86±34 | 450±133 | 3±0.6 | 15±5 |
| | fluid | 4.1±1.3 | 75±49 | 32±13 | 74±17 | 74±22 | 322±87 | 6±1 | 2±2 |