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Anodic stripping voltammetry with gold electrodes as an alternative method for the routine determination of mercury in fish. Comparison with spectroscopic approaches

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Abstract

The applicability to the determination of mercury in tuna of square wave anodic stripping voltammetry (SW-ASV) conducted at both solid gold electrode (SGE) and a gold nanoparticle-modified glassy carbon electrode (AuNPs-GCE) was demonstrated. Mercury content in two certified materials and in ten samples of canned tuna was measured. The performances of the electrodes were compared with one another as well as with two spectroscopic techniques, namely cold vapour atomic absorption spectroscopy (CV-AAS) and a direct mercury analyser (DMA). The results found pointed out that both SW-ASV approaches can be considered both suitable, easy and alternative methods to monitor mercury concentration in tunas, since they allowed to reach accurate quantification at concentration values lower than the maximum admissible level in this matrix ($[\text{Hg}] = 1 \text{ mg/kg}_{\text{wet weight, ww}}$). In particular, mercury detection at the AuNPs-GCE showed a LOQ in fish-matrix of $0.1 \mu\text{g/l}$, corresponding to $0.06 \text{ mg/kg}_{\text{ww}}$, with performance comparable to that of DMA.

Keywords: mercury, gold nanoparticle-modified glassy carbon electrode, solid gold electrode, direct mercury analyser, cold vapour atomic absorption spectroscopy

1 Introduction

30 Heavy metals are considered among the most alarming forms of pollution in the aquatic
31 environment because of their toxicity and accumulation by marine organisms. Fish absorb heavy
32 metals from the surrounding environment depending on a variety of factors such as the
33 characteristics of the species under consideration, metal concentrations in water and exposure
34 period, as well as abiotic factors such as temperature, salinity, pH, and seasonal changes (Ginsberg
35 & Toal, 2009). The distribution of metals varies among fish species, since it depends on age,
36 development status and other physiological factors. Tuna, as a top predator, being able to
37 concentrate high amounts of heavy metals are also used for biomonitoring of environmental
38 contamination (Has-Schon, Bogut, & Strelec, 2006).

39 Several metals, such as iron, copper, zinc, and manganese play an essential role in biological
40 systems; other elements, as mercury, lead, and cadmium are toxic, even in trace amounts, and they
41 have been included in the regulations of the European Union for contaminants in foodstuffs EC
42 (Commission Regulation, 2008). Mercury, in particular, is a known toxicant which is present in the
43 environment as a result of both natural processes and anthropogenic activities. Fish accumulate
44 considerable amount of this metal in their tissues and, in fact, the primary source of mercury
45 contamination in man is through eating fish (Inskip & Piotrowsiki, 1995).

46 In the last years, the consumption of fish has increased in importance due to the high protein supply,
47 and low saturated fat and omega fatty acids content that are known to contribute to good health.
48 Consequently, the health risk associated with consumption of fish contaminated by heavy metals is
49 an important global concern. The techniques generally adopted for Hg determination are cold
50 vapour atomic absorption spectroscopy (CV-AAS) (Souza-Araujo, Giarrizzo, & Lima, 2015), cold
51 vapour atomic fluorescence spectroscopy (CV-AFS) (Fricke, Götz, Schleyer, & Püttmann, 2015)
52 and inductively coupled plasma mass spectrometry (ICP-MS) (Sasmaz, Akgül, Yıldırım, & Sasmaz,
53 2016). These methods are well established, but they are affected by several drawbacks, such as
54 lengthy analysis times, the use of expensive equipment, the lack of multi elemental analysis, the
55 incapacity for speciation studies (Bagheri, Afkami, Saber-Tehrani & Khoshshafar, 2012) and they
56 cannot be used for field-analysis. Furthermore, several complex steps must be performed, and these
57 require specially trained personnel. Thus, the availability of simple, inexpensive and rapid methods
58 suitable for the routine determination of mercury in food samples is highly desirable.

59 Recently, new devices for direct mercury analysis have been developed, which automatically
60 perform both sample decomposition and Hg detection by AAS, with short analysis times and low
61 limit of quantitation, LOQ = 0.010 mg/kg wet weight) (Squadrone, Benedetto, Brizio, Prearo, &
62 Abete, 2015).

63 Also electrochemistry offer quite attractive routine analysis capabilities, because it is sensitive,
64 inexpensive, simple, fast and can be performed with miniaturized, portable instrumentation. Several
65 electrochemical methods have been developed for mercury determination in different matrices,
66 especially in water (Martín-Yerga, González-García & Costa-García, 2013). Focusing the attention
67 to fish analysis, most of them are based on the preconcentration of Hg onto the working electrode
68 and subsequent stripping, primarily using anodic stripping voltammetry (ASV). In particular, the
69 U.S. Environmental Protection Agency (USEPA) has recommended the use of stripping
70 voltammetry for mercury analysis (EPA, Method 7472, 1996).

71 Even if voltammetric methods are the most commonly reported, some potentiometric methods have
72 also been developed for Hg determination (Clevenger, Smith, & Winefordner, 1997). Other authors
73 suggest the use of anodic stripping chronopotentiometry—because of the low contribution of
74 capacitive current—to the measured signal in comparison to ASV (Kurmaz & Gulyai, 2010).

75 Some reported ASV methodologies are based on the use of simple bare electrodes as working
76 electrode (WE), especially based on carbon and gold. Interestingly, gold thin-layer electrodes made
77 from compact discs (CD-ROM) were employed successfully for mercury determination in fish after
78 a digestion step (Radulescu & Danet, 2008). Unfortunately, solid electrodes suffer from memory
79 effects resulting from the difficult removal of mercury from their surface (Martín-Yerga, González-
80 García & Costa-García, 2013). Therefore, reusing the electrode without interferences remains an
81 important challenge to obtain a WE that fulfils the requirements for routine analysis in accredited
82 laboratories. With this purpose a large number of modified WEs have been studied. Screen-printed
83 carbon electrodes (SPCEs) modified with gold films have been employed and have achieved LODs
84 as low as 0.9 µg/L (Meucci, Laschi, Minunni, Pretti, Intorre, Soldani, & Mascini, 2009). Tamer et
85 al. used a platinum electrode modified with poly(3-hexylthiophene) (Tamer, Oymak & Ertaş, 2007).

86 Also carbon paste electrodes (CPEs) modified with several species able to complex and
87 preconcentrate Hg(II) have been used. For example, CPEs have been modified with several species
88 able to complex and preconcentrate Hg(II). Afkhani et al. modified a CPE with nitrobenzoyl
89 diphenylmethylenphosphorane (N-BDMP) for the simultaneous determination of Cd²⁺ and Hg²⁺ in
90 various samples, fish included (Afkhani, Madrakian, Sabounchei, Rezaei, Samiee, & Pourshahbaz,
91 2012). Also different silica species functionalized with complexing groups have been employed for
92 the modification of GCE and CPEs, For example, fish analysis was performed using silica
93 nanoparticles functionalized with a Schiff base (Afkhani, Madrakian, Ghaedi, Rezaeivala, 2013).

94 The same authors also used a CPE modified with multi-walled carbon nanotubes (MWCNTs) and
95 3-(4-methoxybenzylideneamino)-2-thioxothiazolidin-4-one, as a new synthesized Schiff base, for
96 the simultaneous determination of Pb(II) and Hg(II) by ASV in several samples, including tuna fish

97 (Afkhami, Bagheri, Khoshshafar, Saber-Tehrani, Tabatabaee & Shirzadmehr, 2012). The unique
98 properties of nanotubes enhance the effect of the complexing agent improving the analytical signal
99 of this electrode in comparison to the previous example. Bagheri et al. developed a pasting binder
100 for CPE based on a triphenylphosphine-modified carbon nanotube composite with a room
101 temperature ionic liquid (RTIL) for sensitive and simultaneous determination of Cd(II), Pb(II) and
102 Hg(II) in fish (Bagheri, Afkhami, Khoshshafar, Rezaei, Shirzadmehr, 2013). Recently Ramenzani et
103 al. (Ramezani, Mashhadizadeh, Jalilian & Mehdi Aghilic, 2015) develop a CPE modified with
104 MWCNTs, AuNPs and 1,2-bis[5,2-thiolmethyl-sulphide-1,3,4-oxadiazol-2-yl]-ethane (BTMSOE)
105 as a novel ion-carrier: with this MWCNTs/AuNPs/BTMSOE-CPE they reached very low LOD also
106 in fish matrix.

107 Table 1 shows a comparison among the analytical performance of electrochemical methods applied
108 for mercury determination in fish published in the literature previously described.

109 Despite this numerous electroanalytical approaches reported in literature, EPA still suggests the use
110 of a GCE modified with a gold film, since the modification required is very simple and permits to
111 work with a renewable surface. However it is not possible to determine whether bare gold
112 electrodes and gold film electrodes exhibit significant differences in analytical performance
113 (Abollino, Giacomino, Malandrino, Piscionieri, & Mentasti, 2008, Martín-Yerga, González-García
114 & Costa-García, 2013).

115 In view to propose a technique suitable for routine analyses, cheap and easy to be applied, in this
116 study we tested the performance of ASV using a solid gold electrode (SGE) (Abollino, Giacomino,
117 Malandrino, Piscionieri, & Mentasti, 2008) and a home-made gold nanoparticle- modified glassy
118 carbon electrode (AuNPs-GCE), and assessed their applicability for mercury determination in fish.
119 The AuNPs-GCE, had previously been developed in our laboratory, and applied to the
120 quantification of Hg in the low ng/L range in aqueous solutions and in solid certified materials
121 having different matrices (Abollino, Giacomino, Ginepro, Malandrino & Zelano, 2012). The main
122 advantages of this type of electrode are i) the very easy procedure required for electrode
123 modification, ii) the possibility to work with a renewable surface (as in film electrodes), dissolving
124 the gold nanoparticle layer and depositing a new one when a worsening of the response is noticed,
125 and in addition, iii) the large surface area of the deposited nanoparticles, that gives rise to an
126 improvement of the analytical performance (in particular lower detection limit) in comparison to
127 conventional electrodes.

128 Two reference materials, namely *Tuna Fish BCR 463* and *Tuna Fish ISPRA T-22*, and ten
129 commercial samples of canned tunas (CTs) were analysed for mercury by ASV, using SGE and
130 AuNPs-GCE. The results were compared with those obtained with two spectroscopic techniques,

131 namely conventional CV-AAS and a direct mercury analyser (DMA), to better assess the
132 advantages and drawbacks of the electrochemical approach for Hg determination.

133 Finally, for the sake of completeness, we compared mercury concentrations found in the considered
134 CTs with the maximum admissible value for this element established by the European Legislation.

135

136 **2 Experimental**

137 *2.1 Apparatus and Reagents*

138 Digestions of samples were performed in polytetrafluoroethylene (PTFE) bombs, with a Milestone
139 MLS-1200 Mega microwave laboratory unit (Milestone, Sorisole, Italy).

140 Voltammetric analyses were performed with a PGSTAT 10 potentiostat (Eco Chemie, Utrecht, The
141 Netherlands) coupled to a 663 VA Metrohm (Herisau, Switzerland) stand equipped with an AuNPs-
142 GCE working electrode prepared from a commercial Metrohm GCE (see below) or a solid gold
143 electrode (SGE), a glassy carbon counter electrode and an Ag/AgCl/KCl (3M) reference electrode.
144 The analyser was interfaced to a personal computer; the operational conditions were selected and
145 voltammograms were visualised and processed with the aid of GPES 4.9.

146 Morphological characterisation of the electrode surface was performed by scanning electron
147 microscopy, SEM (LEICA Microsystems, Germany) using a Stereo scan 410 SEM Inspect FTM with
148 Field Emission Gun.

149 The roughness of the obtained AuNPs-layer was investigated by Atomic Force Microscopy (AFM)
150 using a Danish Micro Engineering Scanning Probe Microscope, SPM (AFM/Scanning Tunneling
151 Microscope, STM) Microscope, with a DME Igloo stage with 50 µm DS95-50E SPM head for fluid
152 environment, integrated optical axis on cantilever and total positioning and approach control via
153 CCD PSU camera (DME 2350) and a fully digital hold C26 Dualscope/Rasterscope controller.

154 A DMA-80 Direct Analyser (FKV SrL, Torre Boldone, BG, Italy) was employed; the analyses were
155 carried out in IZSPLV laboratory in Torino. The instrument features a circular, stainless steel,
156 interchangeable 40-position autosampler, and can accommodate both nickel and quartz boats
157 depending on the requirements of the application. It requires regular grade oxygen as a carrier and
158 decomposition gas. The instrument is equipped with a Hollow Cathode Lamp ($\lambda_{\text{Hg}} = 253,7 \text{ nm}$) and
159 a Si-photodiode sensor.

160 The sample solutions were also analysed by with a model 1100 B AA Spectrometer (Perkin Elmer,
161 Waltham, MA, USA) equipped with a MHS-20 Mercury Hydride System accessory. Argon was

162 adopted as carrier to conduct the vapours to the atomization cell. The instrument is equipped with
163 an Electrodeless Discharge Lamp ($\lambda_{\text{Hg}} = 253,7 \text{ nm}$) and a Photomultiplier detector. NaBH_4 and
164 KMnO_4 solutions were used for the formation of volatile hydrides and to monitor the reaction
165 respectively.

166 A chemometric processing of the experimental results was performed by ANOVA, with the aid of
167 an XLStat 7 software package, used as a Microsoft Excel plug-in.

168 Analytical grade reagents were used. A 1000 mg/l standard solution of mercury was prepared from
169 HgCl_2 in 0.012 M HCl. More diluted Hg(II) standard solutions were prepared from the concentrated
170 standards.

171 Calibration standards for DME-80 were prepared using a NIST traceable stock solution of 1000
172 mg/l Hg preserved in 5% HNO_3 . Working standards of 0.1 and 1 mg/l were prepared and preserved
173 in 3.7% HCl and stored in amber glass vials.

174 High purity water (HPW) obtained from a Milli-Q apparatus (Millipore, Bedford, USA) was used
175 throughout.

176 100 mg/l stock solutions of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (Sigma, > 99.9% trace metals basis) in HPW were
177 prepared and used for the deposition of gold nanoparticles onto the electrode.

178 For the CV-AAS analysis a 3% sodium borohydride solution in 1% NaOH was used as reducing
179 reagent.

180 2.2 Procedures

181 2.2.1 Samples and sample pretreatments

182 *Tuna Fish BCR 463* ($[\text{Hg}] = 2.85 \pm 0.16 \text{ mg/kg}$) and *Tuna Fish ISPRA T22* ($[\text{Hg}] = 4.43 \pm 0.34$
183 mg/kg) were analysed to value the efficiency and the accuracy of each technique for mercury
184 quantification in this type of matrix. The concentration in *ISPRA T22* is not certified, so we used the
185 value reported in literature by Detcheva and Grobecker as reference concentration (Detcheva &
186 Grobecker, 2006).

187 Ten samples of CT were purchased in local supermarkets. Samples were transported to the
188 laboratory and coded 1-10 for easy identification. Then, each can was opened and the sauce drained.
189 The samples of tuna fish were grinded in a mortar and they were freeze-dried in order to obtain a
190 powdered sample, useful to be analysed in different times and with different techniques.

191 For ASV and CV-AAS analysis, a dissolution of the matrix was required. Aliquots of 0.5 g of *Tuna*
192 *Fish BCR 463* and *Tuna Fish ISPRA T-22* were transferred into the bombs and digested without any
193 pretreatment with a mixture of 3 ml of HNO₃ and 3 ml of H₂O₂. Aliquots of 0.25 g of CTs were
194 treated in the same way. The following heating program of the microwave unit was adopted: 250 W
195 for 1 min; 0 W for 1 min; 250 W for 5 min; 400 W for 5 min; 650 W for 5 min; ventilation for 25
196 min. The bombs were left to cool at room temperature. The resulting solutions were diluted to 15
197 mL with HPW. The digested solutions were freshly prepared for analysis with each technique.

198 For DMA the analysis was performed directly on the freeze-dried powder, since no other sample
199 pretreatment is required.

200 All the experiments were performed in duplicate and blanks were simultaneously run.

201

202 *2.2.2 Deposition of gold nanoparticles on the glassy carbon electrode*

203 Modification with gold nanoparticles layer was performed following the procedure described in our
204 previous work (Abollino, Giacomino, Malandrino, Piscionieri, & Mentasti, 2008)

205 The presence of AuNPs was confirmed by SEM analyses and by cyclic voltammetry (see below).

206 The latter was performed varying the potential from 0 to 1.3 to 0 V in 0.5 M H₂SO₄.

207 An estimation of the roughness of the obtained gold layer was obtained by AFM (see below).

208

209 *2.2.3 Solid gold electrode pretreatment*

210 The SGE was polished and activated following the procedure reported in our previous paper
211 (Giacomino, Abollino, Malandrino, & Mentasti, 2008).

212 During all the analyses hereafter described there was no need to renew electrode surface with
213 alumina powder.

214

215 *2.2.4 Determination of mercury by SW-ASV*

216 Aliquots of 4 or 2 ml of digested solutions were transferred into the voltammetric cell and diluted to
217 20 ml with 0.06 M HCl or 60 mM NaCl, in the case of SGE or AuNPs-GCE respectively.

218 The following procedures were the same for the two electrodes.

219 After 120 s of deposition at 0 V, a voltammetric scan was performed, adopting the parameters,
220 previously optimised in our laboratory (Giacomino, Abollino, Malandrino, & Mentasti, 2008).

221 The medium exchange technique was adopted for the analysis: after the electrodeposition step from
222 the sample solutions, the potential was maintained at 0 V with the optional function “Hold” of the
223 instrument and the sample solution cell was replaced by a solution of 0.06 M HCl or 0.06 M NaCl
224 in which the stripping step was then performed (Abollino, Giacomino, Malandrino, Piscionieri, &
225 Mentasti, 2008).

226 After each determination the electrode was maintained in a mixture of 0.2 M HClO₄/3 mM NaCl/1
227 mM NaEDTA for 30 s at 0.80 V, to remove residues of mercury from its surface (Metrohm,
228 Application Bulletin No 96/4e). The presence of EDTA, probably thanks to its complexing
229 properties, favoured this removal.

230 After recording the voltammogram of the sample solutions, aliquots of Hg were added and the
231 corresponding signals were recorded. The standard addition method was adopted for the evaluation
232 of the concentration of mercury in all investigated samples. We obtained well defined peaks by
233 subtracting the blank signal from the voltammograms of the sample solutions.

234 Each sample was analysed in duplicate.

235

236 *2.2.5 Determination of mercury by CV-AAS and DMA80*

237 DMA-80 was used to determine the concentration of total mercury, as reported in U.S. EPA Method
238 7473 (EPA, Method 7473, 2007). By injecting increasing volumes of standard into quartz sample
239 boats, calibration graphs of 0–20 ng and 20–500 ng of mercury were created using the 0.1 and 1
240 mg/l standards, respectively. The analyses were carried out using the following parameter: drying
241 temperature/time: 90 s to 200°C; decomposition ramp: 120 s to 750°C; decomposition hold: 90 s at
242 750°C; catalyst temperature: 600°C; purge time: 60 s; 12 s at 900°C; recording time: 60 s; oxygen
243 flow: 120 mL/min Aliquots of freeze-dried tuna (about 0.1 g) were transferred into the sample boats
244 and directly analysed.

245 For CV-AAS 1 ml of sample solution was diluted to 15 ml with 1,5% HNO₃ directly in the MHS
246 vessel. A few drops of KMnO₄ solution were added to monitor the reaction of reduction (the colour
247 of the solution changes as a consequence of the reduction MnO₄⁻→MnO₂). The method of external
248 calibration was adopted for the evaluation of mercury content in the sample solutions. Blank

249 solution and five standards were prepared for the instrument calibration (40, 50, 70, 100 and 200 ng
250 of Hg).

251

252 **3 Results and discussion**

253 *3.1 ASV analysis*

254 The performances of both the SGE and the AuNPs-GCE for the determination of Hg in aqueous
255 solutions were shown in our previous works (Abollino, Giacomino, Malandrino, Piscionieri, &
256 Mentasti, 2008; Giacomino, Abollino, Malandrino, & Mentasti, 2008), which report the
257 repeatability, linearity, accuracy, detection limit of the procedure and the interferences of other
258 cations and of anions. Briefly, the height of Hg peak increased with increasing deposition time; a
259 value of 120 s was found to be suitable for concentrations down to 50 µg/L. In synthetic solution,
260 the limit of detection (estimated as $LOD = 3\sigma B/slope$) for SGE was 0.40 µg/l and sensitivity was
261 1.71 µA/µg l⁻¹; the relative error for the determination of 1 µg/l of Hg was -1%. The LOD and
262 sensitivity for AuNPs-GCE were 0.15 ng/l and 3.5 µA/µg l⁻¹ respectively. A concentration as low as
263 10 ng l⁻¹ could be quantified with a relative error of - 0.8%. The interference of several metal ions
264 (As(V), Bi(III), Cd(II), Co(II), Cr(III), Cu(II), Fe(II), Mn(II), Ni(II), Pb(II), and Se(IV)) and some
265 anions (ClO₄⁻, SO₄²⁻, S²⁻, PO₄³⁻, HCOO⁻, BO₃³⁻, IO₃⁻, CH₃COO⁻, CO₃²⁻, ClO₂⁻, F⁻, I⁻, NO₃⁻ and NO₂⁻)
266 on the mercury stripping signal was investigated using both the electrodes in our previous works
267 (Abollino, Giacomino, Malandrino, Piscionieri, & Mentasti, 2008; Abollino, Giacomino, Ginepro,
268 Malandrino & Zelano, 2012). The voltammogram of a solution with 5 µg/l of Hg was recorded in
269 the presence of each ion (added into the vessel in 1:1; 1:10 and 1:100 concentration ratios with
270 respect to the analyte). For all considered cations, no interference was observed on the Hg
271 quantification and the linearity of the calibration curve of Hg was maintained, in good agreement
272 with literature data reported in literature (Augelli, Munoz, Richter, Gouveia & Angnes, 2005; Okcu,
273 Ertas, Gokcel & Tural, 2005). Among the considered anions, no interference was observed with the
274 exception of Br⁻, I⁻ and S²⁻. These ions are known to adsorb strongly on gold surfaces, and among
275 the halogen ions iodides are those with the highest affinity for gold. They have been found to shift
276 analyte signal to more positive potentials as reported by Tamer et al. (Tamer, U., Oymak T., & Ertas
277 N., 2007). The presence of Br⁻ interfered with the quantification of Hg for [Hg] < 3 µg/l or in
278 presence of a ratio Hg:Br ≥ 1:100. The presence of I⁻ and S²⁻ caused a decrease of the background
279 current, a sharp decrease of the mercury signal and a loss of linearity, which hindered the
280 quantification of the analyte. To minimize the excessive adsorption effect a negative potential of -

281 0.80 V was applied for 30 s between the deposition step and the stripping step. At this potential I,
282 S²⁻ and Br⁻ and the other cited anions do not adsorb on the AuNPs-GCS surface. This procedure
283 permits to have well defined voltammetric peaks and a stable voltammetric baseline (Salaun P., &
284 van den Berg C.M.G. 2006).

285 Considering the low analyte concentrations expected in sample solutions, the medium exchange
286 technique was adopted to eliminate the effect of components in the sample matrix that might cause
287 interferences in the stripping step (Detcheva & Grobecker, 2006). Using this method, we observed
288 an improvement of Hg recovery (about + 8%) in the reference materials for both the electrodes.

289 3.2 Experiments with SGE

290 Using SGE, the stripping step was conducted in a solution of either 60 mM HCl or 60 mM NaCl. In
291 our previous work, the measurements in HCl showed higher sensitivity in comparison with NaCl;
292 however, in the case of real sample solutions containing HNO₃ we chose to work in NaCl to avoid
293 the formation of traces of *aqua regia* which could damage the Au surface. In the present study, we
294 have checked the response of the SGE using both the supporting electrolytes.

295 All the results are reported in Table 2. We can observe as, using NaCl instead of HCl, the recoveries
296 increase from 89.6% to 94.4 % for *BCR 643* and from 80.2 % to 99.0 % for *Ispra T22*.

297 As to CT solutions, we found higher concentrations using NaCl in comparison with HCl. This is
298 due to the fact that, using HCl, the baselines of the voltammograms related to each sample (i.e.
299 blank, sample solution before and after standard additions) were not perfectly overlapped: this
300 behavior caused a systematic underestimation of the mercury concentration in all CTs. Using NaCl,
301 the baselines for each sample are exactly overlapped.

302 The LOQ in the considered matrix (computed as the minimum amount determined with a good
303 accuracy) was 0.7 µg/l Hg, corresponding to 0.4 mg/kg in the dried sample.

304 Using SGE, no detectable mercury peaks were observed for samples 3, 4 and 8.

305

306 3.3 Experiments with AuNPs-GCE

307 All the analyses were repeated using AuNPs-GCE adopting the best condition found for SGE, that
308 is NaCl 60 mM as supporting electrolyte (Table 2). In this conditions, we obtained recoveries of
309 96.8 % and 99.8% for *BCR 643* and *Ispra T22* respectively. Using AuNPs-GCE, it was possible to
310 quantify mercury also in the CTs characterised by the lowest analyte concentrations. The LOQ in
311 the investigated matrix is 0.1 µg/l Hg, corresponding to 0.06 mg/kg in the dried sample. These
312 results confirm that the large surface area of the deposited gold nanoparticles permits an
313 improvement of sensitivity in comparison with a traditional solid surface.

314 As-expected, we obtained similar values of concentration for all the considered samples with both
315 electrodes (see section 3.7 for statistical comparison).

316

317 3.4 Effect of supporting electrolyte

318 In our previous study we demonstrated that the performance of HCl and NaCl as supporting
319 electrolytes with AuNPs-GCE is the same. This finding is in agreement with EPA Method 7472,
320 based on ASV with a gold film electrode (GFE), which states that the solutions can be rendered
321 electrically conductive by adding HCl or NaCl indifferently. These reagents permit to obtain the
322 best results, since the formation of complexes between Hg and chloride ions enhances the
323 sensitivity of the stripping signal (Metrohm, Application Bulletin No 96/4e). On the other hand, the
324 two electrolytes have a different behaviour with SGE, as shown above, probably because of the
325 formation of traces of *aqua regia*, and consequently of nitrosyl chloride, which attacks the gold
326 surface forming a passivating layer. In the case of AuNPs-GCE (Abollino, Giacomino, Malandrino,
327 Piscionieri, & Mentasti, 2008) or GFE (EPA, Method 7472, 1996) this effect is not appreciable due
328 to the renovation of active surface. The fact that NaCl provides more stable signals with SGE
329 confirms the observations reported by Bonfil (Bonfil, Brand, & Kirowa-Eisner, 2000), who
330 suggested a mixture of NaCl and HNO₃ as the best supporting electrolyte for the determination of
331 metals using this kind of electrode.

332 Figure 1 shows the voltammograms obtained with SGE using HCl (a) and NaCl (b) as supporting
333 electrolytes for the analysis of *BCR 643*; the baseline considered for the evaluation of peaks height
334 is also reported. As we can see, in the case of NaCl the baseline is well defined by the points in
335 which the voltammograms cross, while in the case of HCl a shift of the baseline on the left of the
336 peaks seems to cause an underestimation of the mercury concentration. For comparison Fig.1c
337 reports the voltammograms recorded at AuNPs-GCE for the same sample: as specified in
338 “Procedure” section in the case of SGE the sample was diluted in the electrolyte with a ratio 1:5,
339 while in the case of AuNPs-GCE the ratio in voltammetric cell is 1:10; even if the concentration in
340 cell is lower, the voltammograms reported in Fig.1 show the higher sensitivity obtained by the
341 nanostructured electrode. The coefficient of correlation obtained was greater than 0.9950 for all the
342 considered samples. Figure 1d reports the voltammograms obtained analysing CT3 with AuNPs
343 using NaCl as electrolyte. In all CT-voltammograms it is possible to see another peak at 0.38 V,
344 caused by the presence of traces of Cu (Abollino, Giacomino, Malandrino, Piscionieri, & Mentasti,
345 2008), present in tuna as a micronutrient; a fraction of this metal might derive from the metallic
346 package, as found by Buculei et al. (Buculei, Amariei, Oroian, Gutt, Gaceu, & Birca, 2014).

347

348 3.5 Monitoring of the electrodes surface

349 The deposition of gold nanoparticles is well detectable through a colour change of the glassy carbon
350 surface from black to red-orange. The status of the gold nanoparticles layer was valued with SEM.
351 From SEM images (Fig. S1, Supplementary material), it is possible to see the presence of a
352 homogeneous gold layer composed of particles, having an average diameter of approximately $100 \pm$
353 25 nm.

354 A very good repeatability in the morphology of the nanostructured layer was obtained starting from
355 different GCEs and different brands of Au salts. A drawback of SEM technique as method to
356 monitor the surface is that once a metal nanoparticles-modified electrode is analysed with SEM, the
357 metal particles are charged by the beam and they could coalesce and form an “Au film”, so another
358 method has to be used for the daily monitoring of the goodness of the active surface.

359 A fast and easy method to do this is cyclic voltammetry (CV). Fig. 2a and 2b show voltammograms
360 recorded in 0.5 M H₂SO₄ with SGE and AuNPs-GCE respectively.

361 The shapes of the voltammograms reported as “line 1” are well known in literature and identify a
362 solid (Fig 2a) or nanostructured (Fig 2b) gold surface (Salaun, Planer-Friedrich, & van den Berg,
363 2007). The anodic peak at 1.25 V is due to oxide formation, probably consisting of hydrated oxides
364 or Au(OH)_n_{ads}. In the CV voltammogram for AuNPs-GCE, we can observe a “shoulder” before the
365 oxidation peak, typical of a nanostructured gold surface, caused by the formation of different multi-
366 oxide species for the gold nanoparticles (Priano et al., 2008). The cathodic peak at +0.90 V, present
367 in both the voltammograms, can be attributed to the reduction of the gold oxide formed during the
368 anodic cycle. The charge recorded under the reduction peak is generally used to characterize and
369 monitor the electroactive sensor area, since it is proportional to the amount of deposited Au (Dai,
370 Nekrassova, Hyde, & Compton, 2004). As expected, a higher signal is obtained in the case of
371 AuNPs-GCE in comparison with SGE, due to the presence of nanoparticles that increase the
372 electrode surface.

373 The voltammogram reported as “Line 2” in Fig.2a represents the CV recorded after a prolonged use
374 of the electrode surface, that causes a worsening in the analytical response. In the case of SGE, the
375 CV reported in Fig. 2a shows additional peaks in comparison to CV obtained from an optimal
376 surface. These peaks are due to the electrochemistry of gold, since are caused by the formation of
377 multilayers of oxides in which gold can exist in different oxidation states (Au⁰/Au^I/Au^{III})
378 (Giacomino, Abollino, Lazzara, Malandrino, & Mentasti, 2011); the presence of these layers causes
379 a worsening of the electrode performance. After the treatments with NaOH, ethanol and water a

380 cyclic voltammogram like that reported in Fig. 2a-line1 is again obtained. We suppose that in the
381 presence of NaOH the surface is covered with a layer of hydroxides, that replace completely or in
382 part the hydration layer; ethanol removes this deposited layer and water removes the traces of
383 ethanol.

384 Regarding AuNPs-GCE, in Fig. 2b-line2 we can observe i) a shift of the anodic peak to more
385 positive potential contemporary to a reduction of the peak height and to the disappearance of the
386 shoulder and ii) a drastic reduction of the cathodic peak due to a loss of available active surface. In
387 this condition the electrode can be used as “Au film electrode”, but it presents a lower sensitivity.
388 With this kind of electrode the treatment of the surface is usually not performed, as it is more
389 convenient to dissolve the gold layer and deposit a new one.

390 Daily, we recorded ten CV-scans in H₂SO₄ to check the repeatability of the electrode response and,
391 consequently, the goodness of the electrode surface. Many researchers use this CV-treatment as an
392 activation step for solid electrodes: for our experience, we suggest to make, after the treatment in
393 H₂SO₄, a further activation step by applying a potential of 0.60 V for 60 s in 0.06 M HCl before
394 mercury determination, in order to maintain the electrode surface active and reproducible and
395 consequently to enhance the quality and reproducibility of the mercury signal.

396 An estimation of the roughness of the obtained gold layer was obtained by AFM. Unfortunately,
397 AFM instruments are not suitable to analyse this type of electrode, because of its size (height = 5
398 cm). For this reason this evaluation was performed with the aid of a glassy carbon plate (height = 3
399 mm), onto which the same procedure of surface cleaning and deposition adopted for GCE was
400 followed. Obviously, the conditions in which the deposition occurs were different because i) during
401 the deposition the electrode is rotated, while it is not possible to move the GC plate: so, in this case,
402 the solution was maintained in agitation with a magnetic stirrer; and ii) the sizes of the GC surfaces
403 are different: the electrode surface can be estimated as $\approx 3,14 \text{ mm}^2$ (as calculated from the radius of
404 the active surface = $1 \text{ mm} \pm 0.01$, provided by the producer), while the plate has an area $\approx 2,5 \text{ cm}^2$
405 (manually estimated by measuring the total surface in contact with Au solution). In any case, AFM
406 analysis (Fig. S2, Supplementary material) confirmed the presence of a homogeneous gold layer: in
407 particular the profile reported in Fig.S2-c shows the regularity of the obtained surface.

408

409 *3.6 Atomic Absorption Spectroscopy Analysis*

410 In the last decade, direct mercury analysers demonstrated to be a reliable alternative to wet
411 chemistry techniques. The DMA automatically performs thermal decomposition, catalytic

412 reduction, amalgamation, desorption and AAS detection to rapidly treat and analyse solid or liquid
413 samples for mercury, with an output result for mercury content in about 5 min (per sample) with no
414 pre-treatments required and no waste generation. For the considered matrix, the limit of quantitation
415 (LOQ) for detection of mercury by means of this method is 0.037 mg/kg (wet weight). The obtained
416 calibration curve, $y = -3.06 \cdot 10^{-7}x^2 + 1.10x \cdot 10^{-3} - 5.5 \cdot 10^{-3}$ shows a good linearity, $R^2 = 0.9998$
417 (Squadrone, Benedetto, Brizio, Prearo, & Abete, 2015).

418 The results are reported in Table 3: as we can observe, a quantitative recovery was obtained
419 analysing BCR material. For this reason, and taking into account that the use of DMA for mercury
420 determination is well-established, we considered the results obtained using this technique as
421 “reference values” to evaluate the data obtained with the other methods. Moreover, since the
422 process does not require the conversion of mercury to mercuric ions, both solid and liquid matrices
423 can be analysed without the need for acid digestion or other sample preparation steps. Therefore,
424 the comparison with DMA results permitted us to quantify the possible losses through volatilization
425 or incomplete digestion which may take place with the other investigated analytical techniques
426 (Haynes, Gragg, Johnson, Robinson, & Orazio, 2006).

427 Table 3 shows also the results obtained using conventional CV-AAS, preceded by sample digestion
428 in microwave oven. The method has a good linearity, as shown by the calibration curve obtained (y
429 $= 0.0012x - 0.0011$, $R^2 = 0.9981$).

430 The concentrations measured in the certified materials show a loss of mercury of about $-20 \pm 5\%$
431 that can be ascribed to a partial volatilization of the metal during the digestion step and/or during
432 the conversion to elemental Hg and transfer to the measurement cell. Then, the analysis of the CTs
433 was carried out: the quantification of Hg concentration was possible in all the samples with the only
434 exception of CT3, in which the signal was too low. The results obtained with CV-AAS were surely
435 negatively influenced by the absence of the gold trap required for determination of Hg at ultratrace
436 level by this technique.

437

438 *3.7 Comparison among the techniques*

439 We compared the results obtained with the considered techniques to understand the real
440 performance of ASV determination, in particular using AuNPs-GCE.

441 Considering the DMA results ($[\text{Hg}]_{\text{DMA}}$) as “true”, the concentrations in CTs found using SGE with
442 NaCl, AuNPs-GCE and CV-AAS were expressed as percentages of recovery (Table 4) with respect
443 to $[\text{Hg}]_{\text{DMA}}$.

444 As it can be seen, the values range between 79% and 112%. The median values increase in the order
445 CVAAS < SGE < AuNPs-GCE.

446 Figure 3 reports scatter plots comparing $[\text{Hg}]_{\text{DMA}}$ with the concentrations measured with SGE in
447 NaCl (3a), AuNPs (3b) and CV-AAS (3c), the equations of the lines so obtained and the
448 corresponding values of R^2 . For SGE only the samples containing higher concentrations (six CTs)
449 were considered, while for CV-AAS it was possible to compare the results obtained for all the
450 samples with the exception of sample CT3. The comparison indicate a good correlation between the
451 mercury amounts measured in the samples with different techniques. The results obtained by ASV
452 demonstrated the highest correlation with $[\text{Hg}]_{\text{DMA}}$; in particular using AuNPs-GCE a correlation of
453 0.9889 was obtained considering the whole data set.

454 A statistical comparison among all the results obtained was also made using ANOVA (level of
455 probability = 95%) considering the concentrations found using the different techniques for each
456 sample. For all the considered samples, the Hg concentrations measured with DMA were not
457 significantly different from those found by ASV using AuNPs-GCE. As to the other techniques,
458 among the quantifiable samples, no significant differences were observed from $[\text{Hg}]_{\text{DMA}}$ with the
459 only exception of CT3 in the case of CV-AA.

460 It is very important to underline the key role of analysing freshly prepared sample solutions
461 (maximum one week), since losses of 10-30 % were observed in solutions analysed two weeks after
462 the digestion. Good repeatability was observed among sample solutions prepared from different
463 aliquots of the same lyophilized tuna demonstrating the homogeneity of the sample.

464 Finally, Table 5 summarizes the analytical performance found in this study for each adopted
465 technique.

466 Both voltammetric and spectroscopic approaches can be considered suitable techniques to monitor
467 Hg concentration in fish, since they allowed to reach accurate Hg quantification at concentration
468 values lower than the maximum admissible level of this element in the considered matrix (1
469 mg/kg_{ww}) (Commission Regulation (EC) No. 629/2008).

470 Considering the two investigated spectroscopic techniques, we can conclude that DMA performs
471 better than conventional CV-AAS and can be considered a fast, accurate and reliable alternative to
472 wet chemistry techniques. Its main advantage is the high sample throughput, as no sample

473 preparation is required The technique permits to determine Hg with high accuracy and precision. Its
474 main drawback is that it is applicable only to the determination of this analyte.

475 Electrochemical techniques are sensitive, relatively inexpensive and they also enable the
476 determination of a number of inorganics and organics at trace or ultratrace concentrations. The
477 drawbacks are the longer time required in comparison with DMA, in particular for the wet digestion
478 step, with possible loss of analyte by vaporisation, and the production of waste solutions. In the case
479 of in-cell concentrations higher than 0.7 $\mu\text{g/l}$ both SGE and AuNPs-GCE provide very accurate
480 determinations. The presence of the nanostructured active surface in the latter permits to quantify
481 lower concentrations with a LOQ comparable to that of DMA. Another great advantage of the
482 electrochemical approach is the possibility to carry out speciation studies: in the case of mercury, it
483 is possible to distinguish between inorganic Hg and methylmercury, the most harmful specie for
484 humans (Abollino, Giacomino, Malandrino, Marro, & Mentasti, 2009)

485

486 *3.8 Evaluation of Hg in examined canned tunas*

487 Since the main purpose of our study is to verify the applicability of AuNPs-GCE for the
488 quantification of Hg in fish in routine controls, we compared the concentrations obtained with the
489 European Legislation limit for this analyte: the maximum admissible level for mercury in fishery
490 products and fish tissues is 0.5 mg/kg_{ww} for most fish species, and 1 mg/kg_{ww} for tuna and some
491 others species (Commission Regulation (EC) No. 629/2008). During the lyophilisation step, the
492 CTs lost about $30 \pm 5\%$ of weight, so we calculated the final concentrations in wet fish. All the
493 examined samples contained Hg concentrations lower than the admissible value (Table S1,
494 Supplementary material).

495

496 **4 Conclusions**

497 In this work the suitability of ASV with gold electrodes for the determination of mercury in fish
498 was demonstrated.

499 Under the optimized conditions, for both the electrochemical approaches, the oxidative current
500 exhibited a linear dependence on Hg(II) ion concentration in wide dynamic ranges of 0.2-100 $\mu\text{g/l}$
501 ($R^2 = 0.9891$) and 0.010-100 $\mu\text{g/l}$ ($R^2 = 0.9922$) with detection limits of 0.02 and 0.001 $\mu\text{g/l}$ for SGE
502 and AuNPs-GCE respectively.

503 The main advantage of the AuNPs-GCE with respect to SGE is the improvement of the analytical
504 performance, thanks to the increase of active surface of the gold nanoparticles. In particular the
505 proposed sensor shows high sensitivity (even with short deposition time, 120 s), good selectivity,
506 long-term stability, great repeatability and a limit of quantification of 0.1 µg/l for Hg in fish matrix.
507 The comparison between DMA and conventional CV-AAS shows that the former has better
508 performances in terms of accuracy, precision and analysis times, owing to the integration of sample
509 pretreatment and analyte detection. The results obtained by DMA and ASV with AuNPs-GCE are
510 not significantly different, confirming that the latter is an effective alternative to other more
511 common techniques for the determination of mercury in fish.

512 The choice of the most suitable analytical method for the determination of Hg is influenced by the
513 number of samples, by the available time and by the number of considered analytes: for a fast
514 determination of Hg in a lot of samples, DMA seems to be the best solution; ASV is particularly
515 suitable for a relatively low sample number in which also speciation studies or the measurement of
516 other (inorganic or organic) species is required, The proposed technique has the main advantages of
517 electrochemical methods i.e. versatility, sensitivity, low costs of instrument purchase and
518 management, availability in most analytical laboratories and the possibility of using portable
519 devices for on-site and in situ measurements. The ease of the procedures requested for the electrode
520 modification could offer a simple, inexpensive and rapid methods for the routine determination of
521 mercury in food samples. The good accuracy and precision demonstrated by ASV with AuNPs-
522 GCE show as this technique can be fruitfully used in ring tests.

523

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