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Additional Information

1 **Influence of potential pulses amplitude sequence in a Voltammetric**

2 **Electronic Tongue (VET) applied to assess antioxidant capacity in Aliso.**

3 Esteban Fuentes[1], Miguel Alcañiz[3], Laura Contat[3], Edwin O. Baldeón[1,2], José M. Barat[1],

4 Raúl Grau[1]

5 [1]Departamento de Tecnología de Alimentos. Grupo CUINA. Universidad Politécnica de

6 Valencia, Spain

7 [2]Departamento de Ingeniería de Alimentos y Productos Agropecuarios. Universidad

8 Nacional Agraria la Molina-UNALM, Av. La Molina s/n, Lima, Peru

9 [3]Instituto de Reconocimiento Molecular y Desarrollo Tecnológico (IDM), Centro Mixto

10 UniversitatPolitècnica de València e Universidad de Valencia, Camino de Vera s/n,

11 46022 Valencia, Spain

12

13

14

15 *Author for correspondence:

16 **Address:**

17

18 Universidad Politécnica de Valencia

19 Camino de Vera, s/n

20 46022 VALENCIA – SPAIN

21 **E-mail:**

22 **Phone:**

23

Abstract

The aim of the study was to evaluate influence of potential pulses amplitude sequence in a Voltammetric Electronic Tongue (VET) applied to assess antioxidant capacity in Aliso. Four signals configurations were studied, two of them built by small increases of potential and two with bigger increments. The highest current values were obtained when pulses with bigger change of potential were used although the best results were shown by the pulse sequence which included an intermediate pulse before the relevant pulse. A mathematical model based on trolox pattern was developed to predict antioxidant capacity of aliso, employing information obtained from all the electrodes, although model validation could be done only employing the information from gold electrode. This result confirms the advantages of using several cross-sensitive sensors in applications where the compound to be quantified is mixed with other compounds.

INTRODUCTION

The popularity of antioxidant substances is related to the attack, or even the neutralisation, of free radicals and reactive oxygen species (Halliwell, 1989), which are the cause of different types of pathologies and illnesses (Halliwell, 1994). Andean regions like those in Peru have an impressive number of endemic species and plant varieties with desirable and unique functional properties (Brack, 1999). Among others plants, Aliso (*Alnusacuminata*) possesses an important antioxidant characteristic, mainly by polyphenolic compounds like catechins (Chirinos, Pedreschi, Rogez, Larondelle, & Campos, 2013). This plant is principally consumed in infusions in tea bags. A low-cost fast measurement method to assess the antioxidant capacity of these infusions would be of much interest.

Numerous studies have suggested the use of voltammetric techniques to determine antioxidant capacity. These electrochemical methods are widely used in analytical chemistry thanks to their sensitivity, versatility, simplicity and robustness. Voltammetry is based on measuring the current that flows through a working electrode when a potential signal is applied to it. The main contributors to this current are the charge transfer due to chemical reactions that occur at the working electrode interface (faradaic processes) and the charge reorganisation at the electrical double layer due to an electrode's potential changes (non-faradaic processes). The type of potential signal applied to the working electrode varies for each voltammetric technique (Bard & Faulkner, 2002).

In linear sweep voltammetry and cyclic voltammetry, a linear potential scan is applied to the electrode while measuring the current. The resulting i-E curve (voltammogram) provides information about the faradaic processes that occur at the working electrode interface. These two electrochemical methods are a very useful tool to characterise electrode materials and to determine the redox properties of the molecules present in

61 dissolution. Cyclic voltammetry has been used in a wide range of sensoring applications,

62 analysis of grapes (Medina-Plaza, 2014), classification of teas (Bhattacharyya et al.,

63 2012), biofilm formation monitoring (Kang, Kim, Tak, Lee, & Yoon, 2012), insulin

64 detection (Habibi, Omidinia, Heidari, & Fazli, 2016), ethanol and methanol determination

65 (Pereira, Sousa, Munoz, & Richter, 2013) and neurotransmitter assessment (Singh,

66 Sawarynski, Dabiri, Choi, & Andrews, 2011). The quantification of total antioxidant

67 capacity in alcoholic beverages (G. Toro, López, & Taipe, 2011), species (Ziyatdinova &

68 Budnikov, 2014), body fluids (Chevion, Berry, Kitrossky, & Kohen, 1997; Psotová,

69 Zahálková, Hrbáč, Šimánek, & Bartek, 2001), animal tissue (S Chevion, Or, & Berry,

70 1999), besides some plants (Chevion, Chevion, Chock, & Beecher, 1999 b) and fruits

71 (Hoyle & Santos, 2010), has also been achieved by means of cyclic voltammetry.

72 In pulse voltammetry the signal applied to the electrode is a sequence of potential pulses.

73 The most relevant pulse voltammetry techniques are Square Wave Voltammetry (SWV),

74 Differential Pulse Voltammetry (DPV) and Normal Pulse Voltammetry (NPV). In all

75 these methods, the current is generally sampled at the beginning or the end of each pulse

76 to obtain the i-E curve. Similar information to cyclic voltammetry is obtained, but these

77 techniques allow sensitivity to increase and measurement times to reduce. Pulse

78 voltammetry is an extremely useful technique for measuring the organic and inorganic

79 species present in samples, even at concentrations of 10^{-8} M (Wang, 2006). Pulse

80 voltammetry is a very viable method to determine phenolic and polyphenolic compounds

81 and allows the assessment of the total antioxidant capacity of analysed samples, and has

82 the benefit of not requiring large quantities of reagents, materials or equipment. Its high

83 selectivity can afford the identification of new compounds (Blasco, González, & Escarpa,

84 2004).

85 A different approach is employed for chronoamperometry, where one or two potential

86 pulses are applied, and the temporal evolution of the resulting current is collected. The

87 advantage of this technique is that the obtained information is related to both faradaic and

88 non-faradaic processes. A modified version of chronoamperometry is used in

89 voltammetric electronic tongues (VET), where a sequence of pulses with arbitrary

90 amplitudes are applied to different working electrodes and the data of the temporal

91 evolution of the current are processed by multivariate analysis tools to classify samples

92 or to quantify compounds in a complex matrix. VET applications include drinking water

93 and wastewater quality assessments (Campos, Alcañiz, Aguado, et al., 2012 a; Eriksson

94 et al., 2011), antioxidant capacity evaluations of camu-camu and tumbo (Baldeón et al.,

95 2015) and the quantification of organic acids malic, citric and ascorbic (Escobar et al.,

96 2013).

97 A very important factor to be considered in VET measurements is the pulses sequence

98 design (Ivarsson, Holmin, Höjer, Krantz-Rülcker, & Winquist, 2001). Some authors have

99 investigated optimising the classification and prediction models obtained with VET by a

100 custom pulses sequence design. In some cases, the pulses sequence design is based on a

101 previous study of the electrochemical processes that occur while measuring (Campos,

102 Alcañiz, Masot, et al., 2012 b). In other cases, the discrimination capability of the

103 classification models is improved by modifying the width of pulses (Tian, Deng, & Chen,

104 2007). The importance of each pulse in the sequence has also been investigated. For

105 example, variable importance in projection (VIP) scores have been used to identify pulses

106 and have provided more relevant information in camu-camu juices VET measurements

107 (Baldeón et al., 2015). A possible application of this identification would be the reduction

108 of the number of pulses by including only the selected pulses in the sequence. However,

109 further research is needed to determine if the elimination of some of pulses in the

110 sequence could affect the information obtained in the relevant pulses.

111 The aim of the study was to evaluate the influence of potential pulses amplitude sequence

112 in a Voltammetric Electronic Tongue (VET) applied to assess antioxidant capacity in

113 Aliso.

114

115 MATERIALS AND METHODS

116 *Sample preparation.*

117 Aliso (*Alnusacuminate*) was obtained in a local market of Huancayo (Junin, Peru).

118 Leaves were washed and dried by hot air at 60°C for 3 h until constant weight. After the

119 drying process, leaves were blended to the size of the leaves in commercial tea bags.

120 Extractions were taken to simulate domestic tea-making by employing commercial tea

121 bags (Almajano, Carbó, Jiménez, & Gordon, 2008; Chan, Lim, Chong, Tan, & Wong,

122 2010; Samaniego-Sánchez et al., 2011). Three grams of leaves were mixed with water at

123 85°C for 5 minutes with constant shaking, and were then filtered.

124 To obtain the voltammetric response of aliso, infusions were prepared at 2.5%, 5%,

125 10% and 20% (v/v) with an electrolyte solution (0.01 M phosphate buffer), made by

126 mixing potassium phosphate (KH_2PO_4) and dihydrogen phosphate trihydrated potassium

127 ($K_2HPO_4.3H_2O$) adjusted at pH 7.4. The electrolyte solution or the buffer was necessary

128 to maintain the minimum energy flow in the highest dilutions.

129 To compare the behaviour of the voltammetric response of the aliso infusion with an

130 antioxidant chemical pattern, dilutions of trolox (0.25, 0.5, 1.25, 2.5 and 5 mM) were also

131 measured in the same buffer phosphate. The voltammetric response of trolox was also

132   employed to develop the mathematical model to be used to predict the antioxidant activity

133   of aliso, expressed as a trolox equivalent.

134   In order to check the accuracy of the antioxidant prediction value obtained and

135   compared with the real one, a chemical ABTS method based on the method of Re (Re et

136   al., 1999), as described by Kuskoski (Kuskoski, Asuero, Troncoso, Mancini-Filho & Fett,

137   2005), was employed as a reference, but with slight modifications. As a result, the

138   antioxidant capacity of aliso, expressed as a trolox equivalent, was 10.58 ± 0.73 mM,

139   which is similar to those obtained by other authors (Chirinos et al., 2013). So, the values

140   for dilutions (2.5, 5, 10, 20 % v/v) were 0.26 mM, 0.53 mM, 1.058 mM and 2.12 mM,

141   respectively.

142   Experimentation with different concentrations of the analyte allowed us to know not

143   only the capacity of the equipment to order and characterise the samples according to

144   their voltammetric responses, but also the possibility of defining behaviours of patterns

145   or a certain chemical compound present in the dissolution to be analysed.

146

147   *Voltammetric Electronic Tongue (VET)*

148   The measurement system used herein was designed and developed by the Institute of

149   Molecular Recognition and Technological Development of the Polytechnic University of

150   Valencia in Spain (Garcia-Breijo et al., 2013). It consists of electronic equipment

151   connected to a PC, a software application that runs on the PC and electrodes. The software

152   application allows the configuration of measurements (including the definition of the

153   signal to be applied to the working electrodes). Having configured the measurement, the

154   corresponding information is sent to the electronic equipment, which takes the

155   measurement and transmits the temporal evolution values of the resulting current signal

156 to the PC. The data collected by the PC are then stored for later processing by multivariate

157 analysis tools.

158   Up to four working electrodes, based on noble metals (Ir, Rh, Pt and Au), were used

159 in this study. Noble electrodes were housed respectively in two stainless steel cylinders.

160 The outer part of these cylinders was used as the counter electrodes, while a standard

161 Ag/KCl electrode was used as the reference electrode.

162

163 *Pulses sequence. Studying the influence of the potential pulses amplitude order in the*

164 *sequence.*

165   In order to evaluate how the potential signal applied to the working electrodes of

166 VET affected the voltammetric response, four different signals were studied. The first

167 evaluated signal was a linear potential scan, which is typically used in cyclic voltammetry

168 (Figure 1-a) with a maximum and minimum voltage of 1,000 mV and -1,000 mV

169 respectively, and a scan rate of 250 mV/s. Due to the digitalisation process which occurs

170 in the measurement equipment, this signal comprised micropulses with an amplitude of 8

171 mV and a pulse width of 32 ms. The second signal (Figure 1-b) was composed of 50

172 micropulses (50+) with a potential increment between pulses of 18mV, which started at

173 0mV and reached a top potential of 800mV. The third sequence (4+) included four pulses

174 of 0mV, 200mV, 500mV and 800mV (Figure 1-c), while the fourth sequence (3+)

175 consisted of three pulses at 0mV, 500mV and 800mV (Figure 1-d). For signals 50+, 4+

176 and 3+, the pulse width was 100 ms.
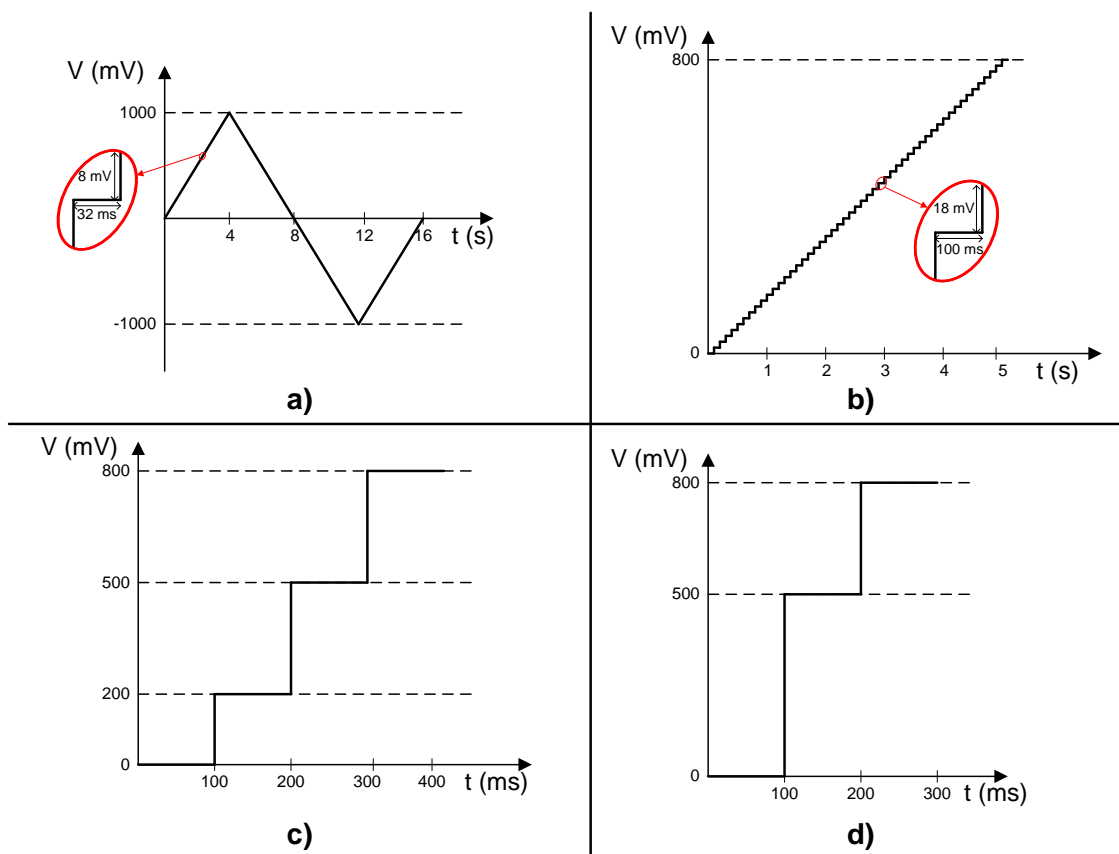
177

178 Figure 1. Signals applied to the working electrodes of VET. a) linear potential scan with

179 a maximum voltage of 1,000 mV, a minimum voltage of -1,000 mV and a scan rate of

180 250 mV/s; b) 50 micropulses with a potential increment between pulses of 18 mV, from

181 0 mV to 800 mV; c) four pulses of 0 mV, 200 mV, 500 mV and 800 mV; d) three pulses

182 of 0 mV, 500 mV and 800 mV. For signals b), c) and d), the pulse width was 100 ms.

183

184 *Statistical analysis.*

185 The effect of dilution on the voltammetric response at different pulse widths (time of

186 pulse application) was subjected to a variance study (ANOVA).

187 To obtain the antioxidant prediction model, locally weighted regression (LWR) was used.

188 LWR calculates a single locally weighted regression model using the given number of

189 principal components regression (PCR) *ncomp* to predict a dependent variable *y* from a

190 set of independent variables x. LWR models are useful for making predictions when

191 dependent variable y has a non-linear relationship with the measured independent

192 variables, x (Centner & Massart, 1998; Cleveland & Devlin, 1988). Two thirds of the total

193 available data were used to develop models to obtain the model, and the remaining data

194 to test it (prediction).

195 The evaluation of the accuracy of the models and antioxidant prediction was made by

196 examining the relative mean square error (RMSEP), bias and R^2 of the prediction

197 regression.

198

199 **RESULTS AND DISCUSSION**

200

201 *Potential signals evaluation*

202 The voltammograms obtained when the cyclic voltammetry signal was applied showed

203 different voltammetry responses for both the trolox chemical pattern and aliso dilutions.

204 For trolox, all the noble electrode (Ir, Pt, Rh and Au) voltammograms included a

205 characteristic electrochemical peak, whose value increased with the dilution

206 concentration. Figure 2 shows the trolox (A) and aliso (B) cyclic voltammograms for Ir,

207 Rh, Pt and Au, respectively. The trolox voltammograms showed quasi-reversible redox

208 reactions with a clearly maximum anodic point at around 750 mV for Ir, 650 mV for Rh,

209 550 mV for Pt and 480 mV for Au. Similar electrochemical behaviour for trolox has been

210 previously observed by other authors (Gulaboski, Mirčeski, & Mitrev, 2013; Pekec,

211 Feketefoldi, Ribitsch, Ortner, & Kalcher, 2013; Pohanka et al., 2011)). In their works a

212 voltammetric signal for trolox that corresponded to an irreversible redox process at

213   potentials of about +0.4 V was obtained with a glassy carbon used as the working

214   electrode or at 670 mV when employing the Pt electrode.

215   This electrochemical behaviour was reproduced for only aliso with the Au electrode, for

216   which a highly attenuated response was detected at 400 mV. No other electrode showed

217   any characteristic electrochemical reaction for aliso at the potential values where the

218   reaction peaks for trolox were obtained. Thus using cyclic voltammetry with Pt, Ir or Rh

219   electrodes did not allow any discrimination between the aliso dilutions at different

220   concentrations. The fact that the voltammetric response obtained for trolox was attenuated

221   or even disappeared when aliso dilutions were measured was probably due to the

222   adsorption of a variety of compounds on the electrode surface, in addition to phenolic and

223   polyphenolic compounds, essential oils and some other compounds present in aliso

224   (Gonzalez, Suarez, Garcés de Granada, & Orozco de Amezquita, 2011).
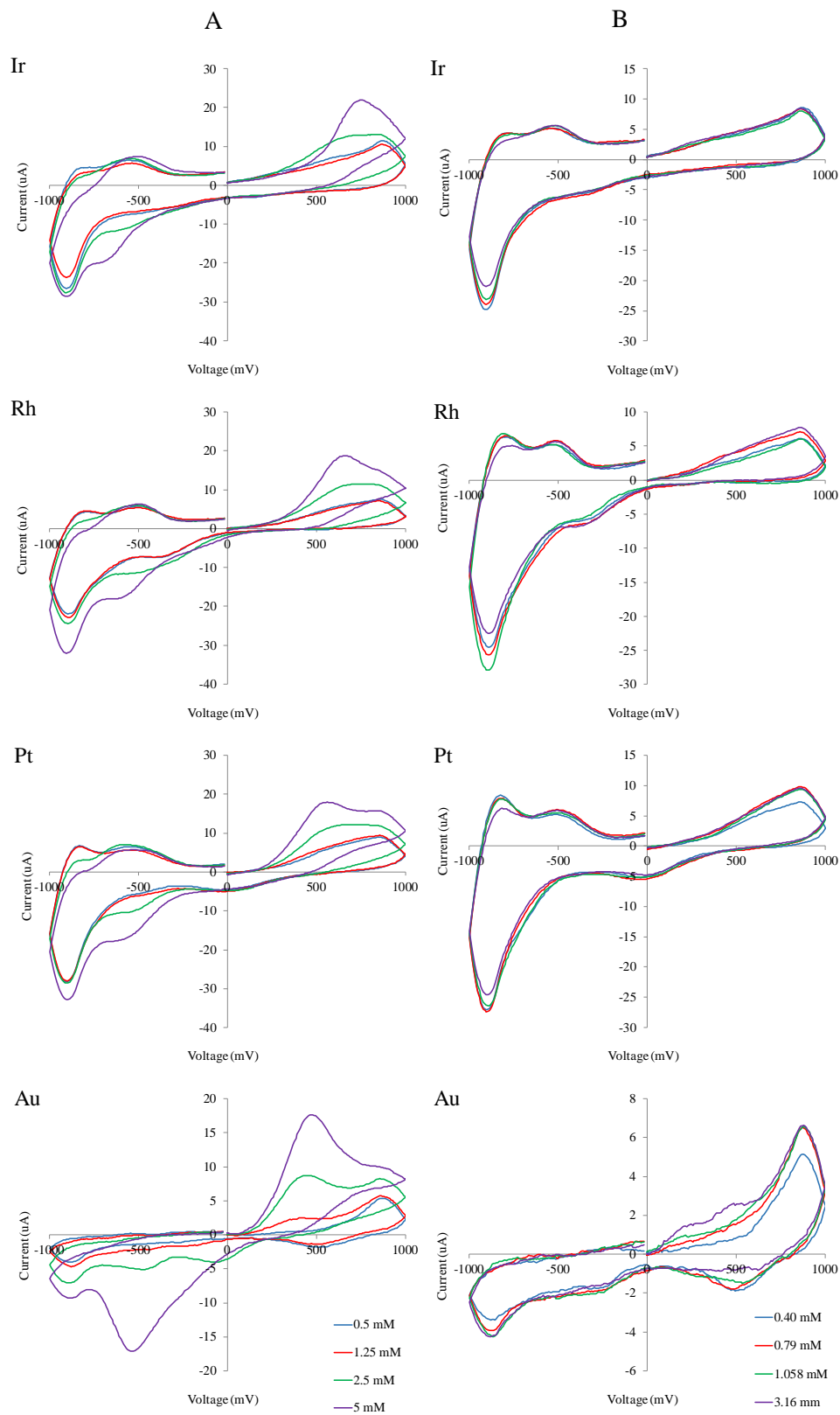
225   Figure 2. A. Trolox and B. Aliso responses measured through Ir, Rh, Pt and Au electrodes

226 Figure 3 shows the values of the temporal evolution of the current measured while

227 applying the 800 mV pulse to Ir and Rh electrodesin aliso dilutions for pulse sequences

228 50+ (A), 3+ (B) and 4+ (C). Figure 5 shows the same information when the 500 mV pulse

229 was applied to electrodes Pt and Au. For simplicity purposes, only five current point in

230 the pulse (at 20, 40, 60, 80 and 100 ms) were collected and the average values of the

231 repetitions with their corresponding standard error were plotted. The selection of the pulse

232 to be analysed per electrode was based on the voltammetric response observed in the

233 trolox measurement.

234 According to these results, different pulse configurations produced distinct

235 electrochemical responses, which were capable or incapable of recognising and

236 discriminating between samples and concentrations (Ivarsson et al., 2001). No

237 discrimination between the aliso concentrations was detected for pulse sequence 50+ for

238 any electrode (Figure 3-A). The cyclic voltammetry potential signal and pulse sequence

239 50+ were strikingly similar, as was the resulting current response for 500mV and 800

240 mV.

241
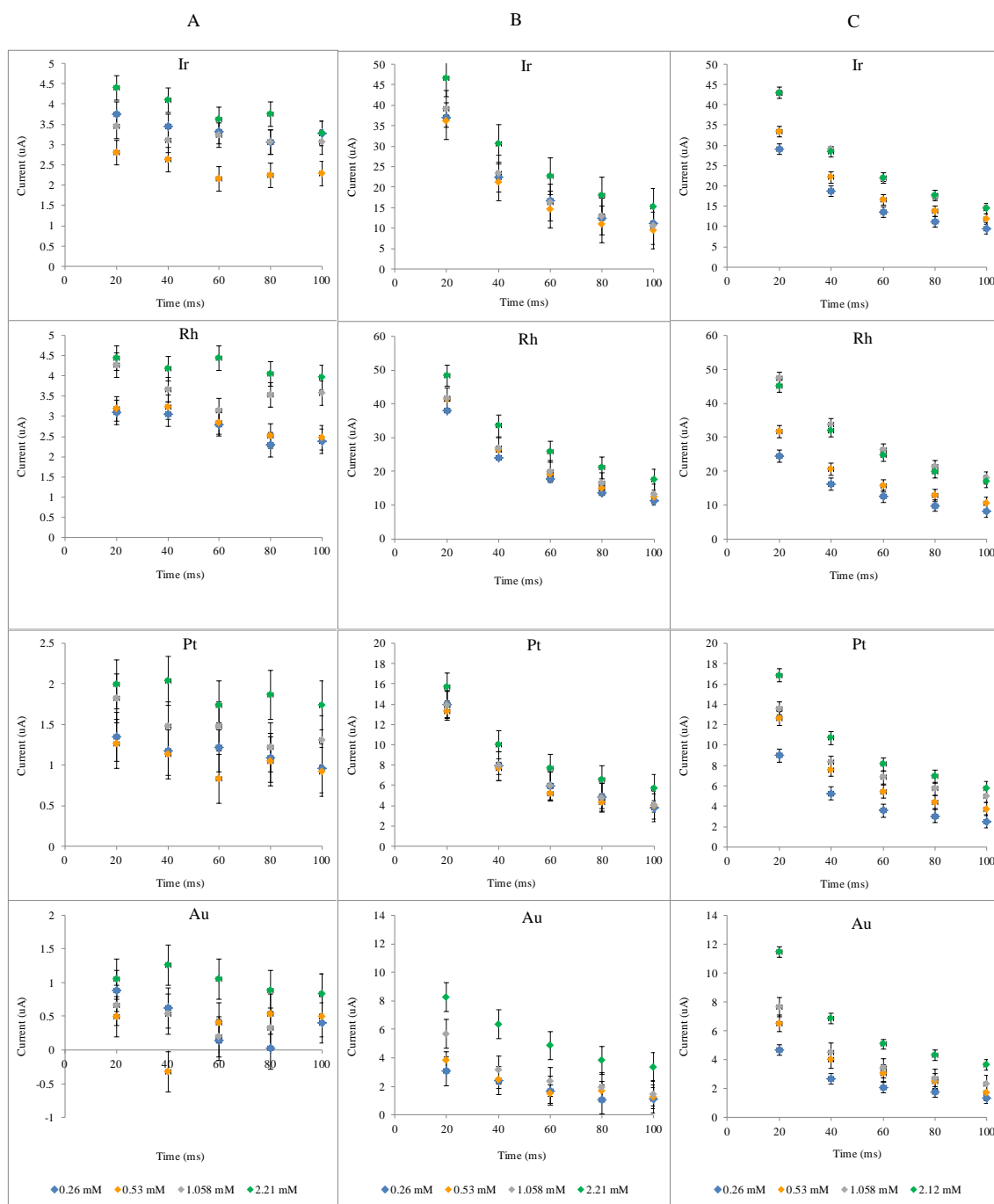
Figure 3.Voltammetric response when the 800 mV pulse was applied to electrodes Ir, Rh,

Pt and Au in the aliso dilutions for pulses sequences 50+ (A), 3+ (B) and 4+ (C).

For all the electrodes, the current values measured with pulse sequences 3+ and 4+ were

much higher than those obtained with cyclic voltammetry and sequence 50+. According

to these results, the increase in the potential difference between two consecutive pulses

248 in the sequence raised the measured current. Part of this current was of faradaic origin,

249 while another part was due to non-faradaic processes (double layer)(Winquist F. 2008).

250 Pulse sequence 4+ showed a very good level of discrimination between the aliso dilutions

251 for Pt and Au, and a moderate discrimination level for Ir and Rh, but only the

252 Au electrode presented an acceptable discrimination level for pulse sequence 3+. The

253 standard deviation of the data obtained for pulse sequence 3+ was greater than for pulse

254 sequence 4+. The cause of these differences could be the importance of non-faradaic

255 processes for each pulse sequence. The transition between 0 V and 500 mV in pulse

256 sequence 3+ was done in one step, while an intermediate pulse of 200 mV was introduced

257 for pulse sequence 4+. The non-faradaic current was related with a charges reorganization

258 on the electrode surface when the potential applied to it was modified. If the potential

259 changed in one step, the charges reorganization process would be more important and the

260 non-faradaic current would be higher. When the potential change was achieved in two

261 steps, the non-faradaic current generated when the final potential value was reached

262 would be lower. For pulse sequence 3+ the non-faradaic component of the measured

263 current was greater than for pulse sequence 4+. So, major increase in the potential

264 difference between two consecutive pulses generates higher current values, but the

265 presence of an intermediate pulse, before the relevant pulse, increase the potential

266 difference even more. On the one hand, this implied a wider dispersion in the

267 measurement for pulse sequence 3+ as the non-faradic currents were affected by many

268 factors, such as cell geometry, sample conductivity and others (Bataller, Gandía, García-

269 Breijo, Alcañiz, & Soto, 2015), and they consequently had lower reproducibility. On the

270 other hand, as pulse sequence 4+ presented fewer non-faradaic components, the main

271 contribution to the measured current came from the faradaic processes, which for

272 electrodes Au and Pt at 500 mV, were related with antioxidant capacity (Figure 2). The

273   best faradaic signal, which is capable of discriminating between concentrations, was also

274   generated at 20 ms. Other authors, who worked with pure chemical patterns and an

275   electrochemical cell similar to that used herein, have reported that the information from

276   faradic processes is more relevant after the first 10 ms (Campos, Alcañiz, Masot, et al.,

277   2012).

278   Figure 4 shows all the aliso concentrations current values after 20 ms of the 500 mV pulse

279   for the three pulse sequences together and the current value obtained at 500 mV in the

280   cyclic voltammograms. As expected, cyclic voltammetry and pulse sequence 50+ did not

281   allow any discrimination among the aliso dilutions. The current values measured at 20

282   ms in pulse 500 mV for sequence 3+ and 4+ presented a linear relation with the aliso

283   concentration. Once again the results obtained for pulse sequence 3+ presented more

284   dispersion and less sensitivity that those obtained for pulse sequence 4+.

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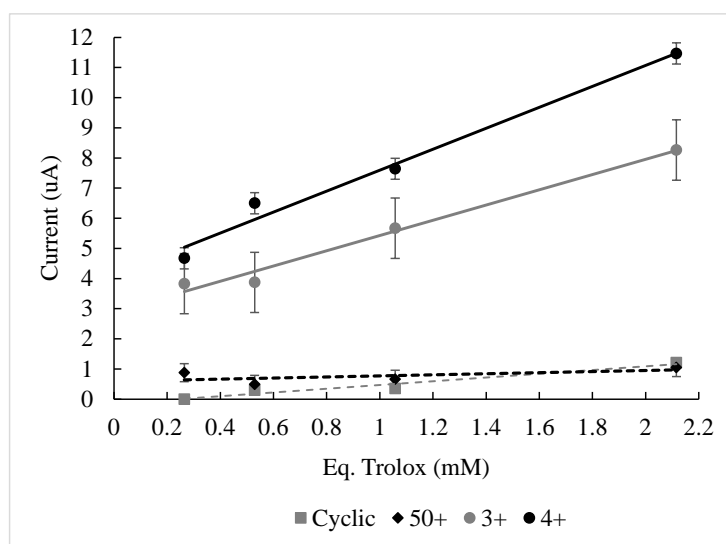
286

287   Figure 4 Voltammetry response at 20 ms during pulse 500 mV for electrode Au in aliso.

288

289

291   As pulsetrain 4+ (0, 200, 500 and 800mV) showed the highest discrimination levels for

292   the aliso electrochemical response, this pulse sequence was used to develop prediction

293   models of aliso antioxidant capacity. The methodology applied to build these models was

294   similar to that used in a previous work with camu-camu (Baldeón et al., 2015), but in this

295   case LWR, and not the partial least squares (PLS) procedure, was employed. Models were

296   built and validated with the trolox data and then applied to predict aliso measurements.

297   In order to evaluate the convenience of using all the voltammetric electronic tongue

298   electrodes or just the electrode with the best individual results, several models were built:

299   one for each noble electrode and another with the information of all the metals. Table 1

300   shows the results of LWR models for trolox. The most robust model was obtained using

301   the Au electrode data, while the obtained models presented lower R^2 and higher RMSEP

302   and bias values for the other electrodes (Ir, Rh and Pt). When all the electrodes were used,

303   the model displayed good behaviour, probably because the LWR algorithm had conferred

304   a higher weight to the Au electrode data. According to these results, the Au electrode

305   voltammetric data sufficed to obtain excellent prediction models for trolox simple

306   dilutions.

307   The trolox prediction models were then applied to predict the aliso antioxidant capacity

308   and these predictions were compared with those obtained by the chemical ABTS method

309   (10.58 ± 0.73 mM of the trolox equivalent). The results are also shown in Table 1. The R^2

310   for Au was good in this case, but the RMSEP and bias values were too high. None of the

311   other three electrodes gave acceptable results but, surprisingly, the combination of the

312   data of all the electrodes led to an excellent aliso antioxidant capacity prediction.

313

314   Table 1 Results of the LWR models generated by employing the voltammetry response

315   to Au electrodes and the noble electrodes, obtained for aliso predictions.

	Electrodes	RMSEP	Bias	R^2
Trolox model	Au	0.101	0.002	0.998
	Pt	0.578	-0.011	0.891
	Rh	1.3	0.372	0.497
	Ir	0.617	-0.0759	0.878
	Noble metals	0.129	-0.035	0.996
Aliso prediction	Au	0.641	-0.508	0.951
	Pt	0.671	-0.065	0.528
	Rh	0.635	-0.197	0.546
	Ir	1.032	-0.159	0.024
	Noble metals	0.117	-0.013	0.968

316

317   Figure 5 shows the theoretical trolox equivalents (mM) of the chemical ABTS method,

318   *vs.* prediction employing the LWR models developed using noble metals (A)and Au

319   electrode (B). As previously mentioned, the trolox prediction was very good in both cases.

320   However, when the antioxidant aliso capacity was predicted employing the Au electrode

321   (Figure 5B), although all the points came very close to the regression line (red line in

322   Figure 5-B), the predicted values were lower than the measured ones so both the RMSEP

323   and bias values presented a high value (RMSEP=0.641 and Bias=-0.508). Instead of the

324   aliso antioxidant capacity prediction using all the electrodes, the resulting plot shows all

325   the points practically aligned with the regression line, which almost fitted the 1:1 line.

326

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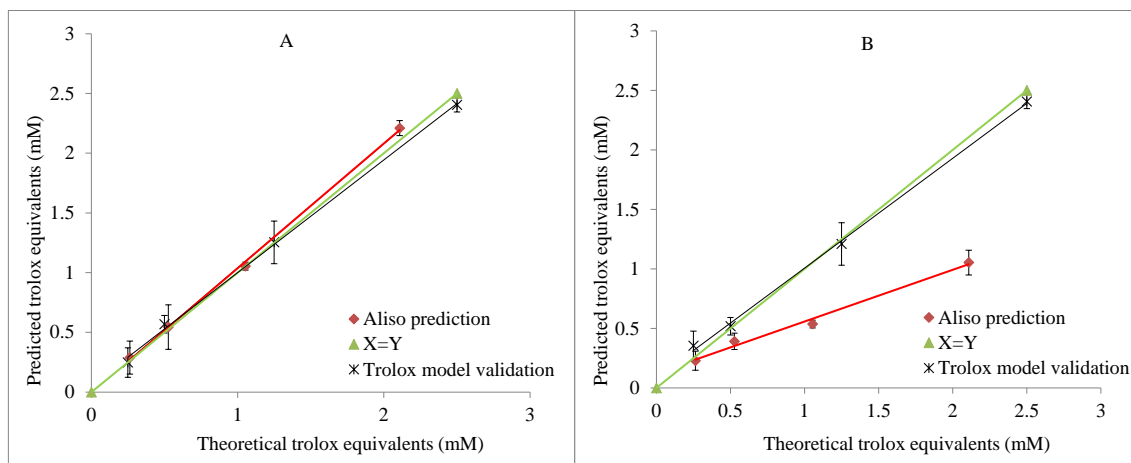
Figure 5.Theorical *vs.* predicted trolox equivalent (mL) for trolox and aliso dilutions

employing the mathematical prediction model obtained using the voltammetry

information from the noble electrodes (A) or the Au electrode (B).

The explanation for the results should be found in the electronic tongue definition.

Selective sensors are very useful in applications where the signal generated by the sensor

is almost exclusively related to the compound to be measured. Yet when a selective sensor

is used to quantify a compound in certain complex matrices, its response may be affected

by the presence of other compounds (interferents). An electronic tongue is described as a

set of non-specific sensors that present a characteristic electronic fingerprint for each

species in the sample (cross-sensitivity). The combination of information provided by all

the sensors is affected by all the species present in the sample. Processing this information

with the right multivariate analysis tool can lead to the quantification of one compound

or more by compensating for the interferences of the other species (Alcañiz et al., 2012).

The Au electrode voltammetric response correlated closely to the trolox concentration.

When simple trolox dilutions were measured, Au electrodes provides an excellent

quantification model. When aliso dilutions were measured, the voltammetric data

obtained from the Au electrode were related with the antioxidant capacity, but were also

346   affected by the interferents contained in the dilution. Combining the Au electrode data

347   with the information provided by the other electrodes improved the prediction capability

348   of the model as it incorporated the voltammetric response into these interferents.

349

350   **CONCLUSION**

351   The antioxidant capacity of aliso was evaluated by the electronic voltammetric tongue

352   (EVT) by means of sensors made with noble metals (Ir, Rh, Pt and Au). The importance

353   of the applied signal was shown to be essential. Higher current values were obtained when

354   a major increase in the potential difference between two consecutive pulses was

355   introduced as a result of a faradaic and a non-faradaic signal. However, the best results

356   were obtained with a potential sequence in which an intermediate pulse (200 mV) was

357   introduced immediately before the relevant pulse (500 mV). The intermediate pulse

358   reduced the non-faradic signal because the charges reorganisation on the surface electrode

359   was minimised. The uses of these sequence potentials allowed us to obtain two

360   mathematical models to predict antioxidant capacity: one employed only the Au

361   electrode, while the other employed all the electrodes. When models were employed to

362   predict antioxidant trolox capacity (models validation), both models gave good accuracy.

363   Only model that used all the electrodes was capable of correctly predicting the antioxidant

364   capacity of the aliso dilutions. This result confirms the advantages of using several cross-

365   sensitive sensors in applications where the compound to be quantified is mixed with other

366   compounds.

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