

1 **A revisited hemolytic assay for palytoxin detection: limitations for its quantitation in**  
2 **mussels**

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4 **Authors**

5 Valentina Brovedani<sup>a</sup>, Silvio Sosa<sup>a</sup>, Mark Poli<sup>b</sup>, Martino Forino<sup>c</sup>, Katia Varello<sup>d</sup>, Aurelia  
6 Tubaro<sup>a</sup>, Marco Pelin<sup>a</sup>

7

8 <sup>a</sup> Department of Life Sciences, University of Trieste, Via A. Valerio 6, 34127 Trieste, Italy

9 <sup>b</sup> U.S. Army Medical Research Institute of Infectious Diseases, Ft Detrick, Maryland, United  
10 States

11 <sup>c</sup> Department of Pharmacy, University of Napoli Federico II, 80131 Napoli, Italy

12 <sup>d</sup> Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, Via Bologna  
13 148, 10154 Torino, Italy

14

15 **E-mail**

16 [valentina.brovedani@phd.units.it](mailto:valentina.brovedani@phd.units.it) (Valentina Brovedani), [ssosa@units.it](mailto:ssosa@units.it) (Silvio Sosa),  
17 [mark.a.poli.civ@mail.mil](mailto:mark.a.poli.civ@mail.mil) (Mark Poli), [forino@unina.it](mailto:forino@unina.it) (Martino Forino),  
18 [katia.varello@izsto.it](mailto:katia.varello@izsto.it) (Katia Varello), [tubaro@units.it](mailto:tubaro@units.it) (Aurelia Tubaro), [mpelin@units.it](mailto:mpelin@units.it)  
19 (Marco Pelin)

20

21

22 **Corresponding author**

23 Prof. Aurelia Tubaro, Department of Life Sciences, University of Trieste, Via A. Valerio 6,  
24 34127 Trieste, Italy; e-mail: [tubaro@units.it](mailto:tubaro@units.it); Tel.: +39.040.5588835; Fax: +39.040.5583165.

25

## 26 **Abstract**

27 Palytoxin (PLTX) and its analogues have been detected as seafood contaminants and  
28 associated with a series of human foodborne poisonings. Due to some fatalities ascribed to the  
29 ingestion of PLTX-contaminated marine organisms, the development of methods for its  
30 detection in seafood is recommended by the European Food Safety Authority (EFSA). Due to  
31 its feasibility, the spectrophotometric hemolytic assay is widely used to detect PLTX in  
32 different matrices, even though a standardized protocol is still lacking.

33 Thus, on the basis of available assay procedures, a new standardized protocol was set up  
34 using purified human erythrocytes exposed to PLTX (working range:  $3.9 \times 10^{-10}$ - $2.5 \times 10^{-8}$  M) in  
35 a  $K^+$ -free phosphate buffered saline solution, for 5 h at 41°C. An intra-laboratory  
36 characterization demonstrated its sensitivity (limit of detection,  $LOD=1.4 \times 10^{-10}$  M and  
37 quantitation,  $LOQ=3.4 \times 10^{-10}$  M), accuracy (*bias*=-0.8%), repeatability ( $RSDr=15\%$  and 6%  
38 for intra- and inter-day repeatability, respectively) and specificity. However, the standardized  
39 method seems not to be suitable for PLTX quantitation in complex matrices, such as mussels  
40 (*Mytilus galloprovincialis*) extracts, below the limit suggested by EFSA (30  $\mu$ g PLTXs/Kg  
41 shellfish meat). Thus, the hemolytic assay for PLTX quantitation in seafood should be used  
42 only after a careful evaluation of the specific matrix effects.

43

## 44 **Keywords**

45 Palytoxin, hemolytic assay, matrix effect, *Mytilus galloprovincialis*

*Abbreviations:* PLTX, palytoxin; LOD, limit of detection; LOQ, limit of quantitation;  $RSDr$ ,  
relative standard deviation of repeatability; 42-OH-PLTX, 42-hydroxy-palytoxin; Ost-D,  
Ostreocin-D; OVTX, ovatoxin; EFSA, European Food Safety Authority; SAGM, Saline-  
Adenine-Glucose-Mannitol solution; ADSOL, Adenine-Dextrose Solution; D-PBS,  
Dulbecco's Phosphate Buffered Saline; EDTA, ethylenediaminetetracetic acid; O.D., optical  
density.

*Abbreviations:* PLTX, palytoxin; LOD, limit of detection; LOQ, limit of quantitation; RSDr, relative standard deviation of repeatability; 42-OH-PLTX, 42-hydroxy-palytoxin; Ost-D, Ostreocin-D; OVTX, ovatoxin; EFSA, European Food Safety Authority; SAGM, Saline-Adenine-Glucose-Mannitol solution; ADSOL, Adenine-Dextrose Solution; D-PBS, Dulbecco's Phosphate Buffered Saline; EDTA, ethylenediaminetetracetic acid; O.D., optical density.

## 47 **1. Introduction**

48 Palytoxin (PLTX) is a highly toxic non-polymeric complex molecule, originally isolated from  
49 zoanthids of the genus *Palythoa* (Moore and Scheuer, 1971), and later identified in benthic  
50 dinoflagellates of the genus *Ostreopsis* and cyanobacteria of the genus *Trichodesmium*  
51 (Ciminiello et al., 2008; Kerbrat et al., 2011). Over the years, several PLTX analogues have  
52 been detected in different marine organisms, including: (i) Ostreocin-D (Ost-D) in *Ostreopsis*  
53 *siamensis* (Usami et al., 1995); (ii) two stereoisomers of 42-hydroxy-PLTX (42-OH-PLTX),  
54 differing only for the configurational inversion at C50, identified in *Palythoa toxica* and *P.*  
55 *tuberculosa* (Ciminiello et al., 2014a); and (iii) ovatoxin-a (OVTX-a), the major toxin  
56 produced by *Ostreopsis cf. ovata* in the Mediterranean Sea (Ciminiello et al., 2012). In  
57 addition, a series of OVTX-a analogues have been identified, such as OVTX-b to -k and  
58 isobaric palytoxin (Brissard et al., 2015; García-Altare et al., 2015; Tartaglione et al., 2016).  
59 These toxins may enter the human food chain through their accumulation into different  
60 marine edible organisms, such as fishes, crustaceans, bivalves, gastropods, cephalopods,  
61 echinoderms, sponges and polychaete worms (Aligizachi et al., 2011; Biré et al., 2013; Gleibs  
62 and Mebs, 1999). In particular, a series of human poisonings characterized by general  
63 malaise, myalgia, cardiac problems, respiratory distress, and sometimes death, have been  
64 ascribed to the ingestion of PLTX-contaminated fishes and crabs in tropical areas (Deeds and  
65 Schwartz, 2010; Tubaro et al., 2011b; Wu et al., 2014).

66 In recent years, microalgae belonging to the genus *Ostreopsis* have frequently bloomed in the  
67 temperate Mediterranean Sea and the relevant toxins (mainly OVTX-a) have been detected in  
68 microalgae, aerosolized seawater and some marine edible organisms. However, no foodborne  
69 poisonings associated with PLTXs have yet been documented in this area, so far (Biré et al.,  
70 2013; Ciminiello et al., 2014a; Del Favero et al., 2012).

71 Although there is no official regulation for PLTXs in seafood, the European Food Safety  
72 Authority (EFSA) suggested a maximum limit of 30  $\mu\text{g}$  PLTXs/Kg of shellfish meat, and  
73 recommended the development of specific, rapid, precise and accurate methods for PLTXs  
74 quantitation in seafood during monitoring programs (EFSA, 2009). Despite several methods  
75 for PLTXs detection have been already published (Riobò et al., 2011; Tubaro et al., 2014),  
76 they are not completely validated to be officially accepted, frequently requiring expensive  
77 equipments and highly qualified operators, and/or presenting limitations in terms of  
78 sensitivity, specificity and matrix effects.

79 Among them, the delayed hemolytic assay is one of the most used screening methods for  
80 PLTX quantitation, due to its simplicity, cheapness, rapidity and sensitivity. It is based on the  
81 toxin ability to convert the  $\text{Na}^+/\text{K}^+$  ATPase of mammal erythrocytes to a nonspecific cationic  
82 channel, leading to a rapid loss of  $\text{K}^+$  ions from cells and a delayed hemoglobin release that  
83 can be easily measured spectrophotometrically (Habermann et al., 1981). The hemolytic assay  
84 is usually carried out following the Bignami's protocol (1993) with or without modifications,  
85 but a standardized and universally accepted procedure has not been defined, so far. Most  
86 literature studies report the original assay with a series of modifications mainly concerning  
87 the origin of erythrocytes, the time and temperature of their incubation with PLTX, and/or the  
88 working buffer composition (Aligizaki et al., 2008; Biré et al., 2013; Brissard et al., 2014;  
89 Gleibs et al., 1995; Kim et al., 2002; Lenoir et al., 2004; Malagoli, 2007; Onuma et al., 1999;  
90 Pezzolesi et al., 2012; Riobò et al., 2006; Riobò et al., 2008; Taniyama et al., 2001; Taniyama  
91 et al., 2003; Volpe et al., 2014; Wachi and Hokama, 2001). Due to these experimental  
92 variables, a standardized protocol for the hemolytic assay to be considered as a reference  
93 procedure in PLTX quantitation is still lacking.

94 Moreover, edible marine species could contain hemolytic compounds different from PLTXs,  
95 tentatively interfering with the assay giving false positive results. For instance, a recently

96 developed biosensor to quantify PLTX in mussels, based on lactate dehydrogenase release  
97 from sheep erythrocytes, appeared to be influenced by a significant mussels matrix effect and  
98 low PLTX recovery (Volpe et al., 2014). Thus, the accuracy and precision of the hemolytic  
99 assay for spectrophotometric PLTX quantitation in shellfish needs to be properly evaluated.  
100 Hence, this study was carried out to set up a novel standardized protocol for the hemolytic  
101 assay useful as reference procedure and to characterize its suitability for PLTX quantitation in  
102 mussels (*Mytilus galloprovincialis*), often heavily contaminated by PLTXs (Brissard et al.,  
103 2014; Aligizaki et al., 2008; Aligizaki et al., 2011; Amzil et al., 2012).

104

## 105 **2. Materials and methods**

### 106 *2.1 Toxins and other materials*

107 PLTX was purchased from Wako Pure Chemicals Industries Ltd. (Osaka, Japan; purity > 90  
108 %). 42*S*-hydroxy-50*S*-palytoxin and 42*S*-hydroxy-50*R*-palytoxin were isolated from *Palythoa*  
109 *tuberculosa* and *P. toxica*, as previously reported (Ciminiello et al., 2009; Ciminiello et al.,  
110 2014b). Ovatoxin-a and the mixture of ovatoxin-a,-d,-e were kindly provided by Prof. P.  
111 Ciminiello (Università di Napoli “Federico II”, Naples, Italy). The mouse monoclonal anti-  
112 PLTX antibody 73D3 (mAb-PLTX) was produced by a hybridoma cell culture, as previously  
113 described (Bignami et al., 1992). All the other reagents were of analytical grade and  
114 purchased from Sigma-Aldrich (Milan, Italy), if not otherwise specified.

115

### 116 *2.2 Red blood cell purification*

117 Blood samples from healthy human volunteers were obtained from the Transfusion Center,  
118 Azienda Ospedaliera Universitaria, Trieste, Italy. All donors signed an approved consent form  
119 giving permission for the collection and use of blood for research purposes (WMA  
120 Declaration of Helsinki). Blood was drawn by venipuncture between 08.00 a.m. and 10.00

121 a.m. to minimize variability due to circadian rhythms, and immediately processed. Blood,  
122 collected into standard triple bag systems, was fractionated following standard procedures to  
123 obtain buffy-coats, used to purify red blood cells. Buffy coats (50 ml) were diluted 1:1 (v/v)  
124 in the erythrocytes preservation solution (specified in section 2.3) and then centrifuged at  
125 2400 rpm for 10 min at 4 °C. Red blood cells pellet (2 ml), suspended in the red blood cells  
126 preservation solution (10 ml), was washed three times by centrifugation at 1500 rpm for 5 min  
127 at 15 °C. Then, the final pellet was re-suspended 1:10 (v/v) in the preservation solution.

128

### 129 *2.3 Red blood cells storage*

130 Three storage solutions were used to evaluate red blood cells preservation at 4° C: Saline-  
131 Adenine-Glucose-Mannitol solution (SAGM, containing  $5 \times 10^{-2}$  M dextrose, 0.15 M NaCl,  
132  $2.4 \times 10^{-3}$  M adenine and  $2.9 \times 10^{-2}$  M d-mannitol) (Zehnder et al., 2008), Adenine-Dextrose  
133 Solution (ADSOL, containing 0.12 M dextrose, 0.15 M NaCl,  $3.9 \times 10^{-3}$  M adenine and  $4.1 \times 10^{-2}$   
134 M d-mannitol) (Moore, 1991) and Dulbecco's Phosphate Buffered Saline (D-PBS)  
135 containing 1mM ethylenediaminetetracetic acid (EDTA) and 5 mM glucose (Lowe et al.,  
136 1973). Red blood cells preservation was evaluated by means of erythrocyte resistance to  
137 spontaneous lysis and erythrocyte concentration. For the first parameter, 0.4 ml of red blood  
138 cells suspension was centrifuged at 1500 rpm for 5 min and the optical density of the  
139 supernatant was then measured at 405/540 nm (Microplate autoreader; Bio-Tek Instruments;  
140 Vinoski, VT). Red blood cell concentration was evaluated by cell counting following the  
141 Trypan Blue Exclusion Test. Acceptable thresholds were constant cells concentration and  
142 optical densities lower than 0.5, a value close to the average optical densities of the negative  
143 controls (125 µl of red blood cell suspension incubated with K<sup>+</sup>-free D-PBS without toxin).  
144 Both parameters were assessed daily for 36 days.

145

#### 146 *2.4 Experimental design*

147 The hemolytic assay was standardized considering the following parameters: the use of  
148 purified human erythrocytes *vs* whole blood, the influence of selected ions (borates, calcium,  
149 sodium, and potassium) on PLTX-induced hemolysis, and the incubation temperature of  
150 erythrocytes exposed to PLTX. The standardized assay was subsequently characterized  
151 according to the international principles, as described by the Eurachem Guide (Magnusson  
152 and Örnemark, 2014).

153

#### 154 *2.5 Standardized hemolytic assay*

155 After washing, red blood cells were pelleted by centrifugation (1500 rpm for 5 min) and re-  
156 suspended in K<sup>+</sup>-free D-PBS at the concentration of  $1 \times 10^8$  cells/ml. In 96-wells plates, 125  $\mu$ l  
157 of PLTX solution and 125  $\mu$ l of the erythrocytes suspension were added to each well and  
158 incubated for 5 h at 41 °C (PLTX final concentrations:  $1.22 \times 10^{-11}$ - $4.00 \times 10^{-7}$  M). As negative  
159 controls, 125  $\mu$ l of red blood cell suspension were incubated with 125  $\mu$ l K<sup>+</sup>-free D-PBS  
160 without toxin. As a positive control, 100% hemolysis was achieved by incubating the  
161 erythrocytes suspension with 125  $\mu$ l of 0.1% Tween 20 (v/v) for 5 h at 41° C. After  
162 incubation, the plate was centrifuged at 1500 rpm for 5 min at 15 °C and, using a  
163 multichannel pipette, the supernatant was carefully transferred into a clear flat bottom 96-  
164 wells plate avoiding pellet braking up. Optical density (O.D.) of supernatant was then  
165 measured at 405/540 nm and the percentage of hemolysis calculated with respect to the  
166 positive control by the following formula:

167 % of hemolysis =  $100 \times (\text{O.D. PLTX exposed sample} - \text{O.D. negative control}) / (\text{O.D. positive}$   
168  $\text{control} - \text{O.D. negative control})$ .

169

#### 170 *2.6 Evaluation of the matrix effect*



171 To assess the hemolytic assay suitability to quantify PLTX in mussels at levels below the  
172 suggested EFSA limit (30  $\mu\text{g}$  PLTXs/kg edible parts, corresponding to about  $11.2 \times 10^{-9}$   
173 mol/kg; EFSA, 2009), different extracts of *Mytilus galloprovincialis* edible parts were  
174 prepared. Each extract was analyzed by liquid chromatography high resolution mass  
175 spectrometry (LC-HRMS) to verify the absence of PLTX before the matrix effect evaluation.  
176 Mussels were collected in the Gulf of Trieste (Italy) and shucked meat (200 g) was  
177 homogenized (14000 rpm, 3 min) using an Ultra-Turrax (Ika-Werk; Staufen, Germany). The  
178 homogenate (1 g) was extracted three times with 3 ml of different solvents (80%, 50% or 20%  
179 aqueous ethanol or aqueous methanol). Each extractive solution was then centrifuged at 5500  
180 rpm for 30 min, the corresponding supernatants were pooled and the volumes adjusted to 10  
181 ml with the relevant extraction solvents to obtain six extracts at a final concentration of 0.1 g  
182 mussels meat equivalents/ml. The hemolytic activity of each extract was then evaluated at  
183 five dilutions (1:1, 1:10, 1:50, 1:100 and 1:1000, v/v) to assess background hemolysis.  
184 At dilutions devoid of background hemolysis, extracts were spiked with different PLTX  
185 concentrations to prepare matrix matched-samples at PLTX levels ranging from  $3.9 \times 10^{-10}$  to  
186  $2.5 \times 10^{-8}$  M. These matched samples were then analyzed using the hemolytic assay. The  
187 relevant hemolytic activity was compared to that induced by the same PLTX concentrations  
188 without matrix.

189

## 190 *2.7 Statistical analyses*

191 Results of the hemolytic assay are presented as mean $\pm$ SE of at least three independent  
192 experiments performed in triplicate. Linearity ( $r^2$ ) of the calibration curve was estimated by  
193 linear regression analysis, using the GraphPad Prism software version 5.0 (GraphPad Prism;  
194 GraphPad Software, Inc.; San Diego, CA). Concentration-effect curves were compared by  
195 two-way ANOVA statistical analysis and Bonferroni post test, and significant differences

196 were considered at  $p < 0.05$ .  $EC_{50}$  (effective concentration giving 50% hemolysis) was  
197 calculated by nonlinear regression using a four parameters curve-fitting algorithm of the  
198 GraphPad Prism software.

199 Hemolytic assay performance was characterized according to the international principles  
200 described by the Eurachem Guide (Magnusson and Örnemark, 2014). Briefly, limit of  
201 detection (LOD) and quantitation (LOQ) were expressed as PLTX concentration  
202 corresponding to the average of 10 blank values plus 3 or 10 times the standard deviations,  
203 respectively. Accuracy was measured as % Bias ( $n = 10$ ), calculated as % difference between  
204 PLTX concentration measured by the assay and the theoretical concentration in the sample  
205 divided by PLTX theoretical concentration. Repeatability was expressed as relative standard  
206 deviation of repeatability (RSDr), measured as % ratio between the standard deviation of  
207 independent results and their mean value. Both independent results obtained by the same  
208 operator in one day (intra-assay RSDr;  $n = 10$ ) and within a 6-month period by different  
209 operators (inter-assay RSDr;  $n = 10$ ) were considered.

210

### 211 **3. Results and discussion**

212

#### 213 *3.1 Storage of purified human erythrocytes*

214 The human erythrocytes model was chosen due to the easy availability of human blood, rapid  
215 isolation of significant cell numbers, and low cost. The use of purified human erythrocytes  
216 poses the need of a medium suitable to preserve the cells. To this aim, three cells storage  
217 solutions were evaluated: Saline-Adenine-Glucose-Mannitol Solution (SAGM), Adenine-  
218 Dextrose Solution (ADSOL) and D-PBS containing 1 mM EDTA and 5 mM glucose (PBS-  
219 EDTA-glucose), as described in section 2.3 (Lowe et al., 1973; Moore, 1991; Zehnder et al.,  
220 2008). Erythrocytes preservation in these solutions was daily evaluated, monitoring two

221 parameters up to 36 days: the spontaneous hemolysis (measured spectrophotometrically as  
222 hemoglobin release) and the erythrocytes concentration determined by visual cell counting.  
223 Acceptable thresholds were constant cell concentration in the storage solution and optical  
224 densities lower than 0.5. Only PBS-EDTA-glucose solution allowed maintenance of red  
225 blood cells suitable for the assay for up to 3 weeks (Fig. S1), probably due to the presence of  
226 crucial constituents providing the proper energy source to erythrocytes (glucose) and  
227 preventing coagulation (EDTA). Hence, these conditions allow to prepare a batch of human  
228 erythrocytes suitable for the hemolytic assay up to 3 weeks, reducing the working time and  
229 avoiding to purify the erythrocytes before each single experiment.

230

### 231 *3.2 Optimization of the hemolytic assay*

232 The hemolytic assay was standardized considering different experimental parameters reported  
233 by previous published studies. In the first series of experiments, the hemolytic assay was  
234 carried out following the most recent published method, using human erythrocytes (Malagoli,  
235 2007). Following Malagoli's protocol, the sensitivity of purified human erythrocytes to  
236 PLTX-induced hemolysis was compared to that of the whole human blood. The hemolytic  
237 activity of PLTX ( $7.8 \times 10^{-10}$ - $5.0 \times 10^{-8}$  M) towards purified human erythrocytes was  
238 significantly higher than that displayed by the whole human blood: 65% and 7% hemolysis  
239 were recorded at  $5.0 \times 10^{-8}$  M PLTX, respectively (Fig. 1A).

240 Previous studies demonstrated that borates, as  $\text{H}_3\text{BO}_3$  or  $\text{Na}_2\text{B}_4\text{O}_7$  ( $> 5.0 \times 10^{-6}$  M), and calcium  
241 ions ( $> 2.0 \times 10^{-5}$  M) in the buffer solution increases PLTX-induced hemolysis, probably by  
242 promoting the interaction between the toxin and its molecular target, the  $\text{Na}^+/\text{K}^+$  ATPase  
243 (Ahnert-Hilger et al., 1982; Habermann, 1983). Thus, the influence of these factors on PLTX-  
244 induced lysis of purified erythrocytes was evaluated. As shown in Fig. 1B, no significant  
245 differences in PLTX-induced hemolysis were observed between buffer solution containing 1

246 mM  $\text{H}_3\text{BO}_3$  and that containing 1 mM  $\text{Na}_2\text{B}_4\text{O}_7$ , in agreement with literature data, where  
247  $\text{H}_3\text{BO}_3$  or  $\text{Na}_2\text{B}_4\text{O}_7$  are interchangeably used. Regarding  $\text{Ca}^{2+}$  ions, no significant differences  
248 were recorded between PLTX-induced hemolysis in D-PBS with or without  $\text{CaCl}_2$  (Fig. 1C).  
249 However, the  $\text{Ca}^{2+}$  concentration in D-PBS solution (137 mM) is higher than that ( $>20 \mu\text{M}$ )  
250 reported to promote the interaction between the toxin and its target (Ahnert-Hilger et al.,  
251 1982). Thus, additional  $\text{Ca}^{2+}$  ions at millimolar concentrations in the D-PBS buffer solution  
252 containing 1mM  $\text{H}_3\text{BO}_3$  are not necessary to increase PLTX hemolytic activity.

253 Based on the mechanism of action of the toxin (i.e. interaction with the  $\text{Na}^+/\text{K}^+$  ATPase), the  
254 influence of  $\text{Na}^+$  and  $\text{K}^+$  ions on PLTX-induced hemolysis was also evaluated. The presence  
255 of  $\text{Na}^+$  ions ( $1.8 \times 10^{-4}\text{M}$  or  $3.6 \times 10^{-4}\text{M}$  NaCl) in the buffer solution was associated to a  
256 significant reduction of PLTX-induced hemolysis, probably due to the medium hypertonicity  
257 causing erythrocytes shrinking and volume reduction (Kregenow, 1971) (Fig. 1D). In  
258 contrast, while the  $\text{K}^+$ -free buffer containing  $\text{H}_3\text{BO}_3$  did not significantly influence the  
259 hemolytic activity of PLTX, the latter was significantly increased using  $\text{K}^+$ -free buffer  
260 without  $\text{H}_3\text{BO}_3$  (Fig. 1E).

261 Finally, the temperature influence on the hemolytic activity was evaluated. After erythrocytes  
262 incubation with PLTX for 5 h at  $41^\circ\text{C}$ , hemolysis was significantly higher than that recorded  
263 at  $37^\circ\text{C}$ , with  $\text{EC}_{50}$  values of  $6.2 \times 10^{-9}\text{M}$  (95% confidence intervals,  $\text{CI} = 5.3 - 7.2 \times 10^{-9}\text{M}$ )  
264 and  $4.9 \times 10^{-8}\text{M}$  (95%  $\text{CI} = 4.1 - 5.9 \times 10^{-8}\text{M}$ ), respectively (Fig. 1F). This result is in  
265 agreement to that reported by Habermann et al. (1981), suggesting that PLTX-induced  
266 hemolysis is temperature-dependent.

267 On the whole, these results allowed to optimize a protocol for the hemolytic assay, using  
268 purified human erythrocytes exposed to PLTX in a  $\text{K}^+$ -free D-PBS buffer at  $41^\circ\text{C}$  for 5 h.

269

270 *3.3 Characterization of the hemolytic assay*

### 271 3.3.1 Calibration curve for PLTX

272 Using the standardized hemolytic assay, the calibration curve for PLTX represented in Fig.  
273 2A was obtained. The working range for PLTX detection was  $3.9 \times 10^{-10}$  -  $2.5 \times 10^{-8}$  M, with a  
274 limit of detection (LOD) and quantitation (LOQ) of  $1.4 \times 10^{-10}$  M and  $3.4 \times 10^{-10}$  M,  
275 respectively. Analyzing the working range by linear regression, plotting the theoretical toxin  
276 concentrations against the PLTX concentrations measured by the hemolytic assay, a good  
277 correlation coefficient was found ( $r^2 = 0.9979$ ;  $n = 10$ ) (Fig. 2B). A mean *Bias* value (%) of -  
278 0.8% (range: -2.0% to 2.4%) highlights the accuracy of the measures (Table 1).

279 Intra-assay and inter-assay repeatability were then evaluated. A good correlation was  
280 observed, with  $r^2$  values of 0.9736 for intra-assay (Fig. 3A) and 0.9977 for inter-assay  
281 repeatability (Fig. 3B). These data were confirmed by the intra-day and inter-day repeatability  
282 coefficients (relative standard deviation of repeatability, RSDr) of 15% ( $n=10$ ) and 6% (six  
283 months period,  $n=10$ ), respectively (Table 1).

284 The haemolytic effect of PLTX in these conditions (described in section 3.2; 94% hemolysis  
285 at  $2.5 \times 10^{-8}$  M) is much higher than that reported by Malagoli (2007) and Taniyama (2001):  
286 they recorded a maximum hemolysis lower than 50% at the highest tested PLTX  
287 concentrations (i.e.  $10^3$  and  $10^2$  ng PLTX/ml, corresponding to  $3.7 \times 10^{-6}$  M and  $3.7 \times 10^{-7}$  M  
288 PLTX, respectively). These concentrations were 1 to 2 orders of magnitude higher than the  
289 highest concentration of the assay working range presented in this study (i.e.  $2.5 \times 10^{-8}$  M,  
290 giving 94% of hemolysis). Thus, an improved PLTX-induced hemolysis was achieved by  
291 lowering the osmolarity of the working buffer ( $K^+$  ions withdrawal) and increasing the assay  
292 temperature (41°C) in the optimized hemolytic assay protocol.

293

### 294 3.3.2 Cross-reactivity with PLTX analogues

295 The hemolytic assay was then evaluated for its ability to detect some PLTX analogues and N-  
296 biotinyl-PLTX within the working range set up for PLTX ( $3.9 \times 10^{-10}$  -  $2.5 \times 10^{-8}$  M). Although  
297 the hemolytic activity of nanomolar concentrations of 42S-OH-50S-PLTX (from *P. toxica*)  
298 was slightly lower than that of the same PLTX concentrations, its hemolytic potency ( $EC_{50} =$   
299  $5.8 \times 10^{-9}$  M, 95% CI =  $4.1 - 8.2 \times 10^{-9}$  M) was comparable to that of PLTX ( $EC_{50} = 6.2 \times 10^{-9}$  M,  
300 95% CI =  $5.3 \times 10^{-9}$  -  $7.2 \times 10^{-9}$  M) (Fig. 4). The latter result is in agreement with a previous  
301 study demonstrating a similar hemolytic activity of the two compounds, using mouse  
302 erythrocytes (Tubaro et al., 2011a). On the other hand, hemolysis induced by 42S-OH-50R-  
303 PLTX (stereoisomer from *P. tuberculosa*) was significantly lower than that of PLTX (Fig. 4),  
304 in agreement with its lower cytotoxic effects, previously observed on HaCaT cells (Ciminiello  
305 et al., 2014b). Also the hemolytic activity of pure OVTX-a and of a mixture of OVTXs  
306 (OVTX-a, -d and -e) was significantly lower than that of PLTX, similarly to their cytotoxicity  
307 toward skin keratinocytes, as recently demonstrated by Pelin et al. (2016). In contrast, the  
308 latter result is not in agreement with other studies hypothesizing that PLTX and OVTXs  
309 possess similar hemolytic activity (Brissard et al., 2014; Pezzolesi et al., 2012). However,  
310 since these studies were carried out using sea urchins and *Ostreopsis ovata* extracts  
311 containing OVTX analogues other than OVTX-a, -d and -e as well as isobaric PLTX  
312 (Brissard et al., 2014; Pezzolesi et al., 2012), we can speculate that those mixtures of toxins  
313 could display a hemolytic activity different than that of OVTX-a or of the other single  
314 OVTXs. Moreover, since complete studies on this matrices were not carried out, a significant  
315 interference of the extract's matrix cannot be excluded.

316 In addition, the hemolytic potency of a semisynthetic PLTX derivative, biotinylated PLTX,  
317 was significantly lower than that of PLTX, inducing only 5% hemolysis at the highest  
318 concentration ( $2.5 \times 10^{-8}$  M). Intriguingly, a biotin linked to the terminal PLTX amino group  
319 reduces PLTX-induced hemolysis, indicating the importance of the primary amine for the

320 hemolytic effect via  $\text{Na}^+/\text{K}^+$ -ATPase interaction. This finding is in agreement with previous  
321 evidences for N-acetyl-PLTX which biological activity via  $\text{Na}^+/\text{K}^+$ -ATPase were over 100  
322 times weaker than those of the parent compound PLTX, tentatively due to a change in the  
323 global toxin conformation that prevents its dimerization (Kudo and Shibata, 1980; Ohizumi  
324 and Shibata, 1980; Inuzuka et al., 2008). Moreover, this result suggests that the functional  
325 hemolytic assay detects only biologically active PLTX analogues. In contrast, other non-  
326 functional analytical methods can detect also biologically inactive PLTX-like compounds  
327 (Boscolo et al., 2013), which might not contribute the whole toxic potential of PLTXs-  
328 contaminated seafood samples.

329 In conclusion, these results demonstrated that the hemolytic assay has a good sensitivity for  
330 PLTX and 42S-OH-50S-PLTX from *P. toxica*. On the contrary, the stereoisomer 42S-OH-  
331 50R-PLTX from *P. tuberculosa*, as well as OVTX-a and OVTXs mixture have lower  
332 hemolytic activity. Anyway, all the tested natural PLTX analogues exert hemolytic effects,  
333 which suggest a common mechanism of action.

334

### 335 3.3.3 Hemolysis neutralization

336 It is known that the cardiac glycoside ouabain, which binds to the same molecular target of  
337 PLTX, inhibits the *in vitro* effects of PLTX (Habermann and Chhatwal, 1982; Pelin et al.,  
338 2013). Thus, to confirm the specific delayed hemolysis by PLTXs, excluding a possible  
339 hemolysis by other constituents of seafood samples, the standardized assay was carried out  
340 pre-incubating the red blood cells with 100  $\mu\text{M}$  ouabain for 30 min at 37 °C (Aligizaki et al.,  
341 2008; Biré et al., 2013; Brissard et al., 2014; Gleibs et al., 1995; Malagoli, 2007; Onuma et  
342 al., 1999; Pezzolesi et al., 2012; Riobo et al., 2006; Riobo et al., 2008; Taniyama et al., 2001;  
343 Taniyama et al., 2003; Volpe et al., 2014). Similarly, the mouse monoclonal anti-PLTX  
344 antibody (mAb-PLTX, 50  $\mu\text{g}/\text{ml}$ ) was used to neutralize the hemolysis induced by PLTX. The

345 PLTX induced hemolysis was completely inhibited by ouabain and only partially by the  
346 mAb-PLTX (Fig. 5), in agreement with previous findings reported by Bignami (1993).

347

### 348 3.4 Matrix effect

349 To assess the interference of mussel matrix on PLTX-induced hemolysis, different extracts of  
350 *Mytilus galloprovincialis* were prepared. Firstly, 80%, 50% and 20% aqueous methanol and  
351 ethanol extracts of toxin-free mussels were tested after 1:1, 1:10, 1:50, 1:100 and 1:1000 (v/v)  
352 dilutions, to verify whether the matrix (mussel extracts) could produce false positive results.  
353 All the aqueous ethanol extracts required dilutions higher than 1:100 to avoid matrix effects in  
354 the hemolytic assay (Fig. S2). On the other hand, no significant interferences were recorded  
355 for the 80% aqueous methanol extract at 1:50 dilution or 50% and 20% aqueous methanol  
356 extracts at 1:10 dilution (Fig. S3). Thus, the subsequent studies were carried out using the  
357 aqueous methanol extracts at these dilutions. Specifically, 80% aqueous methanol extract  
358 (1:50), as well as 50% and 20% aqueous methanol extracts (1:10) were spiked with known  
359 amounts of PLTX (final concentrations ranging from  $3.9 \times 10^{-10}$  M to  $2.5 \times 10^{-8}$  M). Then, the  
360 spiked extracts were analyzed by the hemolytic assay in comparison to the same PLTX  
361 concentrations without matrices. The extract which did not interfere with the hemolytic assay  
362 was the 80% aqueous methanol extract diluted 1:50 (Fig. S4). Notably, this extraction method  
363 is the most suitable for PLTX extraction from mussels, giving 90-100% toxin recovery, by  
364 LC-HRMS and a sandwich ELISA (Ciminiello et al., 2011; Boscolo et al., 2013). Thus, 80%  
365 aqueous methanol at 1:50 dilution was subsequently used for the matrix effect study.

366 Fig. 6 shows the results of the linear regression analysis carried out comparing the theoretical  
367 PLTX concentrations in the spiked extract to those measured by the hemolytic assay. The  
368 LOQ for PLTX in the mussels extract was 1.3 ng/ml, corresponding to 13  $\mu\text{g}/\text{kg}$  meat.  
369 Considering the 1:50 dilution, the LOQ for PLTX in mussels corresponds to 640  $\mu\text{g}/\text{kg}$  meat,



370 a value about 20 times higher than the maximum limit suggested by EFSA (30 µg PLTXs/kg  
371 meat). Moreover, the linear regression analysis yielded a correlation coefficient ( $r^2 = 0.9259$ )  
372 and a *Bias* range from -27.0 to 33.6% (mean *Bias* = 3.7%, Table 2) index of high dispersion  
373 of the data, suggesting low accuracy and precision of the measurement. Thus, the  
374 standardized and characterized hemolytic assay suffers from a significant mussel matrix  
375 effect, which does not allow PLTX quantitation in *Mytilus galloprovincialis* at concentrations  
376 lower than that proposed by EFSA. Interestingly, a significant interference by mussels matrix  
377 was recently observed also for a biosensor exploiting lactate dehydrogenase (LDH) release  
378 from sheep erythrocytes as hemolysis parameter to quantify PLTX (Volpe et al., 2014):  
379 similarly to our study, due to the significant matrix effect, a 1:50 dilution of the mussels  
380 extract was required to quantify PLTX in mussels, not meeting the EFSA requirements  
381 (Volpe et al., 2014). Intriguingly, the observed interference of the mussels extract is not due  
382 to the solvent used for extraction (i.e. 80% aqueous methanol) since the relevant mussels-free  
383 solvent did not induce hemolysis (data not shown). On the contrary, low dilutions of mussels  
384 methanol extract (i.e. 1:1 or 1:10, Fig. S3) displayed an increased hemolytic activity as  
385 compared to that of higher extract dilutions, sometimes comparable to that of the positive  
386 control (0.1% Tween 20). Hence, the significant matrix effect could be due to hemolytic  
387 compounds different from PLTX, that could be at least partially extracted from mussels by  
388 aqueous methanol. Indeed, a series of potentially hemolytic compounds, such as  
389 glycolipids, lysophospholipids and unsaturated fatty acids, are known constituents of  
390 mussel edible parts. The concentration of these compounds in mussel meat could be also  
391 influenced by season, living site of mussels, dietary composition of phytoplankton as well as  
392 temperature and enzymes action during transportation and storage of seafood (Colles and  
393 Chisolm, 2000; Facchini et al., 2016; Ginsburg et al., 1989; Parrish et al., 1998; Pleissner et  
394 al., 2012).

395 All together, these results suggest that PLTX-induced erythrocytes lysis is not a suitable  
396 endpoint for the toxin quantitation in mussels. In addition, since the hemolytic assay has been  
397 worldwide used to detect PLTXs in different field marine samples (also concomitantly to  
398 potential human poisonings ascribable to PLTXs), those data should be carefully considered  
399 because of possible matrices effect that could have affected the analytical outcomes.

400

#### 401 **4. Conclusions**

402 In conclusion, this hemolytic assay employing purified human erythrocytes is characterized  
403 by a good sensitivity, accuracy, specificity and repeatability. However, it does not allow any  
404 PLTX quantitation in mussels (*Mytilus galloprovincialis*) at concentrations below the  
405 maximum limit suggested by EFSA (30 µg/kg; EFSA, 2009), due to the high matrix  
406 interference. In fact, hemolytic substances different from PLTX could be extracted from  
407 mussels together with PLTX (Colles and Chisolm, 2000; Facchini et al., 2016; Ginsburg et  
408 al., 1989; Parrish et al., 1998; Pleissner et al., 2012), causing false positive results. Thus, the  
409 hemolytic assay for PLTX quantitation in seafood could be used only after a careful  
410 evaluation of the specific matrix effects in each natural sample.

411

#### 412 **Acknowledgements**

413 This work was supported by a grant of the Italian Ministry of Health (Progetto Ricerca  
414 Corrente 2012, “Prevenzione delle patologie da biotossine algali: sviluppo di metodi di  
415 screening rapido”, IZSPLV 23/12 RC).

416

#### 417 **Conflict of interest**

418 The authors declare that there are no conflicts of interest.

419

420 **References**

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606

607

608 **Fig. 1.** Optimization of the hemolytic assay. **A:** PLTX-induced hemolysis using whole human  
609 blood or purified human erythrocytes. **B:** PLTX-induced hemolysis on human erythrocytes in  
610 presence of 1 mM boric acid or 1 mM sodium tetraborate. **C:** PLTX-induced hemolysis on  
611 human erythrocytes with and without 1 mM  $\text{CaCl}_2$ . **D:** PLTX-induced hemolysis on human  
612 erythrocytes with and without  $1.8 \times 10^{-4} \text{M}$  or  $3.6 \times 10^{-4} \text{M}$  NaCl, 2x or 4x. **E:** PLTX-induced  
613 hemolysis on human erythrocytes with and without  $\text{K}^+$  ions (2.7 mM KCl, 8.1 mM  $\text{KH}_2\text{PO}_4$ ).  
614 **F:** PLTX-induced hemolysis on human erythrocytes at  $37^\circ \text{C}$  and  $41^\circ \text{C}$ . Each point represents  
615 the mean  $\pm$  SE of 3 different experiments. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  (for D and E as  
616 compared to PBS +  $\text{H}_3\text{BO}_3$  + NaCl 1x; two-way ANOVA and Bonferroni post test).

617

618 **Fig. 2.** **A:** Calibration curve of the hemolytic assay for PLTX. Each point represents the mean  
619  $\pm$  SE of ten different experiments. **B:** Linear regression analysis performed within the working  
620 range of the hemolytic assay ( $3.9 \times 10^{-10} \text{M}$  -  $2.5 \times 10^{-8} \text{M}$ ) by plotting the theoretical PLTX  
621 concentrations against the toxin concentrations measured by the hemolytic assay ( $n = 10$ ).

622

623 **Fig. 3.** Repeatability of the hemolytic assay. Linear regression analysis performed within the  
624 working range of the hemolytic assay ( $3.9 \times 10^{-10} \text{M}$  -  $2.5 \times 10^{-8} \text{M}$ ) by plotting the theoretical  
625 PLTX concentrations against toxin concentrations measured by the hemolytic assay. **A:** Intra-  
626 day repeatability ( $n = 10$ ). **B:** Inter-day repeatability (six months period,  $n = 10$ ).

627

628 **Fig. 4.** Hemolytic activity of N-biotinyl-PLTX, PLTX and its natural analogues within the  
629 working range for PLTX ( $3.9 \times 10^{-10} \text{M}$  -  $2.5 \times 10^{-8} \text{M}$ ). Each point represents the mean  $\pm$  SE of  
630 3 different experiments. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  as compared to PLTX (two-way  
631 ANOVA and Bonferroni post test).

632

633 **Fig. 5.** Neutralization of PLTX-induced hemolysis within the working range of the hemolytic  
634 assay ( $3.9 \times 10^{-10}$  M -  $2.5 \times 10^{-8}$  M) by ouabain and anti-PLTX monoclonal antibody, mAb.  
635 Each point represents the mean  $\pm$  SE of 3 different experiments. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p <$   
636  $0.001$  as compared to PLTX (two-way ANOVA and Bonferroni post test).

637

638 **Fig. 6.** Matrix effect. Linear regression analysis within the working range of the hemolytic  
639 assay ( $3.9 \times 10^{-10}$  M -  $2.5 \times 10^{-8}$  M) performed on 80% aqueous methanol mussels extracts  
640 diluted 1:50. Linear regression analysis was performed within the working range of the  
641 hemolytic assay ( $3.9 \times 10^{-10}$  M -  $2.5 \times 10^{-8}$  M) by plotting the theoretical PLTX concentrations  
642 against toxin concentrations measured by the hemolytic assay.

643 **Table 1.** *Bias* values (%) for PLTX analysis and intra-day (n=10, 1 day) and inter-day (n=10,  
644 6 months) relative repeatability (RSDr %).

645

PLTX (M)	<i>Bias</i> (%)	Intra-day repeatability		Inter-day repeatability	
		Mean	RSDr %	Mean	RSDr %
$3.91 \times 10^{-10}$	-1.2	$4.55 \times 10^{-10}$	17	$3,86 \times 10^{-10}$	10
$7.81 \times 10^{-10}$	-1.7	$7.60 \times 10^{-10}$	12	$7,68 \times 10^{-10}$	3
$1.56 \times 10^{-9}$	-2.0	$1.63 \times 10^{-9}$	20	$1,53 \times 10^{-9}$	8
$3.13 \times 10^{-9}$	2.4	$2.47 \times 10^{-9}$	24	$3,24 \times 10^{-9}$	7
$6.25 \times 10^{-9}$	0.3	$6.51 \times 10^{-9}$	12	$6,27 \times 10^{-9}$	3
$1.25 \times 10^{-8}$	-1.4	$1.28 \times 10^{-8}$	8	$1,24 \times 10^{-8}$	7
$2.50 \times 10^{-8}$	-1.7	$3.18 \times 10^{-8}$	14	$2,50 \times 10^{-8}$	7
Mean	-0.8		15		6

646

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648

649 **Table 2.** *Bias* values (%) for PLTX detected in 80% aqueous methanol mussels extracts  
 650 spiked with the toxin after 1:50 dilution in comparison to the theoretical PLTX concentrations  
 651 (n=6).

652

653

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PLTX (M)	<i>Bias</i> (%)
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654

$3.91 \times 10^{-10}$	-13.8
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655

$7.81 \times 10^{-10}$	-12.8
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656

$1.56 \times 10^{-9}$	12.9
-----------------------	------

657

$3.13 \times 10^{-9}$	31.7
-----------------------	------

658

$6.25 \times 10^{-9}$	-27.0
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659

$1.25 \times 10^{-8}$	33.6
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660

$2.50 \times 10^{-8}$	0.9
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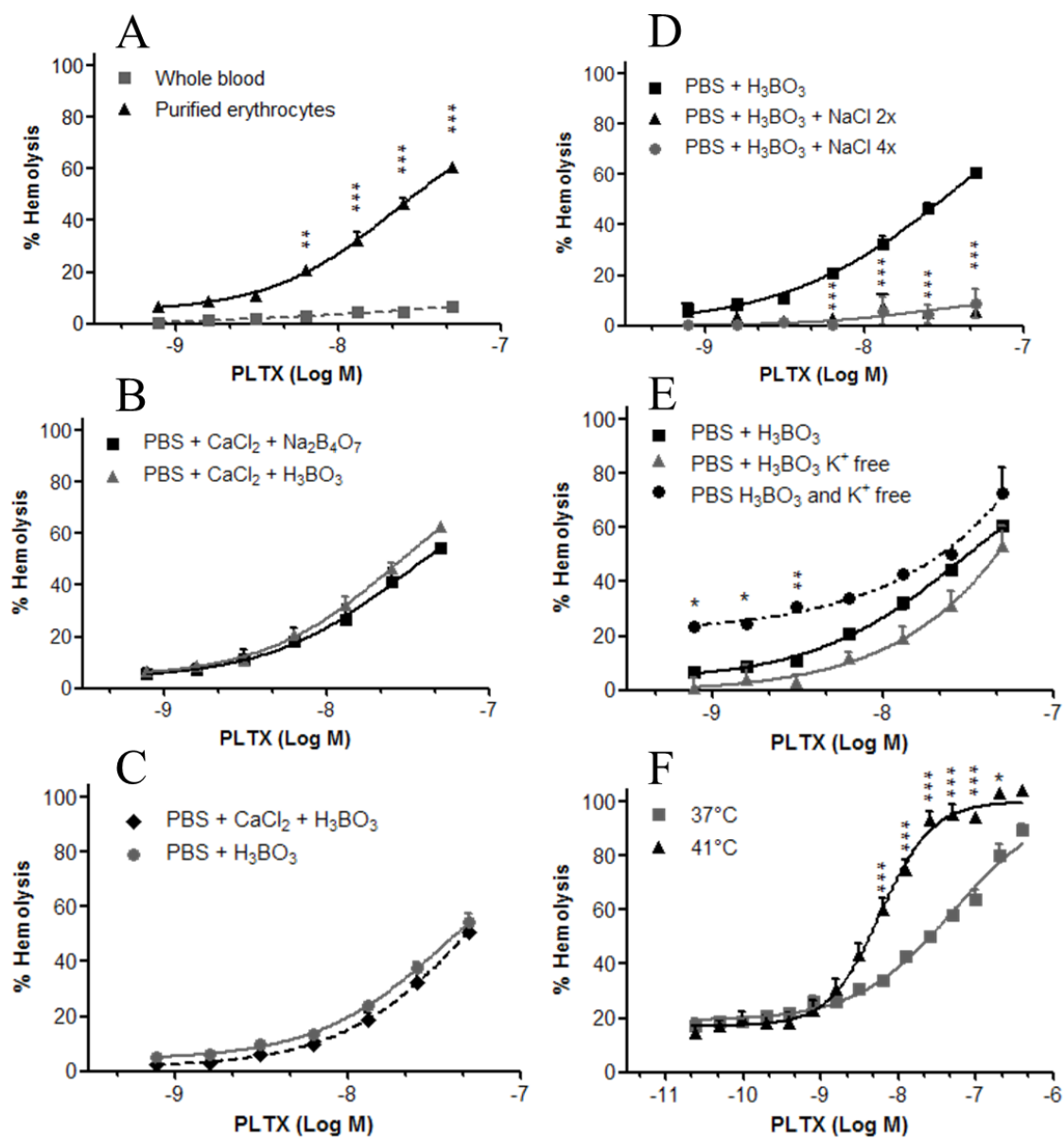
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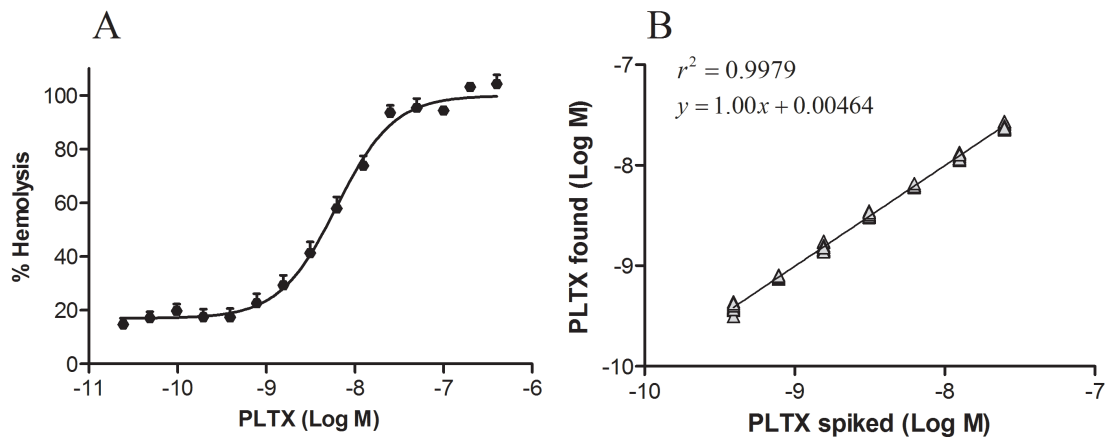
663 Figure 1



664

665

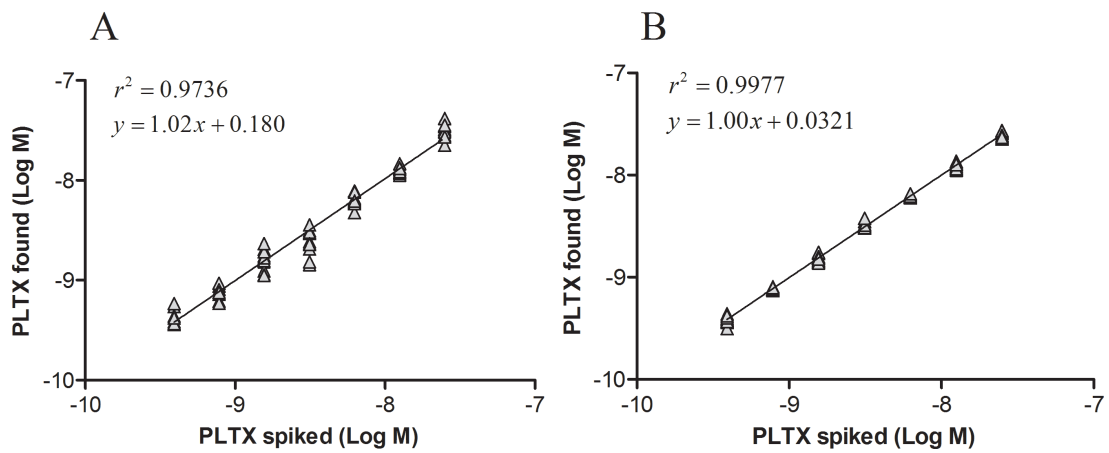
666 Figure 2



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668

669 Figure 3



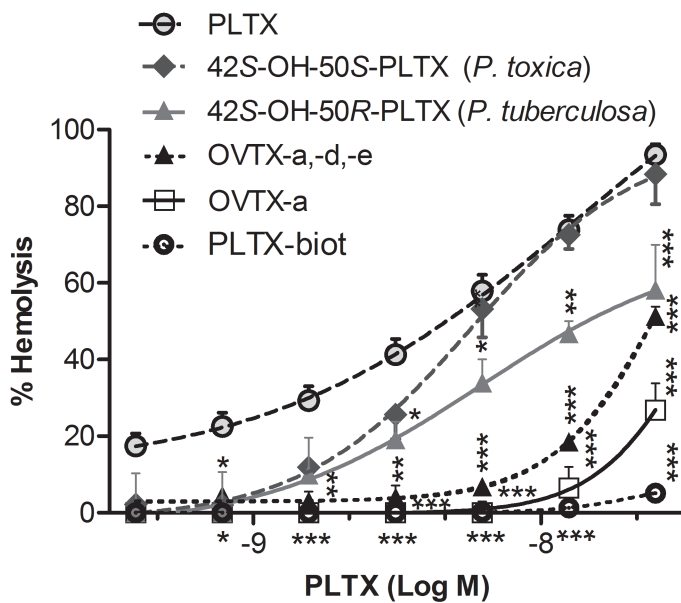
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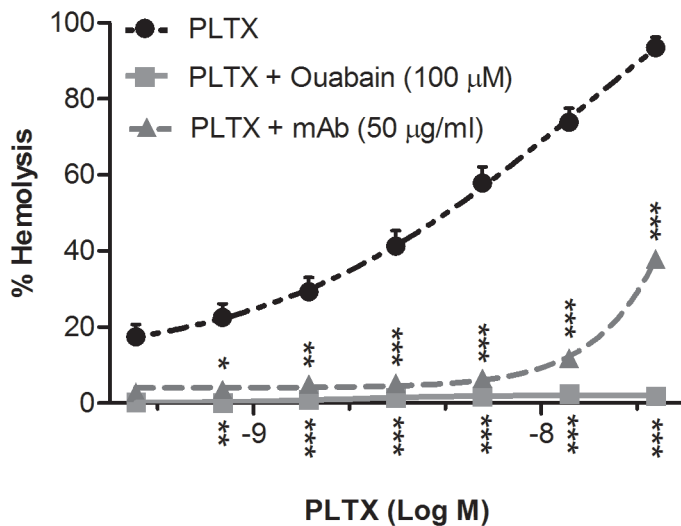
673 Figure 4



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675

676 Figure 5

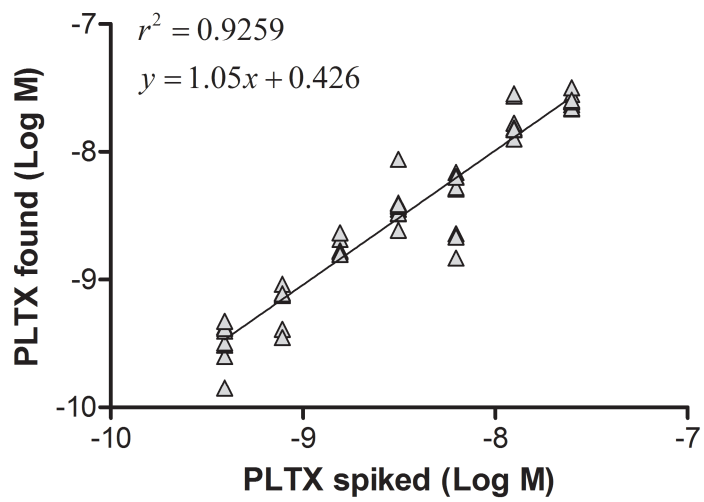


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680 Figure 6



681